Development and evaluation of diagnostics for visceral leishmaniasis focussing on people living with HIV and asymptomatic infections.

'Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor of Philosophy by Sophie I. Owen.'

30th March 2022

Development and evaluation of diagnostics for visceral leishmaniasis focussing on people living with HIV and asymptomatic infections.

Dedication

For my dad and Dan, and in memory of my mum, Mike, and nanny.

Acknowledgements

Firstly, I would like to thank my supervisor Dr Emily Adams. I am forever grateful for your mentorship and friendship.

To the Research Diagnostics group at LSTM - Rachel Byrne, Dr Ana Cubas Atienzar, Prof. Luis Cuevas, Dr Tom Edwards, Dr Louise Ford, Dr Gala Garrod, Grant Kay, Nadia Kontogianni, Kelly Robinson, Dr Vera Unwin, Chris Williams, and members of the group past and present – you have made the last four years the wonderful time that it was, thank you.

Thank you to Dr Sakib Burza and the rest of the team at MSF. I'm incredibly grateful to have worked with you all. The work you do is inspirational.

To the team at the icddr,b in Bangladesh - Dr Dinesh Mondal, Faria Hossain, Dr Prakash Ghosh, Rajashree Chowdhury, Dr Utba Rashid, and the rest of the team – it's been a pleasure working with you.

Dr Albert Picado and Prof. Isra Cruz, your dedication to the study of Leishmaniasis is inspirational, it's been a privilege to learn from you.

To all those I have worked with and met in Patna - Dr Shiril Kumar, Dr Shahwar Kazmi, Sana Sangeeta, and Paramita Sarkar - thank you for making the time I spent in India so enjoyable and memorable.

Dr Chris Jewell, your support and dedication to teaching is greatly appreciated.

Prof. Richard Pleass and Dr Tim O'Dempsey, thank you for your time and support as members of my progress assessment panel.

To the education team at LSTM – Richard Madden, Charlotte Blakeburn, Mary Creegan, and team - thank you for your hard work and patience.

To the friends I've met because of my PhD, thank you for making my time in Liverpool so special.

Finally, a huge thank you to my family and friends, particularly Dan and my dad, for their endless encouragement and support.

Abstract

Development and evaluation of diagnostics for visceral leishmaniasis focussing on people living with HIV and asymptomatic infections.

Sophie I. Owen

The Indian subcontinent (ISC) was targeting visceral leishmaniasis (VL) for elimination by 2020, with Bihar, an endemic state in India a major focus of the campaign. Current diagnostics for VL such as the direct agglutination test (DAT) and rK39 tests detect antibodies, making it difficult to distinguish between past or current infection, particularly in the absence of clinical symptoms or typical VL. Asymptomatic *Leishmania* infections (ALI) outnumber symptomatic infections on the ISC and people living with human immunodeficiency virus (PLHIV) have a higher risk of developing symptoms of VL with high treatment failure, relapse, and mortality, presenting a major challenge for both clinical management and elimination. PLHIV and asymptomatic individuals are of importance to VL elimination, particularly where the reservoir host is thought to be anthroponotic. Diagnostics that enable the detection of ALI, diagnosis of acute VL and *Leishmania*-HIV co-infections, monitoring VL treatment, and surveillance are needed. The *Leishmania* antigen enzyme-linked immunosorbent assay (ELISA) (Clin-Tech, UK) measures antigenuria, therefore identifying antigens excreted in urine during current infections, potentially allowing monitoring of VL treatment response, and predicting treatment failure and relapse in PLHIV. Testing urine is non-invasive and has the potential to replace invasive tissue aspiration. Real-time polymerase chain reaction (qPCR) is a highly sensitive technique to detect *Leishmania donovani* (*L. donovani*) kinetoplast DNA (kDNA). Similarly, loop-mediated isothermal amplification (LAMP) is a technique used to amplify DNA suitable for resource-poor settings. However, the clinical utility of the *Leishmania* antigen ELISA and molecular techniques in asymptomatic and symptomatic *L. donovani* infections with and without HIV has not been fully established in an elimination setting. In this thesis, I describe the prevalence and determinants of ALI, and the utility and diagnostic accuracy of the *Leishmania* antigen ELISA, DAT, LAMP, and qPCR for the detection of ALI in a cohort of 720 contacts of people with VL and PKDL in Bangladesh (chapter 2). Further, I describe the prevalence and determinants of ALI, and examine the utility of the *Leishmania* antigen ELISA, qPCR, rK39 rapid diagnostic test (RDT), and rK39 ELISA for the detection of ALI in a cohort of 1,300 PLHIV in India (chapter 3). The latter cohort were followed for 18 months to evaluate the rate and risk factors for disease progression from ALI to VL in PLHIV and to determine the utility of the *Leishmania* antigen ELISA, qPCR, rK39 RDT, and rK39 ELISA as markers of progression (chapter 4). I then describe the potential use of these assays as diagnostic and test of cure assays for VL in a cohort of HIV co-infected patients in India (chapter 5). In a final chapter, I screened a panel of thirteen monoclonal antibodies for the development of an alternative antigen detection test and evaluated other available antigen detection assays (chapter 6).

