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Protocol: Evaluation of qPCR, peripheral blood buffy coat smear and urine antigen ELISA for diagnosis and test of cure for visceral leishmaniasis in HIV co-infected patients

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Abstract

Introduction: HIV coinfection presents a challenge for diagnosis of visceral leishmaniasis. Invasive splenic or bone marrow aspiration with microscopic visualisation of *Leishmania* parasites remains the gold standard for diagnosis of VL in HIV patients. Furthermore, a test-of-cure by splenic or bone marrow aspiration is required as VL-HIV patients are at a high risk of treatment failure. However, there remain financial, implementation and safety costs to these invasive techniques which severely limit their use under field conditions

Methods and analysis: We aim to evaluate blood and skin qPCR, peripheral blood buffy coat smear microcopy and urine antigen ELISA as non- or minimally-invasive alternatives for diagnosis and post treatment test-of-cure for visceral leishmaniasis in HIV co-infected patients using a sample of 91 confirmed symptomatic VL-HIV patients.

Discussion: This protocol describes the first study to evaluate alternative diagnostic and test of cure tools for patients with VL, co-infected with HIV. If successful, the results would provide an evidence base to move away from invasive and more risky assessment practice to safer more patient friendly diagnostic interventions.

Ethics and dissemination: Ethical approval for this study has been granted by The Liverpool School of Tropical Medicine, The Institute of Tropical Medicine in Antwerp, the University of Antwerp, and the Rajendra Memorial Research Institute of Medical Science in Patna.

Trial registration number: REF/2019/01/023677

Key words

Visceral leishmaniasis, HIV, diagnostics, *Leishmania* antigen ELISA, qPCR

Word count: 3,783

Article Summary

Strengths and limitations of this study

- This study will evaluate non-invasive and minimally invasive alternatives to splenic or bone marrow aspiration in HIV patients for diagnosis of Visceral Leishmaniasis in India.
- If an acceptable alternative diagnostic(s) method is identified as a result of this study, a reduction in the use of invasive sampling methods for diagnosis of VL in HIV patients could be made.
- This study may be limited to HIV patients presenting at hospital with more severe disease.

Introduction

In the absence of treatment, visceral leishmaniasis (VL) caused by a parasitic infection of the *Leishmania* genus also known as kala-azar, is usually fatal [1,2]. The state of Bihar in India remains VL endemic; having failed to meet previous elimination targets, new targets are now set for sustained elimination in India in 2020 [3]. Infection with human immunodeficiency virus (HIV) leads to a loss of immune cells and a susceptibility to comorbidities. VL is recognised as an opportunistic infection in HIV [4,5]. In 2017, between 83,806 (0.12%) and 158,675 (0.23%) individuals were estimated to be infected with HIV in Bihar [6]. Of 2,077 VL patients ≥14 years of age screened in Bihar between 2011 and 2013, 5.6% were found to be HIV positive [7]. HIV-VL coinfected patients have higher rates of treatment failure and relapse than VL mono-infected patients [5].

Current diagnostics for VL are invasive or do not distinguish between past and current infection [8]. VL in India is currently diagnosed by a combination of clinical presentation, rK39 rapid diagnostic test (RDT), and parasitological confirmation of tissue aspirates in those presenting with relapse. Splenic aspirates are the gold standard for diagnosis of VL, with a sensitivity of 93-98%. The procedure is invasive, requires a significant skill set and carries a small risk of fatal hemorrhage (1 in 1,000). Where splenic aspiration is not possible (i.e. unpalpable spleen, low platelet or haemoglobin), bone marrow aspirates (BMA) have a reasonable sensitivity of between 50-78% [9]. Although invasive and painful, BMA does not carry the haemorrhage risk associated with splenic aspiration [10]. Additionally, both require the capacity to conduct microscopic confirmation of *Leishmania donovani* bodies (LD bodies) in macrophages [10].

Test of cure (ToC) is a practice whereby following completion of treatment, a repeat comparative diagnostic test is conducted to ensure effectiveness of treatment. It is necessary in VL-HIV due to the relatively high incidence of treatment failure in this cohort of patients, and the high mortality risk associated with incomplete or ineffective treatment of VL-HIV. ToC for VL in HIV patients is currently carried out by parasitological confirmation at day 29 in splenic aspirates where possible, or bone marrow aspirates where splenic aspiration is not possible and are currently the only established way to assess treatment success. Tests which detect antibodies cannot be used due to the persistent circulation of anti-*Leishmania* antibodies following infection, whether or not the patient has symptoms of disease.

Diagnostics such as the rK39 enzyme-linked immunosorbent assay (ELISA) and the rK39 RDT detect presence of anti-*Leishmania* antibodies to rK39 *Leishmania* antigen. RK39 is a routinely used diagnostic, however, data on the sensitivity of the rK39 RDT in HIV co-infected individuals in India do not exist. Kalon Biological (Guilford, UK) has developed an ELISA which detects *Leishmania* antigen excreted in urine, enabling non-invasive detection of current infection [8]. Case-control evaluation of the urine antigen ELISA carried out by Kalon Biological found a sensitivity of 95.2% (n=105, Bangladesh) and 100% (n=18, Kenya). 48/48 and 17/17 healthy negative control samples from Bangladesh and Kenya respectively were found to be negative (Kalon Biological, UK). *Leishmania* antigen excreted in the urine was previously demonstrated to be effective for measurement of treatment effect in VL mono-infected individuals in Ethiopia and may provide a non-invasive alternative to tissue aspiration for both diagnosis and test of cure [8].

QPCR is a highly sensitive technique to detect current infection, allowing parasite DNA present in blood or other tissue to be detected and quantified. Animal studies have shown that also the skin harvests parasites long after the infectious sand fly bite took place [11,12] and the same is assumed to be the case in humans. Direct comparisons between blood parasite load and skin parasite load in humans, however, are scarce, since skin biopsies are painful and not suitable for large clinical studies. Recently, however, a novel device was developed to take virtually painless microbiopsies from the skin. Finally, the use of peripheral blood buffy coat smear microscopy has been shown to be of value in immunocompetent patients with VL in Bangladesh, where 92% were found to be positive for LD bodies in buffy coat smear microscopy, against splenic aspiration as the gold standard [5]. We can therefore consider a-priori that in VL-HIV co-infected patients, this may be higher.

The diagnostic potential of the urine antigen ELISA, blood and qPCR, and peripheral blood buffy coat smear microscopy for diagnosis of VL in HIV patients in India is yet to be established, therefore we aim to evaluate the sensitivity and specificity of these tests in this population. Additionally, we aim to evaluate the urine antigen ELISA, qPCR and peripheral blood buffy coat smear microscopy as a diagnostic tool for active VL infection and test of cure for patients co-infected with HIV on the Indian subcontinent (ISC) to potentially eliminate the need for repeated and invasive splenic and BM aspiration.

Study objective(s)

Primary objective(s):

- To evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood, and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a diagnostic and test of cure at day 29 for symptomatic VL in HIV positive patients compared to the gold standard parasitological confirmation by splenic aspirate at day 0 and day 29 respectively.

Secondary objective(s):

- To evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a test of cure at day 15 for symptomatic VL in HIV positive patients compared the gold standard of parasitological confirmation of splenic aspirate at day 29.
- To conduct a pilot study to evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood, and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a diagnostic and test of cure at day 29 for symptomatic VL in HIV positive patients compared to bone marrow aspirate in a subset of patients contraindicated for splenic aspiration at day 0 and day 29 respectively.
- To investigate the kinetics of *L. donovani* infection during treatment (days, 0, 3, 8, 15, 29) using urine antigen ELISA, qPCR (blood and skin microbiopsy) and peripheral blood buffy coat smear microscopy.

Methods and Analysis

Participants of the study will be patients admitted at the Rajendra Memorial Research Institute (RMRI), Patna, India. Blood, urine and skin microbiopsy samples will be collected from HIV positive patients with suspected VL (meeting the WHO definition of an rK39 RDT positive test with clinical case definition [13]) at baseline who have undergone parasitological confirmation of VL (splenic aspiration or bone marrow aspiration where splenic aspiration is contraindicated) as per the standard of care (Figure 1).

Approximately 15% of patients are contraindicated for splenic aspiration and require bone marrow aspiration for diagnosis. These patients form a sub-set of patients with atypical presentation or more severe disease who would benefit from less invasive and highly sensitive diagnostics. Patients who are confirmed positive by splenic aspiration will be recruited to the main cohort of the study. Patients who are confirmed positive by bone marrow aspiration will be recruited to the pilot component. All parasitologically confirmed patients will undergo treatment for VL as per the standard of care (Figure 1).

Further blood, urine and skin microbiopsy samples will be collected during treatment and at the end of treatment. Patients who are negative by splenic or bone marrow aspiration will not receive treatment as per the standard of care and will not undergo further sampling. QPCR will be conducted on blood and skin samples, buffy coat smear microscopy on blood samples and urine antigen ELISA on urine samples. Measurements of CD4 counts, full blood counts and screening of TB (GeneXpert and chest X-ray) will be conducted as standard for these patients.

Figure 1. Study workflow.

Main entry criteria

Inclusion criteria:

- WHO definition of a VL suspect: rK39 RDT positive + clinical symptoms
- Has undergone parasitological confirmation (splenic or bone marrow aspiration) as per standard of care
- Diagnosis of HIV as per National AIDS control organization (NACO) guidelines
- Patients found positive by parasitological confirmation (splenic aspiration) in the diagnostic section will be continued to follow-up
- 18 years of age or over
- Given written consent

Exclusion criteria:

- Medical emergency or any other severe chronic medical condition which makes participation in the study medically inadvisable
- Participant refusal
- Splenic and bone marrow aspiration contraindicated

Sample size

The primary objective is to determine the diagnostic accuracy of the urine antigen ELISA, qPCR (blood, spleen and skin microbiopsy), and peripheral blood buffy coat smear microscopy for diagnosis of VL in HIV positive patients compared to the reference standard (clinical examination and parasitological confirmation). Splenic aspirates are the current gold standard for diagnosis of VL and have a sensitivity of between 93-98%. Case-control evaluation of the urine antigen ELISA carried out by Kalon Biological found a sensitivity of 95.2% in a cohort of 105 patients in Bangladesh. We expect the sensitivity of the urine ELISA, qPCR and buffy coat smear microscopy singularly or in combination to be 95% sensitive. Therefore, we calculated sample size based on an expected proportion of positive patients using a sensitivity of 95%. The same number of patients will be used to determine the diagnostic accuracy of the urine antigen ELISA, qPCR (blood, spleen and skin microbiopsy), and peripheral blood buffy coat smear microscopy as a test of cure for VL in HIV positive patients compared to the reference standards (clinical examination and parasitological confirmation tests).

<u>Sample size to estimate a proportion:</u>

Precision = 5%

95% confidence (z statistic = 1.96)

$$n = \frac{Z^2 \, P(1-P)}{d^2}$$

$$n = \frac{3.84 \;\; x \, (0.0475)}{0.0025}$$

$$n = \frac{0.1824}{0.0025}$$

$n = 73$

An additional 4 patients (5%) were added to the sample size to account for patients who may default.

$n = 77$

Of the total patients recruited to the study, approximately 15% are expected to be contraindicated for splenic aspiration and will undergo bone marrow aspiration as per standard practice. In order to meet the required precision for the analysis of the main cohort, an additional 15 patients contraindicated for spleen will be recruited to a pilot study.

$\boldsymbol{n = 91}$

Secondary objective

An additional 4 patients (5%) were added to the sample size to account for patients who may default.