Acronyms

AIDS - acquired immunodeficiency syndrome

ALI - asymptomatic *Leishmania* infection

ART - antiretroviral therapy

ATT - anti-tubercular treatment

C_t - threshold cycle

DAT - direct agglutination test

DDT – dichlorodiphenyltrichloroethane

ELISA - enzyme-linked immunosorbent assay

FIND - Foundation for Innovative New Diagnostics

GGSH - Guru Gobind Singh Hospital

GI – gastrointestinal

HAART - highly active antiretroviral therapy

Hb - Haemoglobin

HIV – human immunodeficiency virus

HRP - horse radish peroxidase

iccdr,b - International Centre for Diarrhoeal Disease Research, Bangladesh

ICMR - Indian Council of Medical Research

ICT - immunochromatographic test

IFAT - indirect fluorescence antibody test

Ig – immunoglobulin

IRS - indoor residual spraying

ISC – Indian subcontinent – referring to India, Nepal, and Bangladesh

L. donovani – *Leishmania donovani*

L. infantum – *Leishmania infantum*

LAMB - liposomal amphotericin B

LAMP - loop-mediated isothermal amplification

LD bodies - *L. donovani* bodies

LFA – lateral flow assay

LST - Leishmanin skin test

LSTM - Liverpool School of Tropical Medicine

mAbs - monoclonal antibodies

MoU - memorandum of understanding

MSF - Médecins Sans Frontières

NAAT - nucleic acid amplification technique

NTD - neglected tropical disease

OIs - opportunistic infections

P. argentipes - *Phlebotomus argentipes*

PBMCs - peripheral blood mononuclear cells

PCR - polymerase chain reaction

PKDL - Post kala-azar dermal leishmaniasis

PLHIV - people living with human immunodeficiency virus

qPCR - quantitative PCR

RDTs - rapid diagnostic tests

RMRIMS - Rajendra Memorial Research Institute of Medical Sciences

SLA - soluble lysate antigen

SSG - sodium stibogluconate

TB – tuberculosis

TMB - 3,3',5,5'-Tetramethylbenzidine

VL – visceral leishmaniasis – disease caused by parasites of the species *Leishmania*, also known in Hindi as kala-azar