Selection of patients

Patients to be recruited will be patients screened at the RMRI, Patna, India. Patients with a suspected diagnosis of HIV-VL as per the inclusion criteria will be invited consecutively to participate. All laboratory tests are to be conducted at the RMRI, Patna, India or an appropriate quality assured laboratory. Informed consent will be taken by study staff who have passed the NIH Protecting Human Research Participants Ethics course (https://phrp.nihtraining.com) or equivalent. A screening and recruitment log will be kept.

Schedule of events

Suspects will be screened with an rK39 RDT and a clinical examination as per standard practice (WHO definition of a VL suspect). Clinical assessment includes temperature (axillary), spleen size (left costal margin on the anterior axillary line to the tip of the spleen medially), liver size (the mid-clavicular line for its total span), body weight, and height. At this point, consent will be taken. Patients who are RDT positive are confirmed through routine parasitological confirmation by splenic aspiration, where not contraindicated. Patients are excluded from splenic aspiration based on an unpalpable spleen, platelet count less than 40,000/µL or haemoglobin (Hb) less than 5g/dL, or with significantly prolonged PT. Patients contraindicated for splenic aspiration will undergo a bone marrow aspiration as per standard practice and will be recruited to the pilot study. Patients will participate in the diagnostic section only, or the diagnostic section and the follow-up section, depending on the result of the splenic or bone marrow aspiration.

Study clinical information will be obtained by a study nurse or doctor on enrolment CRFs:

- Socio-demographic information (e.g. sex)
- HIV-related information (e.g. diagnosis, WHO staging, opportunistic infections)
- ART-related information (e.g. CD4 counts, ART regimen, OI treatment, ART adherence)
- VL/post-kala-azar dermal leishmaniasis (PKDL) related information (e.g. VL/PKDL history, VL/PKDL symptoms)
- Past and current medical conditions (e.g. malaria, chronic comorbidities, concomitant medication)
- VL-focused examination (e.g. vital signs, VL signs and symptoms)

The schedule of sampling is detailed in Table 1. The schedule of tests is detailed in Table 2. Patients with confirmed VL-HIV will be given a course of combination treatment for VL and initiated on ART, where not already on ART, as per standard practice. Further sampling will then be conducted as per the schedule detailed in Table 1, day 0 being day of diagnosis prior to treatment starting. The standard course of treatment for VL in HIV ends on day 14.

A blood sample will be taken for CD4 count, full blood count, qPCR and peripheral blood buffy coat smear microscopy as per the schedule of events (Tables 1 & 2). Patients on the ward undergo routine sampling for tests, where possible sampling will be matched with routine sampling to avoid repeated venepuncture. A urine sample will be taken for urine antigen ELISA. The skin microbiopsy device takes minimally invasive and virtually painless skin samples, and samples will be taken from the nape of the neck as well as on the lower arm for qPCR. The same sample taken for standard microscopic diagnosis of the splenic aspirate will be used for qPCR.

To measure parasitic load by skin qPCR, DNA will be isolated from the microbiopsy device and kinetoplast DNA will be looked for. This will provide a semi-quantitative result in relation to a standard curve of known concentration of cultured parasites.

Formal test of cure (ToC) will be carried out by parasitological confirmation and clinical examination at day 29 as per standard practise. Information regarding treatment failure will be noted.

Table 1. Schedule of sampling.

Day	0	3	8	15	29
Urine	X	X	X	X	X
Blood[1]	X	X	X	X	X
Skin Microbiopsy	X	X	X	X	X
Spleen[1]	X				X*
Bone marrow[1,2]	X				X

[1] Routine samples, all other samples will be matched to routine sample where possible

*In the unlikely event a splenic aspiration is contraindicated on day 29, bone marrow aspirate test of cure will be conducted as per standard practice

[2]In patients who are contraindicated for splenic aspiration a bone marrow aspirate will be taken for diagnosis and test of cure as per standard practice. These patients will be recruited to a pilot study.

Table 2. Schedule of tests.

Day	0	3	8	15	29
Clinical examination	X[1]			X	X[1]
RK39 RDT (blood)[1]	X				
Full blood count[1]	X				X
CD4 count (blood)[1]	X				X
GeneXpert for TB[1]	X				
Chest X-ray for TB[1]	X				
Urine antigen ELISA	X	X	X	X	X
qPCR on blood and skin microbiopsy	X	X	X	X	X
Peripheral blood buffy coat smear	X	X	X	X	X
Spleen parasitological confirmation[1]	X				X*
Bone marrow parasitological confirmation[1,2]	X				X

[1] Routine tests

[2]In patients who are contraindicated for splenic aspiration a bone marrow aspirate will be taken for diagnosis and test of cure as per standard practice. These patients will be recruited to a pilot study.

*In the unlikely event a splenic aspiration is contraindicated on day 29, bone marrow aspirate test of cure will be conducted as per standard practice

Laboratory testing and sample storage

Testing procedures

All samples will be stored at -80◦C until the study ends. This will allow samples to be tested in batch to reduce costs. Testing in batch will also allow blinding of laboratory staff to results of previous time points.

Data analysis and statistical methods

91 consecutive patients meeting the inclusion criteria will be screened for *L. Donovani* infection by qPCR (blood and skin), peripheral blood buffy coat smear microcopy, and urine antigen ELISA at baseline and at times previously shown in the schedule of sampling (Table 1) and schedule of tests (Table 2).

Baseline:

Results of the qPCR, peripheral blood buffy coat smear microcopy, and urine antigen ELISA at baseline will be compared to the gold standard diagnosis (parasitological confirmation).

Sensitivity and specificity with 95% confidence intervals will be calculated as follows:

$$\boldsymbol{Sensitivity} = \frac{\boldsymbol{A}}{(\boldsymbol{A}+\boldsymbol{C})} \times \mathbf{100}$$

$$\boldsymbol{Specificity} = \frac{\boldsymbol{D}}{(\boldsymbol{D}+\boldsymbol{B})} \times \mathbf{100}$$

Where:

	Disease	No Disease	
Positive result	A. True positive	B. False positive	Test positive
Negative result	C. False negative	D. True negative	Test negative
	Total disease	Total no disease	Total

A Kappa coefficient will be used to determine the level of agreement between the evaluation tests and the gold standard.

The continuous variables of the baseline and demographic characteristics will be summarized using number of patients (*n*), mean, SD, median, minimum, maximum, and confidence intervals. The categorical variable gender will be summarized using number of patients (*n*) and percentage (%).

Test of cure:

Patients who test positive for the gold standard at baseline will continue to be screened for *L. Donovani* infection by qPCR (blood and skin), peripheral blood buffy coat smear microcopy, and urine antigen ELISA at times previously shown in the schedule of sampling (Table 1) and schedule of tests (Table 2).

Results of the qPCR (blood and skin), peripheral blood buffy coat smear microcopy, and urine antigen ELISA at day 15 and day 29 will be compared to the gold standard test of cure (parasitological confirmation) at day 29. Sensitivity and specificity with 95% confidence intervals will be calculated as above.

The continuous variables will be summarized using number of patients (*n*), mean, SD, median, minimum, maximum, and confidence intervals.

Risk/benefit assessment

Potential risks related to this study are minimal. Invasive procedures such as splenic aspirate and bone marrow aspirate will only be done as per routine clinical workup; no additional aspirates will be done for the purpose of this study. Urine sampling does not pose any physical risks. Risks during blood or skin sampling are minimal when adhering to standard hygienic rules and include vasovagal reaction, bleeding or infection. Risk of breach in confidentiality will be minimized by using unique personal codes on the case report forms, with the subject's enrolment list linking unique personal codes to the names of the participants in a locked and secured office.

The potential benefit of this study mainly lies in the possibility to identify a diagnostic tool and/or test of cure that makes the current invasive splenic or bone marrow aspirates no longer necessary in the workup of HIV-VL co-infection. This would limit the risks associated to these tests (fatal hemorrhage in case of splenic aspirates of 1/1.000 and painful procedure in case of bone marrow aspirate) and significantly increase the comfort of these patients during their treatment process. Additionally, due to the complexities of these invasive tests, they are only available in specialist centres. If a test of cure at day 15 is equally accurate as the current (parasitological) test of cure at day 29, this would decrease the duration of hospitalization by halve for many patients and eliminate the need to return to the hospital after being released for others, improving access to care for this vulnerable group of patients.

Discussion

Considering the discomfort, iatrogenic risk and level of technical skill involved in parasitological confirmation, development of tests which can diagnose current infection and determine test of cure that are safer, better tolerated and less technically demanding are required. HIV patients diagnosed with VL in India have recently been shown to have better treatment outcomes with a combination therapy of liposomal amphotericin B (LAMB) and miltefosine over 14 days (CTRI/2015/05/005807) [9]. The choice of day 29 for Test of Cure (ToC) is based on older treatment regimens which were traditionally a month long. As such, evidence for the diagnostic accuracy of a test of cure at the end of a shorter treatment (e.g. day 15 in this case) may allow patients to be discharged without the need to return on day 29 for ToC; however it is also possible that the extended time to the day 29 ToC is requires to counter 'slow response' in the viscera to treatment.

The sensitivity of the rK39 RDT is well established in immunocompetent patients with sensitivities identified by systematic review of approximately 97% on the Indian subcontinent and 85% in east Africa [13,14].The rK39 RDT was found to have a sensitivity of 77% in HIV co-infected individuals compared to 87% in HIV negative patients with VL in Ethiopia [15]. As these antibodies remain present even after successful treatment, they cannot be used either as a diagnostic tool in suspected relapse nor as a test of cure following treatment and therefore need to be used in combination with a clinical history or parasitological proof to distinguish between past or current infection [10].

QPCR is considered a proxy for parasite load in an individual but is currently restricted to use for research purposes. The potential for qPCR on blood for use in monitoring of treatment effect has been demonstrated in a cohort in Bangladesh [16]. However, measuring parasite load using qPCR on blood samples might not be the best proxy for measuring parasite load in an individual. A first study in Ethiopia suggested that qPCR in skin was more sensitive in detecting parasite DNA than qPCR in blood. However, more data are needed to validate this skin microbiopsy device [17].

Once complete, the results of this study have the potential to inform alternative minimally- and non-invasive tools for diagnosis and test of cure in VL patients co-infected with HIV. This would allow clinicians to move away from tissue aspirations, methods which carry a risk of discomfort to the patient, and a risk of fatal haemorrhage in the case of splenic aspiration. These interventions may also allow diagnosis within less specialised healthcare facilities.

Ethics and dissemination

This study has been approved by the ethics boards of The Liverpool School of Tropical Medicine, The Institute of Tropical Medicine in Antwerp, the University of Antwerp, and the Rajendra Memorial Research Institute of Medical Science in Patna. The results of the study of this study will be published in an open source, peer reviewed journal. Results will also be presented to policy makers at national and international level. In particular, the WHO GRC who are due to provide global updated management guidelines for VL-HIV co-infection in 2020. Reporting of results will follow STARD guidelines. Data will be made available upon request.

Patient and Public Involvement

No patient involved.

References

1 Cameron MM, Acosta-Serrano A, Bern C, *et al.* Understanding the transmission dynamics of Leishmania donovani to provide robust evidence for interventions to eliminate visceral leishmaniasis in Bihar, India. *Parasit Vectors* 2016;**9**:25. doi:10.1186/s13071-016-1309-8

2 Diro E, Ritmeijer K, Boelaert M, *et al.* Long-term Clinical Outcomes in Visceral Leishmaniasis/Human Immunodeficiency Virus-Coinfected Patients During and After Pentamidine Secondary Prophylaxis in Ethiopia: A Single-Arm Clinical Trial. *Clin Infect Dis* 2018;**66**:444–51. doi:10.1093/cid/cix807

3 Le Rutte EA, Coffeng LE, Bontje DM, *et al.* Feasibility of eliminating visceral leishmaniasis from the Indian subcontinent: explorations with a set of deterministic age-structured transmission models. *Parasit Vectors* 2016;**9**:24. doi:10.1186/s13071-016-1292-0

4 National AIDS Control Organisation, Ministry of Health and Family Welfare G of I. National Strategic Plan for HIV/AIDS and STI 2017 - 2024. 2017. http://naco.gov.in/sites/default/files/Paving the Way for an AIDS 15122017.pdf (accessed 11 Apr 2018).

5 Jarvis JN, Lockwood DN. Clinical aspects of visceral leishmaniasis in HIV infection. *Curr Opin Infect Dis* 2013;**26**:1–9. doi:10.1097/QCO.0b013e32835c2198

6 National AIDS Control Organization & ICMR-National Institute of Medical Statistics. India HIV Estimations 2017: Technical Report. 2018. http://naco.gov.in/sites/default/files/HIV Estimations 2017 Report_1.pdf (accessed 27 Nov 2018).

7 Burza S, Mahajan R, Gonzalez Sanz M, *et al.* HIV and Visceral Leishmaniasis Coinfection in Bihar, India: An Underrecognized and Underdiagnosed Threat Against Elimination. *Clin Infect Dis* 2014;**59**:552–5. doi:10.1093/cid/ciu333

8 Vallur AC, Tutterrow YL, Mohamath R, *et al.* Development and comparative evaluation of two antigen detection tests for Visceral Leishmaniasis. *BMC Infect Dis* 2015;**15**:1–10. doi:10.1186/s12879-015-1125-3

9 Burza S, Croft SL, Boelaert M. Leishmaniasis. Published Online First: 2018. doi:10.1016/S0140-6736(18)31204-2

10 Srivastava P, Dayama A, Mehrotra S, *et al.* Diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 2011;**105**:1–6. doi:10.1016/j.trstmh.2010.09.006

11 Courtenay O, Carson C*, Calvo-Bado L, *et al.* Heterogeneities in Leishmania infantum Infection: Using Skin Parasite Burdens to Identify Highly Infectious Dogs. doi:10.1371/journal.pntd.0002583

12 Aslan H, Oliveira F, Meneses C, *et al.* New Insights Into the Transmissibility of *Leishmania infantum* From Dogs to Sand Flies: Experimental Vector-Transmission Reveals Persistent Parasite Depots at Bite Sites. *J Infect Dis* 2016;**213**:1752–61. doi:10.1093/infdis/jiw022

13 World Health Organization. Visceral Leishmaniasis Rapid Diagnostic Test Performance WHO Diagnostic Evaluation Series. 2011. http://www.who.int/tdr/publications/documents/vl-rdt-evaluation.pdf.%5Cnpapers2://publication/uuid/285CD644-B096-4FED-AB60-1D3330E6D024

14 Boelaert M, Verdonck K, Menten J, *et al.* Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. *Cochrane Database Syst Rev* Published Online First: 20 June 2014. doi:10.1002/14651858.CD009135.pub2

15 Horst R ter, Tefera T, Assefa G, *et al.* Field evaluation of rK39 test and direct agglutination test for diagnosis of visceral leishmaniasis in a population with high prevalence of human immunodeficiency virus in Ethiopia. *Am J Trop Med Hyg* 2009;**80**:929–34. doi:80/6/929 [pii]

16 Hossain F, Ghosh P, Khan MAA, *et al.* Real-time PCR in detection and quantitation of Leishmania donovani for the diagnosis of Visceral Leishmaniasis patients and the monitoring of their response to treatment. *PLoS One* 2017;**12**:e0185606. doi:10.1371/journal.pone.0185606

17 Kirstein OD, Abbasi I, Horwitz BZ, *et al.* Minimally invasive microbiopsies: a novel sampling method for identifying asymptomatic, potentially infectious carriers of Leishmania donovani. *Int J Parasitol* 2017;**47**:609–16. doi:10.1016/j.ijpara.2017.02.005

Author Statement

All authors contributed to study design.

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Conflicts of Interests

The authors of this protocol declare no competing interests.

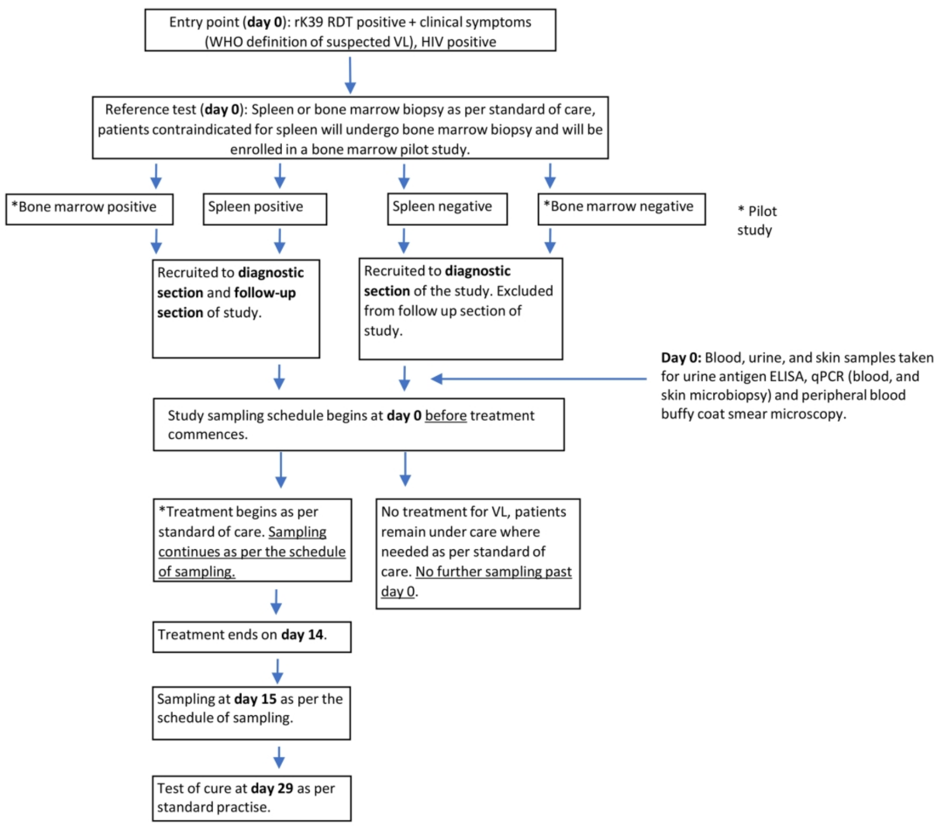

Figure 1. Study workflow.

178x156mm (300 x 300 DPI)

Protocol: Evaluation of qPCR on blood and skin microbiopsies, peripheral blood buffy coat smear, and urine antigen ELISA for diagnosis and test of cure for visceral leishmaniasis in HIV co-infected patients in India, a prospective cohort study.

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Protocol: Evaluation of qPCR on blood and skin microbiopsies, peripheral blood buffy coat smear, and urine antigen ELISA for diagnosis and test of cure for visceral leishmaniasis in HIV co-infected patients in India, a prospective cohort study.

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Abstract

Introduction: HIV coinfection presents a challenge for diagnosis of visceral leishmaniasis. Invasive splenic or bone marrow aspiration with microscopic visualisation of *Leishmania* parasites remains the gold standard for diagnosis of VL in HIV patients. Furthermore, a test-of-cure by splenic or bone marrow aspiration is required as VL-HIV patients are at a high risk of treatment failure. However, there remain financial, implementation and safety costs to these invasive techniques which severely limit their use under field conditions.

Methods and analysis: We aim to evaluate blood and skin qPCR, peripheral blood buffy coat smear microscopy and urine antigen ELISA as non- or minimally invasive alternatives for diagnosis and post treatment test-of-cure for visceral leishmaniasis in HIV co-infected patients in India, using a sample of 91 parasitologically confirmed symptomatic VL-HIV patients.

Ethics and dissemination: Ethical approval for this study has been granted by The Liverpool School of Tropical Medicine, The Institute of Tropical Medicine in Antwerp, the University of Antwerp, and the Rajendra Memorial Research Institute of Medical Science in Patna.

CTRI Trial registration number: CTRI/2019/03/017908

Key words

Visceral leishmaniasis, HIV, diagnostics, *Leishmania* antigen ELISA, qPCR

Word count: 3,783

Article Summary

Strengths and limitations of this study

- This study will evaluate non-invasive and minimally invasive alternatives to splenic or bone marrow aspiration in HIV patients for diagnosis of visceral leishmaniasis in India.
- If an effective alternative diagnostic(s) method is identified as a result of this study, a reduction in the use of invasive sampling methods for diagnosis and test of cure of VL in HIV patients could be made.
- The study addresses both issues of initial diagnosis and test of cure.
- This study is limited to HIV patients presenting at hospital who are likely to be presenting with more advanced disease.
- The use of the minimally invasive techniques do not have standardised approaches.

Introduction

In the absence of treatment, visceral leishmaniasis (VL) caused in India by a parasitic infection of *Leishmania* donovani, also known as kala-azar, is usually fatal [1,2]. The state of Bihar in India remains VL endemic; having failed to meet previous elimination targets, new targets were set for sustained elimination in India by 2020 [3]. Infection with human immunodeficiency virus (HIV) leads to a loss of immune cells and a susceptibility to comorbidities. VL is recognised as an opportunistic infection in HIV [4,5]. In 2017, between 83,806 (0.12%) and 158,675 (0.23%) individuals were estimated to be infected with HIV in Bihar [6]. Of 2,077 VL patients ≥14 years of age screened in Bihar between 2011 and 2013, 5.6% were found to be HIV positive, while upto 20% of reported VL patients from highly endemic districts in Bihar are co-infected with HIV [7,8]. HIV-VL coinfected patients have much higher rates of treatment failure and relapse than those without HIV [5].

Current diagnostics for VL are invasive or do not distinguish between past and current infection [9]. VL in India is currently diagnosed by a combination of clinical presentation, rK39 rapid diagnostic test (RDT), and parasitological confirmation of tissue aspirates in those presenting with relapse. Splenic aspirates are the gold standard for diagnosis of VL, with a sensitivity of 93-98%. The procedure is invasive, requires a significant skill set and carries a small risk of fatal hemorrhage (1 in 1,000). Where splenic aspiration is not possible (i.e. unpalpable spleen, low platelet or haemoglobin), bone marrow aspirates (BMA) have a reasonable sensitivity of between 50-78% [10]. Although invasive and painful, BMA does not carry the haemorrhage risk associated with splenic aspiration [11]. Additionally, both require the capacity to conduct microscopic confirmation of *Leishmania donovani* bodies (LD bodies) in macrophages [11].

Test of cure (ToC) is a practice whereby following completion of treatment; a repeat comparative diagnostic test is conducted to ensure effectiveness of treatment. It is necessary in VL-HIV due to the relatively high incidence of treatment failure in this cohort of patients, and the high mortality risk associated with incomplete or ineffective treatment of VL-HIV. ToC for VL in HIV patients is currently carried out by parasitological confirmation at day 29 in splenic aspirates where possible, or bone marrow aspirates where splenic aspiration is not possible and remain the only established way to determine treatment success. Tests which detect antibodies cannot be used due to the persistent circulation of anti-*Leishmania* antibodies following infection, whether or not the patient has symptoms of disease.