VL-HIV – VL and HIV coinfection

WCL - whole cell lysate

WHO – World Health Organization

WHO-TDR - WHO's Special Programme for Research and Training in Tropical Diseases

Contents

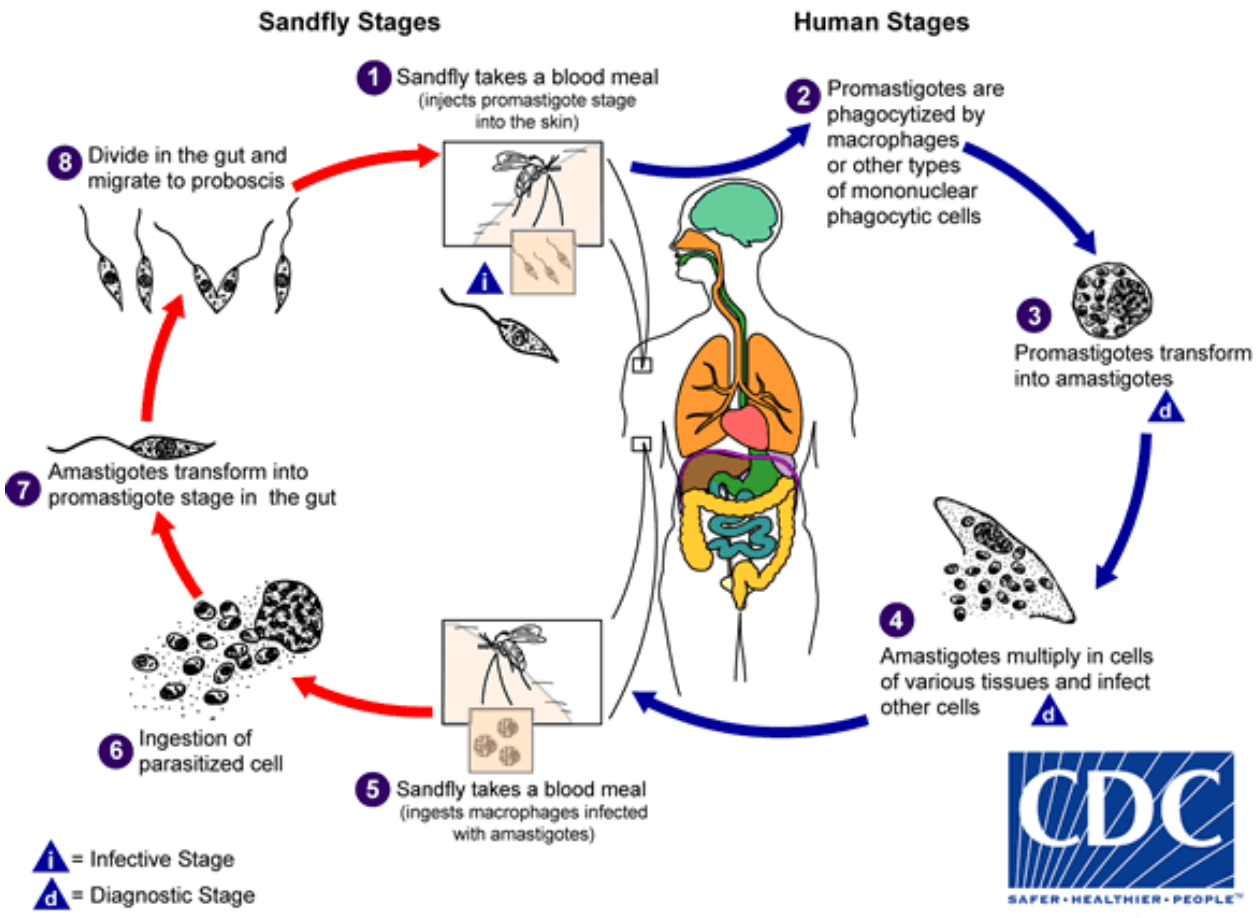
Chapter 1. General Introduction

1.1 Visceral leishmaniasis (VL)

1.1.1 Aetiology

Visceral leishmaniasis (VL) is a neglected tropical disease (NTD) [1]. Parasites of the *Leishmania* genus are transmitted by the bite of an infected female blood feeding sand fly [2]. Infection with *Leishmania* parasites can lead to a range of clinical manifestations, VL being the most severe form [1]. Clinical manifestation is dependent on a complex combination of factors including the parasite species, of which there are approximately 20 transmittable to humans, and the host immune response [3]. *Leishmania donovani* (*L. donovani*) is the causative agent in Asia and Africa. In South America, parts of Asia, the Mediterranean, and the Middle East, VL is caused by *Leishmania infantum* (*L. infantum*) [3,4].

Briefly, following the bite of an infected sand fly, parasites in the promastigote stage are taken up by host mononuclear phagocytes such as macrophages and neutrophils, where they mature to the amastigote stage and replicate [5,6]. Infected host mononuclear phagocytes move to the spleen, lymph nodes and bone marrow where amastigotes further disseminate and replicate [5,6]. Subsequently when a sand fly takes a blood meal of an infected host, the fly takes up amastigotes which transform to the promastigote stage, and the cycle continues [6]. Absence of a sufficient immune response can lead to clinical disease [3]. The lifecycle of *Leishmania* is shown in Figure 1.



306

307 **Figure 1. The lifecycle of *Leishmania* infection [7].**

308

309 *1.1.2 Epidemiology*

310 Of the approximately 200 countries that reported data on VL to the World Health Organization (WHO) in
311 2020, 79 were endemic for VL [8]. There are estimated to be between 50,000 to 90,000 new VL cases
312 each year, and its broad global distribution is shown in Figure 2 [9,10]. VL is endemic to the Indian
313 subcontinent (ISC), East Africa, and Brazil with India, Sudan, South Sudan, Kenya, China, Yemen, Eritrea,
314 Ethiopia, Somalia, and Brazil making up over 90% of new reported cases in 2020 [9]. Reservoirs of
315 *Leishmania* parasites can be both anthroponotic and zoonotic, dependent on parasite species and
316 geographical location [3].

Overall, the incidence of VL has been declining worldwide [3]. Outbreaks in East Africa are often linked to the mass migration of non-immune individuals to endemic regions due to conflict or seasonal work [11,12]. On the ISC, regional outbreaks occur approximately every 15 years [13]. Local outbreaks have been linked to the increasing rates of coinfection of VL and people living with human immunodeficiency virus (PLHIV), referred to as VL-HIV from this point, except for the Mediterranean where the incidence is now decreasing due to high coverage of highly active antiretroviral therapy (HAART) [14,15].