Diagnostics such as the rK39 enzyme-linked immunosorbent assay (ELISA) and the rK39 RDT detect presence of anti-*Leishmania* antibodies to rK39 *Leishmania* antigen. RK39 is a routinely used diagnostic, however, data on the sensitivity of the rK39 RDT in HIV co-infected individuals in India do not exist. Kalon Biological (Guilford, UK) has developed an ELISA which detects *Leishmania* antigen excreted in urine, enabling non-invasive detection of current infection [9]. Case-control evaluation of the urine antigen ELISA carried out by Kalon Biological found a sensitivity of 95.2% (n=105, Bangladesh) and 100% (n=18, Kenya). 48/48 and 17/17 healthy negative control samples from Bangladesh and Kenya respectively were found to be negative (Kalon Biological, UK). *Leishmania* antigen excreted in the urine was previously demonstrated to be effective for measurement of treatment effect in non-HIV infected patients with VL in Ethiopia and may provide a non-invasive alternative to tissue aspiration for both diagnosis and test of cure [9].

QPCR is a highly sensitive technique to detect current infection, allowing parasite DNA present in blood or other tissue to be detected and quantified. In this study, we will use the qPCR assay targeting *Leishmania* kinetoplast DNA as previously described by Adams et al. [12]. Animal studies have shown that also the skin harvests parasites long after the infectious sand fly bite took place [13,14] and the same is assumed to be the case in humans. Direct comparisons between blood parasite load and skin parasite load in humans, however, are scarce, since skin biopsies are painful and not suitable for large clinical studies. Recently, however, a novel device was developed to take virtually painless microbiopsies from the skin. Finally, the use of peripheral blood buffy coat smear microscopy has been shown to be of value in immunocompetent patients with VL in Bangladesh, where 92% were found to be positive for LD bodies in buffy coat smear microscopy, against splenic aspiration as the gold standard [5]. We can therefore consider *a-priori* that in VL-HIV co-infected patients, this may be similar if not higher.

The diagnostic potential of the urine antigen ELISA, blood and qPCR, and peripheral blood buffy coat smear microscopy for diagnosis of VL in HIV patients in India is yet to be established, therefore we aim to evaluate the sensitivity and specificity of these tests in this population. Additionally, we aim to evaluate the urine antigen ELISA, qPCR and peripheral blood buffy coat smear microscopy as a diagnostic tool for active VL infection and test of cure for patients co-infected with HIV on the Indian subcontinent (ISC) to potentially eliminate the need for repeated and invasive splenic and bone marrow aspiration.

Study objective(s)

Primary objective(s):

- To evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood, and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a diagnostic and test of cure at day 29 for symptomatic VL in HIV positive patients compared to the gold standard parasitological visualisation by splenic aspirate at day 0 and day 29 respectively.

Secondary objective(s):

- To evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a test of cure at day 15 for symptomatic VL in HIV positive patients compared the gold standard of parasitological confirmation of splenic aspirate at day 29.
- To conduct a pilot study to evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood, and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a diagnostic and test of cure at day 29 for symptomatic VL in HIV positive patients compared to bone marrow aspirate in a subset of patients contraindicated for splenic aspiration at day 0 and day 29 respectively.
- To investigate the kinetics of *L. donovani* infection during treatment (days, 0, 3, 8, 15, 29) using urine antigen ELISA, qPCR (blood and skin microbiopsy) and peripheral blood buffy coat smear microscopy.

Methods and Analysis

Participants of the study will be patients admitted at the Rajendra Memorial Research Institute of Medical Sciences(RMRIMS), Patna, India. Blood, urine and skin microbiopsy samples will be collected from HIV positive patients with suspected VL (meeting the WHO definition of an rK39 RDT positive test with clinical case definition [15]) at baseline who have undergone parasitological confirmation of VL (splenic aspiration or bone marrow aspiration where splenic aspiration is contraindicated) as per the standard of care (Figure 1).

Approximately 15% of patients are contraindicated for splenic aspiration and require bone marrow aspiration for diagnosis. These patients form a sub-set of patients with atypical presentation or more severe disease who would benefit from less invasive and highly sensitive diagnostics. Patients who are confirmed positive by splenic aspiration will be recruited to the main cohort of the study. Patients who are confirmed positive by bone marrow aspiration will be recruited to the pilot component. All parasitologically confirmed patients will undergo treatment for VL as per the standard of care (Figure 1).

Further blood, urine and skin microbiopsy samples will be collected during treatment and at the end of treatment. Patients who are negative by splenic or bone marrow aspiration will not receive treatment as per the standard of care and will not undergo further sampling. QPCR will be conducted on blood and skin samples, buffy coat smear microscopy on blood samples and urine antigen ELISA on urine samples. Measurements of CD4 counts, full blood counts and screening of TB (GeneXpert and chest X-ray) will be conducted as standard for these patients.

Figure 1. Study workflow.

Main entry criteria

Inclusion criteria:

- WHO definition of a VL suspect: rK39 RDT positive + clinical symptoms
- Has undergone parasitological confirmation (splenic or bone marrow aspiration) as per standard of care
- Diagnosis of HIV as per National AIDS control organization (NACO) guidelines
- Patients found positive by parasitological confirmation (splenic aspiration) in the diagnostic section will be continued to follow-up
- 18 years of age or over
- Given written consent

Exclusion criteria:

- Medical emergency or any other severe chronic medical condition which makes participation in the study medically inadvisable
- Participant refusal
- Splenic and bone marrow aspiration contraindicated

Sample size

The primary objective is to determine the diagnostic accuracy of the urine antigen ELISA, qPCR (blood, spleen and skin microbiopsy), and peripheral blood buffy coat smear microscopy for diagnosis of VL in HIV positive patients compared to the reference standard (clinical examination and parasitological confirmation). Splenic aspirates are the current gold standard for diagnosis of VL and have a sensitivity of between 93-98%. Case-control evaluation of the urine antigen ELISA carried out by Kalon Biological found a sensitivity of 95.2% in a cohort of 105 patients in Bangladesh. We expect the sensitivity of the urine ELISA, qPCR and buffy coat smear microscopy singularly or in combination to be 95% sensitive. Therefore, we calculated sample size based on an expected proportion of positive patients using a sensitivity of 95%. The same number of patients will be used to determine the diagnostic accuracy of the urine antigen ELISA, qPCR (blood, spleen and skin microbiopsy), and peripheral blood buffy coat smear microscopy as a test of cure for VL in HIV positive patients compared to the reference standards (clinical examination and parasitological confirmation tests).

Sample size to estimate a proportion:

Precision = 5%

95% confidence (z statistic = 1.96)

$$n = \frac{Z^2\ P(1-P)}{d^2}$$

$$n = \frac{3.84 \quad x\ (0.0475)}{0.0025}$$

$$n = \frac{0.1824}{0.0025}$$

$n = 73$

An additional 4 patients (5%) were added to the sample size to account for patients who may default.

$n = 77$

Of the total patients recruited to the study, approximately 15% are expected to be contraindicated for splenic aspiration and will undergo bone marrow aspiration as per standard practice. In order to meet the required precision for the analysis of the main cohort, an additional 15 patients contraindicated for spleen will be recruited to a pilot study.

$\boldsymbol{n = 91}$

Selection of patients

Patients to be recruited will be patients screened at the RMRIMS, Patna, India. Consecutive patients with a suspected diagnosis of HIV-VL as per the inclusion criteria will be invited to participate. All laboratory tests are to be conducted at the RMRI, Patna, India or an appropriate quality assured laboratory. Informed consent will be taken by study staff who have passed the NIH Protecting Human Research Participants Ethics course (https://phrp.nihtraining.com) or equivalent. A screening and recruitment log will be kept.

Schedule of events

Suspects will be screened with an rK39 RDT and a clinical examination as per standard practice (WHO definition of a VL suspect). Clinical assessment includes temperature (axillary), spleen size (left costal margin on the anterior axillary line to the tip of the spleen medially), liver size (the mid-clavicular line for its total span), body weight, and height. At this point, consent will be taken. Patients who are RDT positive are confirmed through routine parasitological confirmation by splenic aspiration, where not contraindicated. Patients are excluded from splenic aspiration based on an unpalpable spleen, platelet count less than 40,000/µL or haemoglobin (Hb) less than 5g/dL, or with significantly prolonged PT. Patients contraindicated for splenic aspiration will undergo a bone marrow aspiration as per standard practice and will be recruited to the pilot study. Patients will participate in the diagnostic section only, or the diagnostic section and the follow-up section, depending on the result of the splenic or bone marrow aspiration.

Study clinical information will be obtained by a study nurse or doctor on enrolment CRFs:

- Socio-demographic information (e.g. sex)
- HIV-related information (e.g. diagnosis, WHO staging, opportunistic infections)
- ART-related information (e.g. CD4 counts, ART regimen, OI treatment, ART adherence)
- VL/post-kala-azar dermal leishmaniasis (PKDL) related information (e.g. VL/PKDL history, VL/PKDL symptoms)
- Past and current medical conditions (e.g. malaria, chronic comorbidities, concomitant medication)
- VL-focused examination (e.g. vital signs, VL signs and symptoms)

The schedule of sampling is detailed in Table 1. The schedule of tests is detailed in Table 2. Patients with confirmed VL-HIV will be given a course of combination treatment for VL and initiated on ART, where not already on ART, as per standard practice. Further sampling will then be conducted as per the schedule detailed in Table 1, day 0 being day of diagnosis prior to treatment starting. The standard course of treatment for VL in HIV ends on day 14.

A blood sample will be taken for CD4 count, full blood count, qPCR and peripheral blood buffy coat smear microscopy as per the schedule of events (Tables 1 & 2). Patients on the ward undergo routine sampling for tests, where possible sampling will be matched with routine sampling to avoid repeated venepuncture. A urine sample will be taken for urine antigen ELISA. The skin microbiopsy device takes minimally invasive and virtually painless skin samples, and samples will be taken from the nape of the neck as well as on the lower arm for qPCR. The same sample taken for standard microscopic diagnosis of the splenic aspirate will be used for qPCR.

To measure parasitic load by skin qPCR, DNA will be isolated from the microbiopsy device and kinetoplast DNA will be looked for. This will provide a semi-quantitative result in relation to a standard curve of known concentration of cultured parasites.

Formal test of cure (ToC) will be carried out by parasitological confirmation and clinical examination at day 29 as per standard practise. Information regarding treatment failure will be noted.

Table 1. Schedule of sampling.

Day	0	3	8	15	29
Urine	X	X	X	X	X
Blood[1]	X	X	X	X	X
Skin Microbiopsy	X	X	X	X	X
Spleen[1]	X				X*
Bone marrow[1,2]	X				X

[1] Routine samples, all other samples will be matched to routine sample where possible

*In the unlikely event a splenic aspiration is contraindicated on day 29, bone marrow aspirate test of cure will be conducted as per standard practice

[2]In patients who are contraindicated for splenic aspiration a bone marrow aspirate will be taken for diagnosis and test of cure as per standard practice. These patients will be recruited to a pilot study.

Table 2. Schedule of tests.

Day	0	3	8	15	29
Clinical examination	X[1]			X	X[1]
RK39 RDT (blood) [1]	X				
Full blood count[1]	X				X
CD4 count (blood) [1]	X				X
GeneXpert for TB[1]	X				
Chest X-ray for TB[1]	X				
Urine antigen ELISA	X	X	X	X	X
qPCR on blood and skin microbiopsy	X	X	X	X	X
Peripheral blood buffy coat smear	X	X	X	X	X
Spleen parasitological confirmation[1]	X				X*
Bone marrow parasitological confirmation[1,2]	X				X

[1] Routine tests

[2]In patients who are contraindicated for splenic aspiration a bone marrow aspirate will be taken for diagnosis and test of cure as per standard practice. These patients will be recruited to a pilot study.

*In the unlikely event a splenic aspiration is contraindicated on day 29, bone marrow aspirate test of cure will be conducted as per standard practice

Laboratory testing and sample storage

Testing procedures

All samples will be stored at -80◦C until the study ends. This will allow samples to be tested in batch to reduce costs. Testing in batch will also allow blinding of laboratory staff to results of previous time points.

Data analysis and statistical methods

91 consecutive patients meeting the inclusion criteria will be screened for *L. donovani* infection by qPCR (blood and skin), peripheral blood buffy coat smear microscopy, and urine antigen ELISA at baseline and at times previously shown in the schedule of sampling (Table 1) and schedule of tests (Table 2).

Baseline:

Results of the qPCR, peripheral blood buffy coat smear microscopy, and urine antigen ELISA at baseline will be compared to the gold standard diagnosis (parasitological confirmation).

Sensitivity and specificity with 95% confidence intervals will be calculated as follows:

$$\boldsymbol{Sensitivity} = \frac{\boldsymbol{A}}{(\boldsymbol{A}+\boldsymbol{C})} \times \mathbf{100}$$

$$\boldsymbol{Specificity} = \frac{\boldsymbol{D}}{(\boldsymbol{D}+\boldsymbol{B})} \times \mathbf{100}$$

Where the above values are shown in Table 3.

Table 3. Contingency table to calculate sensitivity and specificity.

	Disease	No Disease	
Positive result	A. True positive	B. False positive	Test positive
Negative result	C. False negative	D. True negative	Test negative
	Total disease	Total no disease	Total

A Kappa coefficient will be used to determine the level of agreement between the evaluation tests and the gold standard.

The continuous variables of the baseline and demographic characteristics will be summarized using number of patients (*n*), mean, SD, median, minimum, maximum, and confidence intervals. The categorical variable gender will be summarized using number of patients (*n*) and percentage (%).

Test of cure:

Patients who test positive for the gold standard at baseline will continue to be screened for infection by qPCR (blood and skin), peripheral blood buffy coat smear microscopy, and urine antigen ELISA at times previously shown in the schedule of sampling (Table 1) and schedule of tests (Table 2).

Results of the qPCR (blood and skin), peripheral blood buffy coat smear microscopy, and urine antigen ELISA at day 15 and day 29 will be compared to the gold standard test of cure (parasitological confirmation) at day 29. Sensitivity and specificity with 95% confidence intervals will be calculated as above.

The continuous variables will be summarized using number of patients (*n*), mean, SD, median, minimum, maximum, and confidence intervals.

Further analysis

Where possible, analysis will also be stratified by markers of disease severity and other sub-groups such as HIV viral load, CD4+ T cell counts, and the use of HAART.

Risk/benefit assessment

Potential risks related to this study are minimal. Invasive procedures such as splenic aspirate and bone marrow aspirate will only be done as per routine clinical workup; no additional aspirates will be done for the purpose of this study. Urine sampling does not pose any physical risks. Risks during blood or skin sampling are minimal when adhering to standard hygienic rules and include vasovagal reaction, bleeding or infection. Some discomfort may be felt during the skin microbiopsy; however, the device has been designed to be virtually painless. Risk of breach in confidentiality will be minimized by using unique personal codes on the case report forms, with the subject's enrolment list linking unique personal codes to the names of the participants in a locked and secured office.

The potential benefit of this study mainly lies in the possibility to identify a diagnostic tool and/or test of cure that makes the current invasive splenic or bone marrow aspirates no longer necessary in the workup of HIV-VL co-infection. This would limit the risks associated to these tests (fatal hemorrhage in case of splenic aspirates of 1/1.000 and painful procedure in case of bone marrow aspirate) and significantly increase the comfort of these patients during their treatment process. Additionally, due to the complexities of these invasive tests, they are only available in specialist centres. If a test of cure at day 15 is equally accurate as the current (parasitological) test of cure at day 29, this would decrease the duration of hospitalization by half for many patients and eliminate the need to return to the hospital after being released for others, improving access to care for this vulnerable group of patients.

Patient and Public Involvement

There was no patient or public involvement in the development of research questions and the study design.

Discussion

Considering the discomfort, iatrogenic risk and level of technical skill involved in parasitological confirmation, development of tests which can diagnose current infection and determine test of cure that are safer, better tolerated and less technically demanding are required. HIV patients diagnosed with VL in India have recently been shown to have better treatment outcomes with a combination therapy of liposomal amphotericin B (LAMB) and miltefosine over 14 days (CTRI/2015/05/005807) [10]. The choice of day 29 for Test of Cure (ToC) is based on older treatment regimens which were traditionally a month long. As such, evidence for the diagnostic accuracy of a test of cure at the end of a shorter treatment (e.g. day 15 in this case) may allow patients to be discharged without the need to return on day 29 for ToC; however it is also possible that the extended time to the day 29 ToC is requires to counter 'slow response' in the viscera to treatment.

The sensitivity of the rK39 RDT is well established in immunocompetent patients with sensitivities identified by systematic review of approximately 97% on the Indian subcontinent and 85% in east Africa [15,16].The rK39 RDT was found to have a sensitivity of 77% in HIV co-infected individuals compared to 87% in HIV negative patients with VL in Ethiopia [17]. As these antibodies remain present even after successful treatment, they cannot be used either as a diagnostic tool in suspected relapse nor as a test of cure following treatment and therefore need to be used in combination with a clinical history or parasitological proof to distinguish between past or current infection [11].

QPCR is considered a proxy for parasite load in an individual but is currently restricted to use for research purposes. The potential for qPCR on blood for use in monitoring of treatment effect has been demonstrated in a cohort in Bangladesh [18]. However, measuring parasite load using qPCR on blood samples might not be the best proxy for measuring parasite load in an individual. A first study in Ethiopia suggested that qPCR in skin was more sensitive in detecting parasite DNA than qPCR in blood. However, more data are needed to validate this skin microbiopsy device [19].

Once complete, the results of this study have the potential to inform alternative minimally- and non-invasive tools for diagnosis and test of cure in VL patients co-infected with HIV. This would allow clinicians to move away from tissue aspirations, methods which carry a risk of discomfort to the patient, and a risk of fatal haemorrhage in the case of splenic aspiration. These interventions may also allow diagnosis within less specialised healthcare facilities.

Ethics and dissemination

This study has been approved by the ethics boards of The Liverpool School of Tropical Medicine, The Institute of Tropical Medicine in Antwerp, the University of Antwerp, and the Rajendra Memorial Research Institute of Medical Science in Patna. The results of the study of this study will be published in an open source, peer reviewed journal. Results will also be presented to policy makers at national and international level. In particular, the WHO GRC who are due to provide global updated management guidelines for VL-HIV co-infection in 2021. Reporting of results will follow STARD guidelines. Data will be made available upon request.

References

1 Cameron MM, Acosta-Serrano A, Bern C, *et al.* Understanding the transmission dynamics of Leishmania donovani to provide robust evidence for interventions to eliminate visceral leishmaniasis in Bihar, India. *Parasit Vectors* 2016;**9**:25. doi:10.1186/s13071-016-1309-8

2 Diro E, Ritmeijer K, Boelaert M, *et al.* Long-term Clinical Outcomes in Visceral Leishmaniasis/Human Immunodeficiency Virus-Coinfected Patients During and After Pentamidine Secondary Prophylaxis in Ethiopia: A Single-Arm Clinical Trial. *Clin Infect Dis* 2018;**66**:444–51. doi:10.1093/cid/cix807

3 Le Rutte EA, Coffeng LE, Bontje DM, *et al.* Feasibility of eliminating visceral leishmaniasis from the Indian subcontinent: explorations with a set of deterministic age-structured transmission models. *Parasit Vectors* 2016;**9**:24. doi:10.1186/s13071-016-1292-0

4 National AIDS Control Organisation, Ministry of Health and Family Welfare G of I. National Strategic Plan for HIV/AIDS and STI 2017 - 2024. 2017. http://naco.gov.in/sites/default/files/Paving the Way for an AIDS 15122017.pdf (accessed 11 Apr 2018).

5 Jarvis JN, Lockwood DN. Clinical aspects of visceral leishmaniasis in HIV infection. *Curr Opin Infect Dis* 2013;**26**:1–9. doi:10.1097/QCO.0b013e32835c2198

6 National AIDS Control Organization & ICMR-National Institute of Medical Statistics. India HIV Estimations 2017: Technical Report. 2018. http://naco.gov.in/sites/default/files/HIV Estimations 2017 Report_1.pdf (accessed 27 Nov 2018).

7 Burza S, Mahajan R, Gonzalez Sanz M, *et al.* HIV and Visceral Leishmaniasis Coinfection in Bihar, India: An Underrecognized and Underdiagnosed Threat Against Elimination. *Clin Infect Dis* 2014;**59**:552–5. doi:10.1093/cid/ciu333

8 Akuffo H, Costa C, van Griensven J, *et al.* New insights into leishmaniasis in the immunosuppressed. *PLoS Negl Trop Dis* 2018;**12**:e0006375. doi:10.1371/journal.pntd.0006375

9 Vallur AC, Tutterrow YL, Mohamath R, *et al.* Development and comparative evaluation of two antigen detection tests for Visceral Leishmaniasis. *BMC Infect Dis* 2015;**15**:1–10. doi:10.1186/s12879-015-1125-3

10 Burza S, Croft SL, Boelaert M. Leishmaniasis. Published Online First: 2018. doi:10.1016/S0140-6736(18)31204-2

11 Srivastava P, Dayama A, Mehrotra S, *et al.* Diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 2011;**105**:1–6. doi:10.1016/j.trstmh.2010.09.006

12 Adams ER, Schoone G, Versteeg I, *et al.* Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Assay for Diagnosis of Cutaneous and Visceral Leishmaniasis. *J Clin Microbiol* 2018;**56**:e00386-18. doi:10.1128/JCM.00386-18

13 Courtenay O, Carson C*, Calvo-Bado L, *et al.* Heterogeneities in Leishmania infantum Infection: Using Skin Parasite Burdens to Identify Highly Infectious Dogs. *PLoS Negl Trop Dis* 2014;**8**:e2583. doi:10.1371/journal.pntd.0002583

14 Aslan H, Oliveira F, Meneses C, *et al.* New Insights Into the Transmissibility of *Leishmania infantum* From Dogs to Sand Flies: Experimental Vector-Transmission Reveals Persistent Parasite Depots at Bite Sites. *J Infect Dis* 2016;**213**:1752–61. doi:10.1093/infdis/jiw022

15 World Health Organization. Visceral Leishmaniasis Rapid Diagnostic Test Performance WHO Diagnostic Evaluation Series. 2011. http://www.who.int/tdr/publications/documents/vl-rdt-evaluation.pdf.%5Cnpapers2://publication/uuid/285CD644-B096-4FED-AB60-1D3330E6D024

16 Boelaert M, Verdonck K, Menten J, *et al.* Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. *Cochrane Database Syst Rev* Published Online First: 20 June 2014. doi:10.1002/14651858.CD009135.pub2

17 Horst R ter, Tefera T, Assefa G, *et al.* Field evaluation of rK39 test and direct agglutination test for diagnosis of visceral leishmaniasis in a population with high prevalence of human immunodeficiency virus in Ethiopia. *Am J Trop Med Hyg* 2009;**80**:929–34. doi:80/6/929 [pii]

18 Hossain F, Ghosh P, Khan MAA, *et al.* Real-time PCR in detection and quantitation of Leishmania donovani for the diagnosis of Visceral Leishmaniasis patients and the monitoring of their response to treatment. *PLoS One* 2017;**12**:e0185606.

doi:10.1371/journal.pone.0185606

19 Kirstein OD, Abbasi I, Horwitz BZ, *et al.* Minimally invasive microbiopsies: a novel sampling method for identifying asymptomatic, potentially infectious carriers of Leishmania donovani. *Int J Parasitol* 2017;**47**:609–16. doi:10.1016/j.ijpara.2017.02.005

Author Statement

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Conflicts of Interests

The authors of this protocol declare no competing interests.