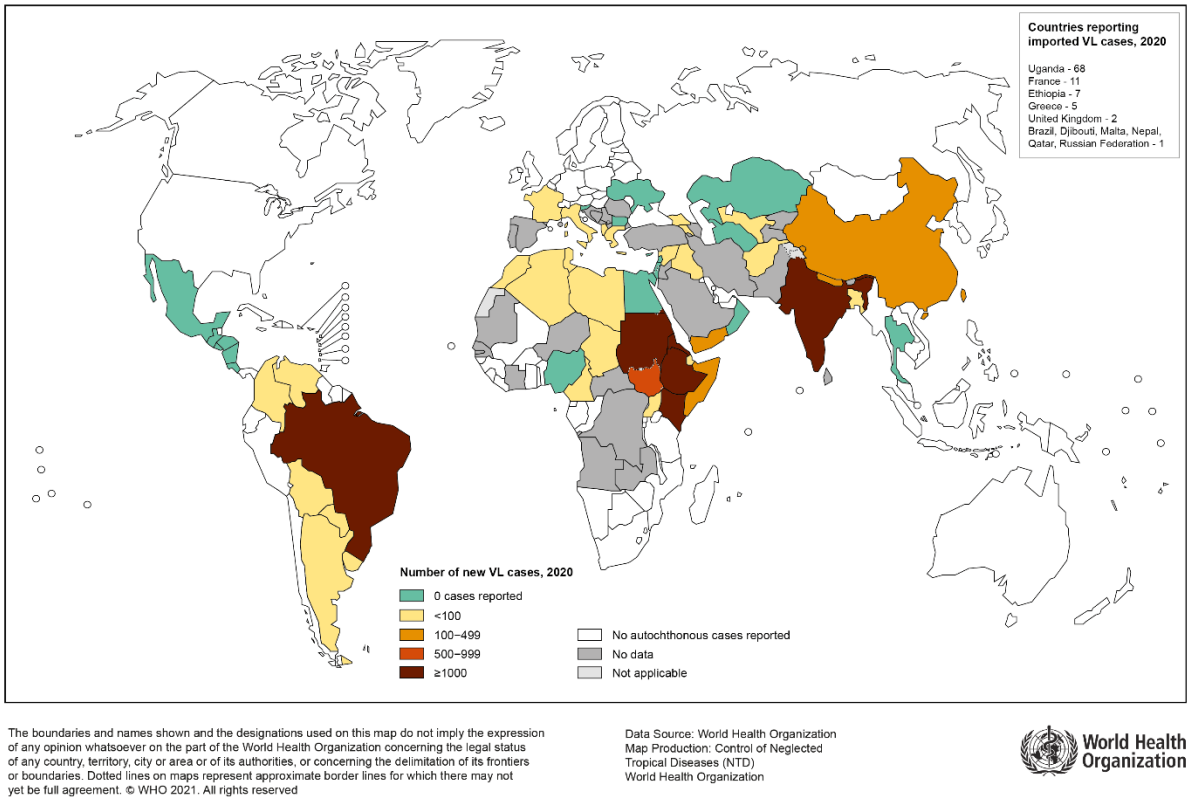


Figure 2. WHO data on the status of endemicity of VL worldwide in 2020 [9].

1.1.3 Clinical presentation

In the absence of VL treatment, secondary infection and anaemia can lead to death within two years, making accurate diagnosis essential [3]. The incubation time for VL is reported to be between two weeks and eight months with both slow and rapid onset of symptoms observed [3]. VL is generally well-defined clinically, and some symptoms are non-specific and overlap with other endemic diseases, such as

malaria, schistosomiasis, and typhoid fever, making diagnosis difficult in areas of co-endemicity [16]. Further misdiagnoses include non-communicable autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [17].

People with VL can present with a combination of the symptoms described in the following paragraphs. A common symptom includes a prolonged and irregular fever of more than two weeks [3]. Further common symptoms include cachexia and lethargy due to an increased metabolism and a decrease in food intake and malabsorption, which is often complicated by presence of concurrent infections such as tuberculosis (TB) and human immunodeficiency virus (HIV) [18]. Splenomegaly is commonly reported and less frequently hepatomegaly, both of which can lead to a painful and distended abdomen [18]. Patients may present with weakness and fatigue due to anaemia, leukopenia, thrombocytopenia, and hypergammaglobulinemia [5]. Patients can also present with atypical symptoms, further complicating diagnosis, as these may be the result of underlying complication such as HIV coinfection, other immunosuppression, or concurrent infections [17,19].