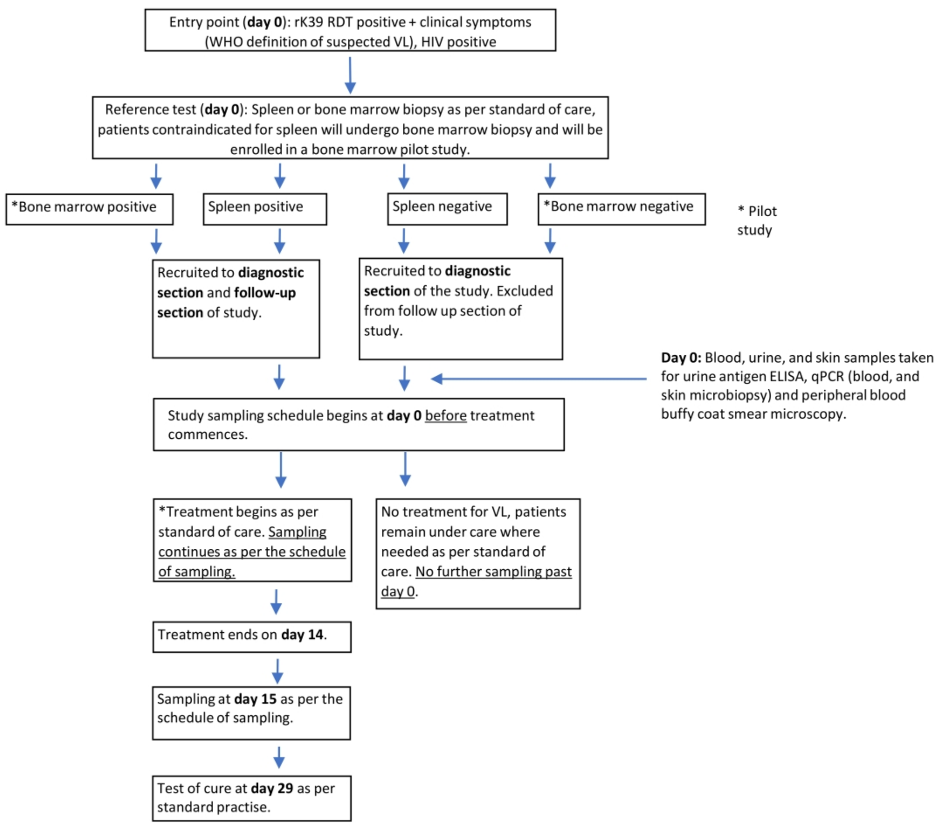

Figure 1. Study workflow.

179x157mm (500 x 500 DPI)

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Protocol: Evaluation of qPCR on blood and skin microbiopsies, peripheral blood buffy coat smear, and urine antigen ELISA for diagnosis and test of cure for visceral leishmaniasis in HIV co-infected patients in India, a prospective cohort study.

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Abstract

Introduction: HIV coinfection presents a challenge for diagnosis of visceral leishmaniasis. Invasive splenic or bone marrow aspiration with microscopic visualisation of *Leishmania* parasites remains the gold standard for diagnosis of VL in HIV patients. Furthermore, a test-of-cure by splenic or bone marrow aspiration is required as VL-HIV patients are at a high risk of treatment failure. However, there remain financial, implementation and safety costs to these invasive techniques which severely limit their use under field conditions.

Methods and analysis: We aim to evaluate blood and skin qPCR, peripheral blood buffy coat smear microscopy and urine antigen ELISA as non- or minimally invasive alternatives for diagnosis and post treatment test-of-cure for visceral leishmaniasis in HIV co-infected patients in India, using a sample of 91 parasitologically confirmed symptomatic VL-HIV patients.

Ethics and dissemination: Ethical approval for this study has been granted by The Liverpool School of Tropical Medicine, The Institute of Tropical Medicine in Antwerp, the University of Antwerp, and the Rajendra Memorial Research Institute of Medical Science in Patna. Any future publications will be published in open access journals.

CTRI Trial registration number: CTRI/2019/03/017908

Key words

Visceral leishmaniasis, HIV, diagnostics, *Leishmania* antigen ELISA, qPCR

Word count: 3,783

Article Summary

Strengths and limitations of this study

- This study will evaluate non-invasive and minimally invasive alternatives to splenic or bone marrow aspiration in HIV patients for diagnosis of visceral leishmaniasis in India.
- If an effective alternative diagnostic(s) method is identified as a result of this study, a reduction in the use of invasive sampling methods for diagnosis and test of cure of VL in HIV patients could be made.
- The study addresses both issues of initial diagnosis and test of cure.
- This study is limited to HIV patients presenting at hospital who are likely to be presenting with more advanced disease.
- The use of the minimally invasive techniques do not have standardised approaches.

Introduction

In the absence of treatment, visceral leishmaniasis (VL) caused in India by a parasitic infection of *Leishmania* donovani, also known as kala-azar, is usually fatal [1,2]. The state of Bihar in India remains VL endemic; having failed to meet previous elimination targets, new targets were set for sustained elimination in India by 2020 [3]. Infection with human immunodeficiency virus (HIV) leads to a loss of immune cells and a susceptibility to comorbidities. VL is recognised as an opportunistic infection in HIV [4,5]. In 2017, between 83,806 (0.12%) and 158,675 (0.23%) individuals were estimated to be infected with HIV in Bihar [6]. Of 2,077 VL patients ≥14 years of age screened in Bihar between 2011 and 2013, 5.6% were found to be HIV positive, while up to 20% of reported VL patients from highly endemic districts in Bihar are co-infected with HIV [7,8]. HIV-VL coinfected patients have much higher rates of treatment failure and relapse than those without HIV [5].

Current diagnostics for VL are invasive or do not distinguish between past and current infection [9]. VL in India is currently diagnosed by a combination of clinical presentation, rK39 rapid diagnostic test (RDT), and parasitological confirmation of tissue aspirates in those presenting with relapse. Splenic aspirates are the gold standard for diagnosis of VL, with a sensitivity of 93-98%. The procedure is invasive, requires a significant skill set and carries a small risk of fatal hemorrhage (1 in 1,000). Where splenic aspiration is not possible (i.e. unpalpable spleen, low platelet or haemoglobin), bone marrow aspirates (BMA) have a reasonable sensitivity of between 50-78% [10]. Although invasive and painful, BMA does not carry the haemorrhage risk associated with splenic aspiration [11]. Additionally, both require the capacity to conduct microscopic confirmation of *Leishmania donovani* bodies (LD bodies) in macrophages [11].

Test of cure (ToC) is a practice whereby following completion of treatment; a repeat comparative diagnostic test is conducted to ensure effectiveness of treatment. It is necessary in VL-HIV due to the relatively high incidence of treatment failure in this cohort of patients, and the high mortality risk associated with incomplete or ineffective treatment of VL-HIV. ToC for VL in HIV patients is currently carried out by parasitological confirmation at day 29 in splenic aspirates where possible, or bone marrow aspirates where splenic aspiration is not possible and remain the only established way to determine treatment success. Tests which detect antibodies cannot be used due to the persistent circulation of anti-*Leishmania* antibodies following infection, whether or not the patient has symptoms of disease.

Diagnostics such as the rK39 enzyme-linked immunosorbent assay (ELISA) and the rK39 RDT detect presence of anti-*Leishmania* antibodies to rK39 *Leishmania* antigen. RK39 is a routinely used diagnostic, however, data on the sensitivity of the rK39 RDT in HIV co-infected individuals in India do not exist. Kalon Biological (Guilford, UK) has developed an ELISA which detects *Leishmania* antigen excreted in urine, enabling non-invasive detection of current infection [9]. Case-control evaluation of the urine antigen ELISA carried out by Kalon Biological found a sensitivity of 95.2% (n=105, Bangladesh) and 100% (n=18, Kenya). 48/48 and 17/17 healthy negative control samples from Bangladesh and Kenya respectively were found to be negative (Kalon Biological, UK). *Leishmania* antigen excreted in the urine was previously demonstrated to be effective for measurement of treatment effect in non-HIV infected patients with VL in Ethiopia and may provide a non-invasive alternative to tissue aspiration for both diagnosis and test of cure [9].

QPCR is a highly sensitive technique to detect current infection, allowing parasite DNA present in blood or other tissue to be detected and quantified. In this study, we will use the qPCR assay targeting *Leishmania* kinetoplast DNA as previously described by Adams et al. [12]. Animal studies have shown that also the skin harvests parasites long after the infectious sand fly bite took place [13,14] and the same is assumed to be the case in humans. Direct comparisons between blood parasite load and skin parasite load in humans, however, are scarce, since skin biopsies are painful and not suitable for large clinical studies. Recently, however, a novel device was developed to take virtually painless microbiopsies from the skin. Finally, the use of peripheral blood buffy coat smear microscopy has been shown to be of value in immunocompetent patients with VL in Bangladesh, where 92% were found to be positive for LD bodies in buffy coat smear microscopy, against splenic aspiration as the gold standard [5]. We can therefore consider *a-priori* that in VL-HIV co-infected patients, this may be similar if not higher.

The diagnostic potential of the urine antigen ELISA, blood and qPCR, and peripheral blood buffy coat smear microscopy for diagnosis of VL in HIV patients in India is yet to be established, therefore we aim to evaluate the sensitivity and specificity of these tests in this population. Additionally, we aim to evaluate the urine antigen ELISA, qPCR and peripheral blood buffy coat smear microscopy as a diagnostic tool for active VL infection and test of cure for patients co-infected with HIV on the Indian subcontinent (ISC) to potentially eliminate the need for repeated and invasive splenic and bone marrow aspiration.

Study objective(s)

Primary objective(s):

- To evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood, and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a diagnostic and test of cure at day 29 for symptomatic VL in HIV positive patients compared to the gold standard parasitological visualisation by splenic aspirate at day 0 and day 29 respectively.

Secondary objective(s):

- To evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a test of cure at day 15 for symptomatic VL in HIV positive patients compared the gold standard of parasitological confirmation of splenic aspirate at day 29.
- To conduct a pilot study to evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood, and skin microbiopsy) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a diagnostic and test of cure at day 29 for symptomatic VL in HIV positive patients compared to bone marrow aspirate in a subset of patients contraindicated for splenic aspiration at day 0 and day 29 respectively.
- To investigate the kinetics of *L. donovani* infection during treatment (days, 0, 3, 8, 15, 29) using urine antigen ELISA, qPCR (blood and skin microbiopsy) and peripheral blood buffy coat smear microscopy.

Methods and Analysis

Participants of the study will be patients admitted at the Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Patna, India. Blood, urine and skin microbiopsy samples will be collected from HIV positive patients with suspected VL (meeting the WHO definition of an rK39 RDT positive test with clinical case definition [15]) at baseline who have undergone parasitological confirmation of VL (splenic aspiration or bone marrow aspiration where splenic aspiration is contraindicated) as per the standard of care (Figure 1).

Approximately 15% of patients are contraindicated for splenic aspiration and require bone marrow aspiration for diagnosis. These patients form a sub-set of patients with atypical presentation or more severe disease who would benefit from less invasive and highly sensitive diagnostics. Patients who are confirmed positive by splenic aspiration will be recruited to the main cohort of the study. Patients who are confirmed positive by bone marrow aspiration will be recruited to the pilot component. All parasitologically confirmed patients will undergo treatment for VL as per the standard of care (Figure 1).

Further blood, urine and skin microbiopsy samples will be collected during treatment and at the end of treatment. Patients who are negative by splenic or bone marrow aspiration will not receive treatment as per the standard of care and will not undergo further sampling. QPCR will be conducted on blood and skin samples, buffy coat smear microscopy on blood samples and urine antigen ELISA on urine samples. Measurements of CD4 counts, full blood counts and screening of TB (GeneXpert and chest X-ray) will be conducted as standard for these patients.

Figure 1. Study workflow.

Main entry criteria

Inclusion criteria:

- WHO definition of a VL suspect: rK39 RDT positive + clinical symptoms
- Has undergone parasitological confirmation (splenic or bone marrow aspiration) as per standard of care
- Diagnosis of HIV as per National AIDS control organization (NACO) guidelines
- Patients found positive by parasitological confirmation (splenic aspiration) in the diagnostic section will be continued to follow-up
- 18 years of age or over
- Given written consent

Exclusion criteria:

- Medical emergency or any other severe chronic medical condition which makes participation in the study medically inadvisable
- Participant refusal
- Splenic and bone marrow aspiration contraindicated

Sample size

The primary objective is to determine the diagnostic accuracy of the urine antigen ELISA, qPCR (blood, spleen and skin microbiopsy), and peripheral blood buffy coat smear microscopy for diagnosis of VL in HIV positive patients compared to the reference standard (clinical examination and parasitological confirmation). Splenic aspirates are the current gold standard for diagnosis of VL and have a sensitivity of between 93-98%. Case-control evaluation of the urine antigen ELISA carried out by Kalon Biological found a sensitivity of 95.2% in a cohort of 105 patients in Bangladesh. We expect the sensitivity of the urine ELISA, qPCR and buffy coat smear microscopy singularly or in combination to be 95% sensitive. Therefore, we calculated sample size based on an expected proportion of positive patients using a sensitivity of 95%. The same number of patients will be used to determine the diagnostic accuracy of the urine antigen ELISA, qPCR (blood, spleen and skin microbiopsy), and peripheral blood buffy coat smear microscopy as a test of cure for VL in HIV positive patients compared to the reference standards (clinical examination and parasitological confirmation tests).

Sample size to estimate a proportion:

Precision = 5%

95% confidence (z statistic = 1.96)

$$n = \frac{Z^2\,P(1-P)}{d^2}$$

$$n = \frac{3.84 \quad x\ (0.0475)}{0.0025}$$

$$n = \frac{0.1824}{0.0025}$$

$n = 73$

An additional 4 patients (5%) were added to the sample size to account for patients who may default.

$n = 77$

Of the total patients recruited to the study, approximately 15% are expected to be contraindicated for splenic aspiration and will undergo bone marrow aspiration as per standard practice. In order to meet the required precision for the analysis of the main cohort, an additional 15 patients contraindicated for spleen will be recruited to a pilot study.

$\boldsymbol{n = 91}$

Selection of patients

Patients to be recruited will be patients screened at the RMRIMS, Patna, India. Consecutive patients with a suspected diagnosis of HIV-VL as per the inclusion criteria will be invited to participate. All laboratory tests are to be conducted at the RMRI, Patna, India or an appropriate quality assured laboratory. Informed consent will be taken by study staff who have passed the NIH Protecting Human Research Participants Ethics course (https://phrp.nihtraining.com) or equivalent. A screening and recruitment log will be kept.

Schedule of events

Suspects will be screened with an rK39 RDT and a clinical examination as per standard practice (WHO definition of a VL suspect). Clinical assessment includes temperature (axillary), spleen size (left costal margin on the anterior axillary line to the tip of the spleen medially), liver size (the mid-clavicular line for its total span), body weight, and height. At this point, consent will be taken. Patients who are RDT positive are confirmed through routine parasitological confirmation by splenic aspiration, where not contraindicated. Patients are excluded from splenic aspiration based on an unpalpable spleen, platelet count less than 40,000/µL or haemoglobin (Hb) less than 5g/dL, or with significantly prolonged PT. Patients contraindicated for splenic aspiration will undergo a bone marrow aspiration as per standard practice and will be recruited to the pilot study. Patients will participate in the diagnostic section only, or the diagnostic section and the follow-up section, depending on the result of the splenic or bone marrow aspiration.

Study clinical information will be obtained by a study nurse or doctor on enrolment CRFs:

- Socio-demographic information (e.g. sex)
- HIV-related information (e.g. diagnosis, WHO staging, opportunistic infections)
- ART-related information (e.g. CD4 counts, ART regimen, OI treatment, ART adherence)
- VL/post-kala-azar dermal leishmaniasis (PKDL) related information (e.g. VL/PKDL history, VL/PKDL symptoms)
- Past and current medical conditions (e.g. malaria, chronic comorbidities, concomitant medication)
- VL-focused examination (e.g. vital signs, VL signs and symptoms)

The schedule of sampling is detailed in Table 1. The schedule of tests is detailed in Table 2. Patients with confirmed VL-HIV will be given a course of combination treatment for VL and initiated on ART, where not already on ART, as per standard practice. Further sampling will then be conducted as per the schedule detailed in Table 1, day 0 being day of diagnosis prior to treatment starting. The standard course of treatment for VL in HIV ends on day 14.

A blood sample will be taken for CD4 count, full blood count, qPCR and peripheral blood buffy coat smear microscopy as per the schedule of events (Tables 1 & 2). Patients on the ward undergo routine sampling for tests, where possible sampling will be matched with routine sampling to avoid repeated venepuncture. A urine sample will be taken for urine antigen ELISA. The skin microbiopsy device takes minimally invasive and virtually painless skin samples, and samples will be taken from the nape of the neck as well as on the lower arm for qPCR. The same sample taken for standard microscopic diagnosis of the splenic aspirate will be used for qPCR.

To measure parasitic load by skin qPCR, DNA will be isolated from the microbiopsy device and kinetoplast DNA will be looked for. This will provide a semi-quantitative result in relation to a standard curve of known concentration of cultured parasites.

Formal test of cure (ToC) will be carried out by parasitological confirmation and clinical examination at day 29 as per standard practise. Information regarding treatment failure will be noted.

Table 1. Schedule of sampling.

Day	0	3	8	15	29
Urine	X	X	X	X	X
Blood[1]	X	X	X	X	X
Skin Microbiopsy	X	X	X	X	X
Spleen[1]	X				X*
Bone marrow[1,2]	X				X

[1] Routine samples, all other samples will be matched to routine sample where possible

*In the unlikely event a splenic aspiration is contraindicated on day 29, bone marrow aspirate test of cure will be conducted as per standard practice

[2]In patients who are contraindicated for splenic aspiration a bone marrow aspirate will be taken for diagnosis and test of cure as per standard practice. These patients will be recruited to a pilot study.

Table 2. Schedule of tests.

Day	0	3	8	15	29
Clinical examination	X[1]			X	X[1]
RK39 RDT (blood)[1]	X				
Full blood count[1]	X				X
CD4 count (blood)[1]	X				X
GeneXpert for TB[1]	X				
Chest X-ray for TB[1]	X				
Urine antigen ELISA	X	X	X	X	X
qPCR on blood and skin microbiopsy	X	X	X	X	X
Peripheral blood buffy coat smear	X	X	X	X	X
Spleen parasitological confirmation[1]	X				X*
Bone marrow parasitological confirmation[1,2]	X				X

[1] Routine tests

[2]In patients who are contraindicated for splenic aspiration a bone marrow aspirate will be taken for diagnosis and test of cure as per standard practice. These patients will be recruited to a pilot study.

*In the unlikely event a splenic aspiration is contraindicated on day 29, bone marrow aspirate test of cure will be conducted as per standard practice

Laboratory testing and sample storage

Testing procedures

All samples will be stored at -80◦C until the study ends. This will allow samples to be tested in batch to reduce costs. Testing in batch will also allow blinding of laboratory staff to results of previous time points.

Data analysis and statistical methods

91 consecutive patients meeting the inclusion criteria will be screened for *L. donovani* infection by qPCR (blood and skin), peripheral blood buffy coat smear microscopy, and urine antigen ELISA at baseline and at times previously shown in the schedule of sampling (Table 1) and schedule of tests (Table 2).

Baseline:

Results of the qPCR, peripheral blood buffy coat smear microscopy, and urine antigen ELISA at baseline will be compared to the gold standard diagnosis (parasitological confirmation).

Sensitivity and specificity with 95% confidence intervals will be calculated as follows:

$$\boldsymbol{Sensitivity} = \frac{\boldsymbol{A}}{(\boldsymbol{A}+\boldsymbol{C})} \times \mathbf{100}$$

$$\boldsymbol{Specificity} = \frac{\boldsymbol{D}}{(\boldsymbol{D}+\boldsymbol{B})} \times \mathbf{100}$$

Where the above values are shown in Table 3.

Table 3. Contingency table to calculate sensitivity and specificity.

	Disease	No Disease	
Positive result	A. True positive	B. False positive	Test positive
Negative result	C. False negative	D. True negative	Test negative
	Total disease	Total no disease	Total

A Kappa coefficient will be used to determine the level of agreement between the evaluation tests and the gold standard.

The continuous variables of the baseline and demographic characteristics will be summarized using number of patients (*n*), mean, SD, median, minimum, maximum, and confidence intervals. The categorical variable gender will be summarized using number of patients (*n*) and percentage (%).

Test of cure:

Patients who test positive for the gold standard at baseline will continue to be screened for infection by qPCR (blood and skin), peripheral blood buffy coat smear microscopy, and urine antigen ELISA at times previously shown in the schedule of sampling (Table 1) and schedule of tests (Table 2).

Results of the qPCR (blood and skin), peripheral blood buffy coat smear microscopy, and urine antigen ELISA at day 15 and day 29 will be compared to the gold standard test of cure (parasitological confirmation) at day 29. Sensitivity and specificity with 95% confidence intervals will be calculated as above.

The continuous variables will be summarized using number of patients (*n*), mean, SD, median, minimum, maximum, and confidence intervals.

Further analysis

Where possible, analysis will also be stratified by markers of disease severity and other sub-groups such as HIV viral load, CD4+ T cell counts, and the use of HAART.

Risk/benefit assessment

Potential risks related to this study are minimal. Invasive procedures such as splenic aspirate and bone marrow aspirate will only be done as per routine clinical workup; no additional aspirates will be done for the purpose of this study. Urine sampling does not pose any physical risks. Risks during blood or skin sampling are minimal when adhering to standard hygienic rules and include vasovagal reaction, bleeding or infection. Some discomfort may be felt during the skin microbiopsy; however, the device has been designed to be virtually painless. Risk of breach in confidentiality will be minimized by using unique personal codes on the case report forms, with the subject's enrolment list linking unique personal codes to the names of the participants in a locked and secured office.