A less common symptom includes cough caused by respiratory infection, interstitial pneumonia or other concurrent infection [18]. In less frequent cases, intestinal inflammation and concurrent infection may occur leading to diarrhoea [18,20]. Jaundice is sometimes seen, and has been associated with a higher risk of VL-related deaths in Brazil and East Africa [21,22]. Rarely, neurological symptoms can be seen, including: a burning sensation in the feet; foot drop; and hearing loss [23]. The hyperpigmentation which gives VL it's alternative name, black disease or kala-azar in Hindi, is only seen in certain regions of India [3]. Other skin manifestations can include cutaneous lesions [24]. Further atypical presentations include ascites, and eye inflammation associated with immunosuppressants given following an organ transplantation [3,22,25].

Following cure, VL patients can remain seropositive for several years after treatment, presenting a challenge for accurate diagnosis and a need for diagnostic algorithms which include a clinical history and a VL specific clinical examination. A study of 42 patients with imported leishmaniasis found that antibody titres declined after treatment, however it took between 51 weeks and six years for patients to become seronegative [26].

1.2 VL elimination on the Indian subcontinent (ISC)

VL is currently the target of an elimination campaign on the ISC, with the governments of India, Nepal, and Bangladesh signing a memorandum of understanding (MoU) in 2005 [27–29]. The campaign is split into several phases. Firstly, the attack phase aims to bring incidence down to <1 case per 10,000 people per year at district level in Nepal, subdistrict or block level in India, and upazila level in Bangladesh [29,30]. Once the targets for elimination are reached in the attack phase, the campaign will enter a consolidation phase of three years of active surveillance, during which time there can be no increase in incidence at the geographical levels described previously [30]. The consolidation phase is followed by a maintenance phase, where surveillance efforts continue and the incidence remains <1 case per 10,000 people at the geographical levels described previously [30]. During these phases diagnostic tests will play a key role [31].

Targets were set for removal of VL as a public health concern by 2015 [29]. However, many areas failed to meet the elimination goal, and 2017 and 2020 were set by the WHO as subsequent deadlines to meet elimination targets in remaining areas [29,32,33]. The 2020 deadline was not met in certain areas of India, but validation is needed to confirm status in Nepal and Bangladesh [33]. More recently, the 'road map for neglected tropical diseases 2021–2030' was published by the WHO, which highlights the need for user-friendly, less invasive diagnostic tests with high diagnostic accuracy to aid early diagnosis and test of cure, among other priorities [33].

Transmission on the ISC occurs when the human host encounters the sand fly vector, *Phlebotomus argentipes (P. argentipes).* Those at highest risk of disease are the poorest members of society in rural areas [34]. A study of 2,013 households in the highly VL endemic district of Muzaffarpur, in Bihar, India, found that 83.3% of households were in the lowest two quintiles of wealth distribution and that the main risk factors for transmission included poor sanitation and housing [35]. The major foci of the elimination programme on the ISC include vector control, diagnosis, and treatment. Models predicted that in the absence of other interventions, sub-optimal indoor residual spraying (IRS) would not achieve the elimination targets in highly endemic areas [36]. Potential challenges for elimination include asymptomatic infections and PLHIV who have a 100-2,300 times greater risk of contracting VL than immunocompetent individuals in endemic areas [15].

1.2.1 The elimination campaign in Nepal

The number of cases in Nepal decreased between 2003 and 2017 to near elimination [37]. Seroprevalence, a proxy for transmission of *L. donovani*, decreased in approximately 6,000 individuals >2 years of age undergoing repeat screening in 2016 compared to 10 years earlier [38]. In 2014, the hilly Okhaldhunga and Bhojpur districts reported transmission, regions not previously thought to be areas of transmission compared to the low altitude setting of the Ganga plains [39]. Similarly, a district thought to be non-endemic reported an increase in VL above the elimination target in 2017 [40]. As of 2020, 100% of districts reported reaching the elimination threshold of <1 case per 10,000 of the population, with validation still needed [33].

1.2.2 The elimination campaign in India

India reported an overall decline in cases and mortality rates, from 80,000 cases in 1992 to under 9,000 cases recorded in 2015 [32]. The states of Bihar, Uttar Pradesh, Jharkhand, and West Bengal carry the highest burden of VL in India (Figure 3) [28,41]. Approximately 90% of India's cases occur in Bihar state, which remained endemic in 33 (87%) of its 38 districts in 2017 [28,32]. The WHO's NTD roadmap for 2021-2030 reported 8% of subdistricts in India remained above the elimination threshold in 2020 [33]. Bihar is the focus of all work conducted in India for this thesis. Further details of study sites are given in section 1.15.