The potential benefit of this study mainly lies in the possibility to identify a diagnostic tool and/or test of cure that makes the current invasive splenic or bone marrow aspirates no longer necessary in the workup of HIV-VL co-infection. This would limit the risks associated to these tests (fatal hemorrhage in case of splenic aspirates of 1/1.000 and painful procedure in case of bone marrow aspirate) and significantly increase the comfort of these patients during their treatment process. Additionally, due to the complexities of these invasive tests, they are only available in specialist centres. If a test of cure at day 15 is equally accurate as the current (parasitological) test of cure at day 29, this would decrease the duration of hospitalization by half for many patients and eliminate the need to return to the hospital after being released for others, improving access to care for this vulnerable group of patients.

Patient and Public Involvement

There was no patient or public involvement in the development of research questions and the study design.

Discussion

Considering the discomfort, iatrogenic risk and level of technical skill involved in parasitological confirmation, development of tests which can diagnose current infection and determine test of cure that are safer, better tolerated and less technically demanding are required. HIV patients diagnosed with VL in India have recently been shown to have better treatment outcomes with a combination therapy of liposomal amphotericin B (LAMB) and miltefosine over 14 days (CTRI/2015/05/005807) [10]. The choice of day 29 for Test of Cure (ToC) is based on older treatment regimens which were traditionally a month long. As such, evidence for the diagnostic accuracy of a test of cure at the end of a shorter treatment (e.g. day 15 in this case) may allow patients to be discharged without the need to return on day 29 for ToC; however it is also possible that the extended time to the day 29 ToC is requires to counter 'slow response' in the viscera to treatment.

The sensitivity of the rK39 RDT is well established in immunocompetent patients with sensitivities identified by systematic review of approximately 97% on the Indian subcontinent and 85% in east Africa [15,16].The rK39 RDT was found to have a sensitivity of 77% in HIV co-infected individuals compared to 87% in HIV negative patients with VL in Ethiopia [17]. As these antibodies remain present even after successful treatment, they cannot be used either as a diagnostic tool in suspected relapse nor as a test of cure following treatment and therefore need to be used in combination with a clinical history or parasitological proof to distinguish between past or current infection [11].

QPCR is considered a proxy for parasite load in an individual but is currently restricted to use for research purposes. The potential for qPCR on blood for use in monitoring of treatment effect has been demonstrated in a cohort in Bangladesh [18]. However, measuring parasite load using qPCR on blood samples might not be the best proxy for measuring parasite load in an individual. A first study in Ethiopia suggested that qPCR in skin was more sensitive in detecting parasite DNA than qPCR in blood. However, more data are needed to validate this skin microbiopsy device [19].

Once complete, the results of this study have the potential to inform alternative minimally- and non-invasive tools for diagnosis and test of cure in VL patients co-infected with HIV. This would allow clinicians to move away from tissue aspirations, methods which carry a risk of discomfort to the patient, and a risk of fatal haemorrhage in the case of splenic aspiration. These interventions may also allow diagnosis within less specialised healthcare facilities.

Ethics and dissemination

This study has been approved by the ethics boards of The Liverpool School of Tropical Medicine, The Institute of Tropical Medicine in Antwerp, the University of Antwerp, and the Rajendra Memorial Research Institute of Medical Science in Patna. The results of the study of this study will be published in an open source, peer reviewed journal. Results will also be presented to policy makers at national and international level. In particular, the WHO GRC who are due to provide global updated management guidelines for VL-HIV co-infection in 2021. Reporting of results will follow STARD guidelines. Data will be made available upon request.

References

1 Cameron MM, Acosta-Serrano A, Bern C, *et al.* Understanding the transmission dynamics of Leishmania donovani to provide robust evidence for interventions to eliminate visceral leishmaniasis in Bihar, India. *Parasit Vectors* 2016;**9**:25. doi:10.1186/s13071-016-1309-8

2 Diro E, Ritmeijer K, Boelaert M, *et al.* Long-term Clinical Outcomes in Visceral Leishmaniasis/Human Immunodeficiency Virus-Coinfected Patients During and After Pentamidine Secondary Prophylaxis in Ethiopia: A Single-Arm Clinical Trial. *Clin Infect Dis* 2018;**66**:444–51. doi:10.1093/cid/cix807

3 Le Rutte EA, Coffeng LE, Bontje DM, *et al.* Feasibility of eliminating visceral leishmaniasis from the Indian subcontinent: explorations with a set of deterministic age-structured transmission models. *Parasit Vectors* 2016;**9**:24. doi:10.1186/s13071-016-1292-0

4 National AIDS Control Organisation, Ministry of Health and Family Welfare G of I. National Strategic Plan for HIV/AIDS and STI 2017 - 2024. 2017. http://naco.gov.in/sites/default/files/Paving the Way for an AIDS 15122017.pdf (accessed 11 Apr 2018).

5 Jarvis JN, Lockwood DN. Clinical aspects of visceral leishmaniasis in HIV infection. *Curr Opin Infect Dis* 2013;**26**:1–9. doi:10.1097/QCO.0b013e32835c2198

6 National AIDS Control Organization & ICMR-National Institute of Medical Statistics. India HIV Estimations 2017: Technical Report. 2018. http://naco.gov.in/sites/default/files/HIV Estimations 2017 Report_1.pdf (accessed 27 Nov 2018).

7 Burza S, Mahajan R, Gonzalez Sanz M, *et al.* HIV and Visceral Leishmaniasis Coinfection in Bihar, India: An Underrecognized and Underdiagnosed Threat Against Elimination. *Clin Infect Dis* 2014;**59**:552–5. doi:10.1093/cid/ciu333

8 Akuffo H, Costa C, van Griensven J, *et al.* New insights into leishmaniasis in the immunosuppressed. *PLoS Negl Trop Dis* 2018;**12**:e0006375. doi:10.1371/journal.pntd.0006375

9 Vallur AC, Tutterrow YL, Mohamath R, *et al.* Development and comparative evaluation of two antigen detection tests for Visceral Leishmaniasis. *BMC Infect Dis* 2015;**15**:1–10. doi:10.1186/s12879-015-1125-3

10 Burza S, Croft SL, Boelaert M. Leishmaniasis. Published Online First: 2018. doi:10.1016/S0140-6736(18)31204-2

11 Srivastava P, Dayama A, Mehrotra S, *et al.* Diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 2011;**105**:1–6. doi:10.1016/j.trstmh.2010.09.006

12 Adams ER, Schoone G, Versteeg I, *et al.* Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Assay for Diagnosis of Cutaneous and Visceral Leishmaniasis. *J Clin Microbiol* 2018;**56**:e00386-18. doi:10.1128/JCM.00386-18

13 Courtenay O, Carson C*, Calvo-Bado L, *et al.* Heterogeneities in Leishmania infantum Infection: Using Skin Parasite Burdens to Identify Highly Infectious Dogs. *PLoS Negl Trop Dis* 2014;**8**:e2583. doi:10.1371/journal.pntd.0002583

14 Aslan H, Oliveira F, Meneses C, *et al.* New Insights Into the Transmissibility of *Leishmania infantum* From Dogs to Sand Flies: Experimental Vector-Transmission Reveals Persistent Parasite Depots at Bite Sites. *J Infect Dis* 2016;**213**:1752–61. doi:10.1093/infdis/jiw022

15 World Health Organization. Visceral Leishmaniasis Rapid Diagnostic Test Performance WHO Diagnostic Evaluation Series. 2011. http://www.who.int/tdr/publications/documents/vl-rdt-evaluation.pdf.%5Cnpapers2://publication/uuid/285CD644-B096-4FED-AB60-1D3330E6D024

16 Boelaert M, Verdonck K, Menten J, *et al.* Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. *Cochrane Database Syst Rev* Published Online First: 20 June 2014. doi:10.1002/14651858.CD009135.pub2

17 Horst R ter, Tefera T, Assefa G, *et al.* Field evaluation of rK39 test and direct agglutination test for diagnosis of visceral leishmaniasis in a population with high prevalence of human immunodeficiency virus in Ethiopia. *Am J Trop Med Hyg* 2009;**80**:929–34. doi:80/6/929 [pii]

18 Hossain F, Ghosh P, Khan MAA, *et al.* Real-time PCR in detection and quantitation of Leishmania donovani for the diagnosis of Visceral Leishmaniasis patients and the monitoring of their response to treatment. *PLoS One* 2017;**12**:e0185606.

doi:10.1371/journal.pone.0185606

19 Kirstein OD, Abbasi I, Horwitz BZ, *et al.* Minimally invasive microbiopsies: a novel sampling method for identifying asymptomatic, potentially infectious carriers of Leishmania donovani. *Int J Parasitol* 2017;**47**:609–16. doi:10.1016/j.ijpara.2017.02.005

Author Statement

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Conflicts of Interests

The authors of this protocol declare no competing interests.

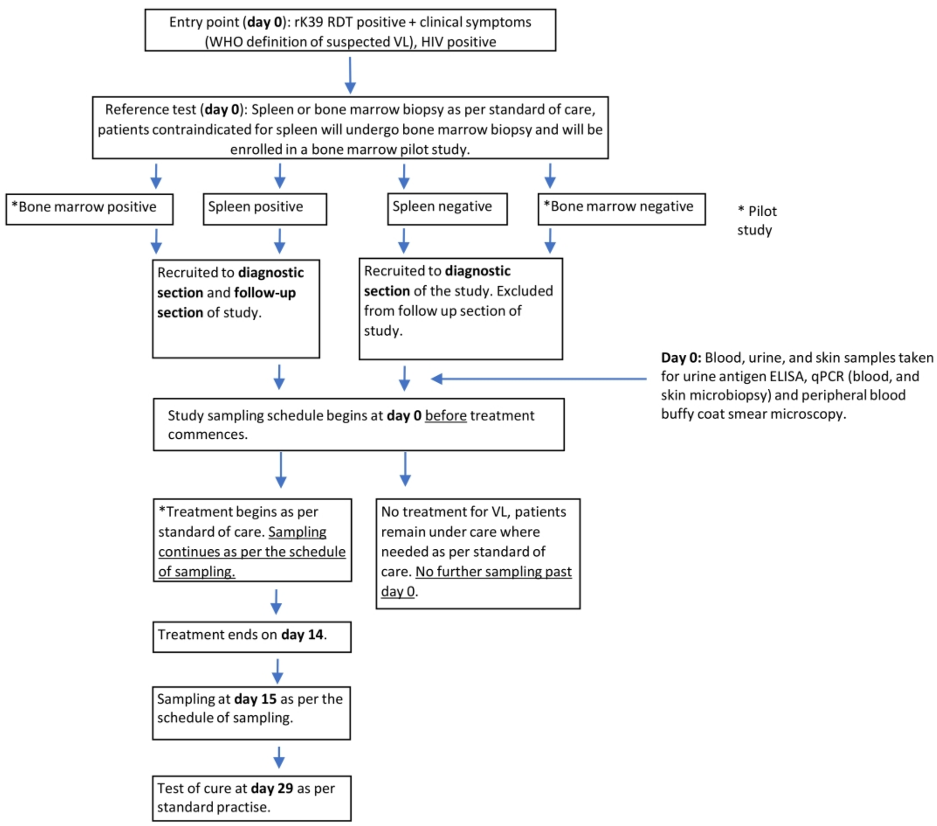

Figure 1. Study workflow.

179x157mm (500 x 500 DPI)