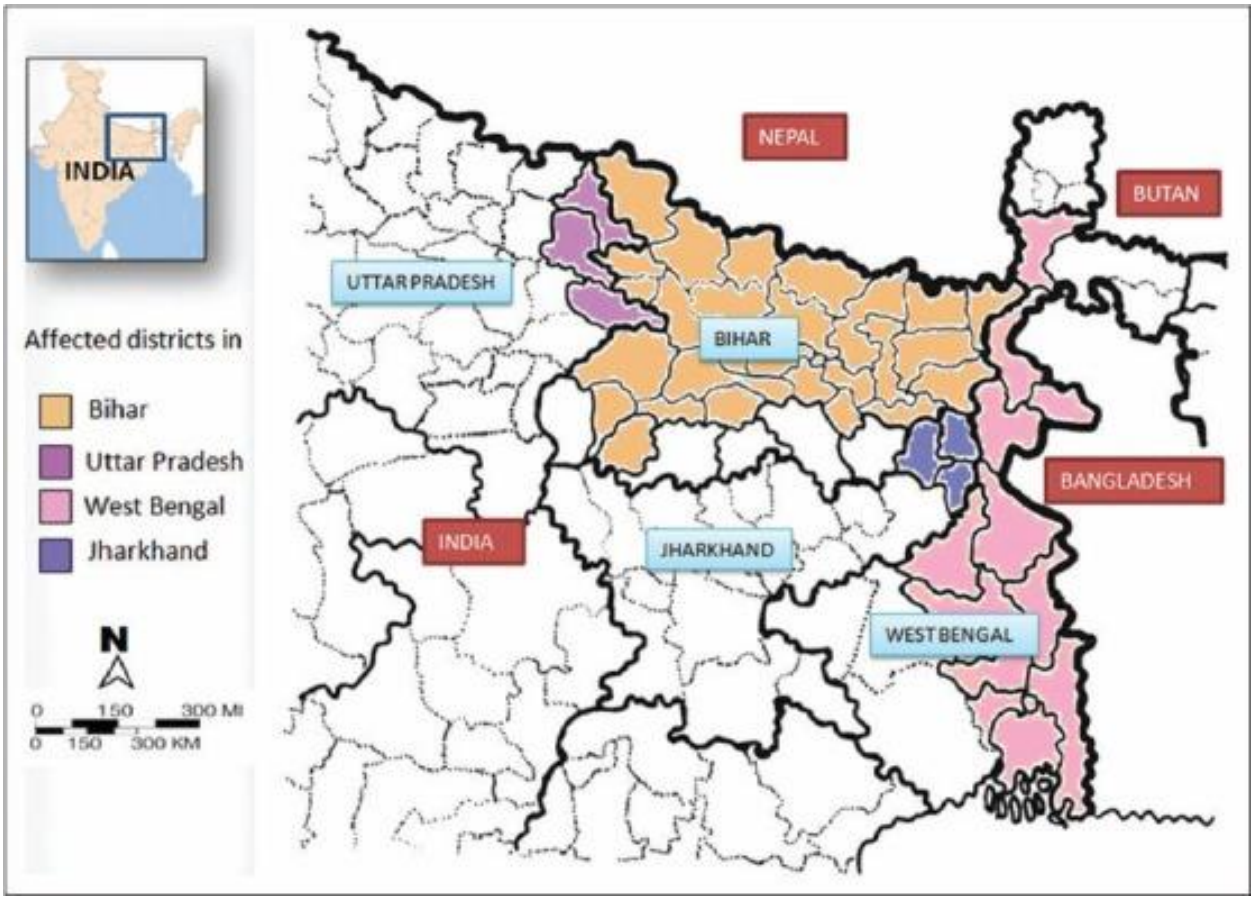


Figure 3. Districts reporting VL in Bihar, Uttar Pradesh, Jharkhand, and West Bengal [13].

1.2.3 The elimination campaign in Bangladesh

An epidemic during the 1800s in Jessore was estimated to kill around 75,000 people [42]. Similarly to India and Nepal, Bangladesh has seen a decline in cases since the 1950s [42]. This decline has been linked to the spraying of dichlorodiphenyltrichloroethane (DDT) as part of the malaria control programmes [43], but reversed once the spraying programme was stopped in approximately the 1970s [44]. Between 2006 and 2013, Mymensingh district had the highest number of cases with hardly any difference seen in the years pre- and post-signing of the 2005 MoU for elimination [42]. In 2017, Bangladesh reached the target of <1 case per 10,000 people at upazila level, having previously had 21% of its upazilas endemic for the disease [31]. As with Nepal, 100% of upazilas in Bangladesh reported reaching the elimination threshold, with validation still needed [33].

1.2.4 Post kala-azar dermal leishmaniasis (PKDL)

Post kala-azar dermal leishmaniasis (PKDL) occurs in 10-20% of cases approximately 2-3 years following treatment for *L. donovani*, and is of importance to the elimination campaign on the ISC where PKDL may serve as an anthroponotic reservoir of infection [45,46]. The development of PKDL is determined by the immune responses to infection [45]. The condition, which is not life-threatening, manifests as a macular, papular, and/or a nodular dermal rash, with macular rashes being the most common presentation in Asia [45]. Generally, PKDL requires treatment in Asia, as self-healing rashes are not commonly seen [3]. Sand flies feeding on an individual with nodular-ulcerative PKDL acquired parasites and were suspected to be the source of an outbreak in West Bengal, along with three cases in other locations [47]. People with PKDL are recognised as a potential reservoir of VL infection in the WHO's NTD roadmap for 2021-2030 [33].

1.3 Lessons learnt from surveillance of other diseases in elimination settings

1.3.1 The campaign to eliminate Leprosy

Lessons may be learnt from other target driven infectious disease elimination campaigns, such as the campaign to eliminate leprosy as a public health concern. Repeated leprosy elimination campaigns were carried out in endemic countries, with the aim to reduce prevalence, much like the elimination campaign for VL on the ISC [48,49]. Effective diagnosis and treatment of incident cases are also key interventions within these campaigns. Within India, individuals with symptoms of leprosy were sensitised via door to door visits and media campaigns, and encouraged to seek diagnosis and treatment at designated medical centres [48]. Case detection increased during the campaigns compared to previous years, and where there were subsequent campaigns, a reduction in annual case detection was seen possibly due to the campaigns removing the back log of cases [48].

The repeated leprosy campaigns, and the outcomes measured as a proxy for the interruption of transmission, were met with some criticism [49,50]. For example, it was perceived that campaigns that integrated leprosy care into health-care systems were less stigmatising, and allowed for more effective treatment than campaigns conducting resource-intensive active case-finding approaches [51]. Furthermore, the use of the term 'elimination' may have led some stakeholders to perceive targets to be complete elimination instead of a reduction in prevalence, and prevented the implementation of a post-elimination strategy which included monitoring and surveillance [50,51]. Diagnostics which are

straightforward and can distinguish between past and current infection were suggested to be a requirement for an elimination campaign, among other conditions [50].

1.4 Asymptomatic *Leishmania* infection (ALI)

L. donovani and *L. infantum* infections can be asymptomatic with detectable parasitaemia [5,52,53]. In Southern France, Le Fichoux *et al*. found that 76 of 565 (13.4%) asymptomatic blood donors with no history of VL had antibodies against *L. infantum* [52]. Parasite culture and polymerase chain reaction (PCR) assays detected parasite and/or parasite DNA in 16 of the 76 (21.1%) donors with antibodies, suggesting intermittent and low density *L. infantum* parasitaemia [52]. A study of 656 blood donors in Spain found 16 (2.4%) and 50 (7.6%) donors had anti-*Leishmania* antibodies by enzyme-linked immunosorbent assay (ELISA) or western blot, respectively. In two subsets of patients in which peripheral blood mononuclear cells (PBMCs) or buffy coats were separated from peripheral blood, parasites and parasite DNA were detected in 3 of 67 (4.5%) and 27 of 122 (22.1%) donors respectively [54].

Ratios of asymptomatic to symptomatic infection vary across the globe, with the ratio for *L. donovani* estimated to be 4-10:1 in areas of Bangladesh and India [55,56]. A study in the endemic regions of India and Nepal found 375 of 9,034 (4.2%) seronegative individuals with no current or previous diagnosis of VL seroconverted and 42 became symptomatic over the 12 subsequent months, 41 of whom had seroconverted, with a ratio of 9:1 asymptomatic to clinical cases [57]. The ratio of asymptomatic to symptomatic infection in Brazil can be as high as 19:1 [58], while in East Africa the ratios range between 1:2.4 and 5.6:1 in Sudan and Ethiopia respectively [59,60].

There remains no xenodiagnostic evidence to show that asymptomatic carriers of *Leishmania* are infectious to sand flies [57]. Seropositive individuals have an increased risk of disease progression [61], with 5-10% of asymptomatic individuals progressing to symptomatic disease per year [37].

1.5 Human immunodeficiency virus (HIV)

Infection with HIV leads to a loss of immunity and a susceptibility to comorbidities. HIV selectively infects and replicates within $CD4^+$ leukocytes, including T cells and macrophages, depending on virus tropism [62]. Patients are at increased risk of opportunistic infections (OIs) such as TB and VL. Generally,

the early stages of infection see a sharp peak in HIV detectable in plasma and a decline in CD4+ T cell counts [63–65]. Over time the depletion of $CD4^+$ T cells begins to slow, viral load declines, and the disease enters clinical latency. This latency can be extended by the administration of HAART. In the absence of HAART, HIV leads to acquired immunodeficiency syndrome (AIDS) characterised by a sharp increase in viraemia, further decline in CD4+ T cells, multiple OIs and ultimately death [65].

1.5.1 HIV in India

Despite a low percentage of the population being infected with HIV in India, due to its large population size the overall numbers of PLHIV are high, particularly in at risk groups [66]. Approximately 2,349,000 people were estimated to be PLHIV in 2019, of which 69,220 (95% CI: 37,030-121,500) were incident infections, and 58,960 (95% CI: 33,610-102,160) AIDS-related deaths [66]. In 2019, an estimated 134,490 (95% CI: 83,020-185,610) individuals were PLHIV in Bihar, with a prevalence of 0.18% (95% CI: 0.11-0.24%) [66]. Bihar's prevalence was below the national average which stands at 0.22% (95% CI: 0.17-0.29%) [66]. Overall, the prevalence of HIV and the number of AIDS-related deaths are declining [66]. However, the trend in Bihar was not declining and the state remained one of eight states where AIDS-related deaths had not declined [67].

1.6 VL-HIV coinfection

VL is an OI in HIV [19,68]. Currently, severe atypical disseminated leishmaniasis is classed as an AIDS-defining illness. VL-HIV coinfected patients have higher treatment failure, relapse, and mortality than immunocompetent individuals [69,70]. HIV increases susceptibility to primary *Leishmania* infection or reactivation of asymptomatic infections, with the latter thought to be more common [19]. The two infections influence each other, with VL infection increasing HIV replication and HIV infection creating a favourable immune environment for replication of *Leishmania* parasites [71].

VL patients with HIV coinfection can present with atypical symptoms, including an absence of splenomegaly and fever, making the diagnosis more challenging [17]. Parasitaemia is higher in these patients, potentially making these individuals 'super spreaders' of VL [72,73]. Parasites can also persist after treatment leading to relapse [15]. Prevalence of VL-HIV coinfection is increasing [17] and coinfections have been reported in over 35 countries from the Mediterranean, South America, East

Africa, and the ISC [74]. Men have a higher incidence of coinfection, which may be due to behavioural patterns, with displacement of males of working age from rural to urban or urban to rural areas for work, putting them at risk of VL-HIV [11].

1.6.1 VL-HIV coinfection in the Mediterranean region

Following the near elimination of VL in the Mediterranean region, outbreaks of VL began to occur anew with the advent of the HIV epidemic, with the first VL-HIV case recorded in 1985 [75]. Most cases were reported from Spain, Italy, and France [14]. VL is transmissible by sharing of contaminated needles [76] and the risk of HIV and VL-HIV coinfection was linked to intravenous drug use with 66% and 79% of HIV and VL-HIV patients being intravenous drug users, respectively [14]. Since the introduction of HAART to the region in 1997 and the subsequent high adherence, the HIV epidemic has since been controlled and only sporadic outbreaks of VL now occur in the Mediterranean [15,75,77].

1.6.2 VL-HIV coinfection in Brazil

The number of PLHIV in Brazil continues to rise [78]. A retrospective study of 917 individuals with VL in Sergipe, Brazil between 1999 and 2015 found 41 (4.5%) patients were coinfected with HIV. The median age of coinfected patients was 36 years compared to 14 years for VL mono-infected individuals [79]. Additionally, 78.0% of coinfected patients were male compared to 64.3% in the full cohort [79].

1.6.3 VL-HIV coinfection in East Africa

HIV testing is recommended for all individuals with VL [80]. However, there is a lack of data on the proportion of individuals with VL that are tested for HIV in East Africa [80]. Rates of coinfection were estimated to be approximately 18% in Ethiopia, with some areas in the northwest as high as 40% [81,82]. In the same area, 31.5% of coinfected individuals had poor treatment outcomes compared to 5.6% of VL mono-infected individuals [82]. A study of 141 patients admitted to Felege Hiwot Hospital between 2016 and 2019 in Ethiopia, found no differences in clinical presentation between VL mono-infections and VL-HIV coinfected individuals, apart from lymphadenopathy (p=0.009) in individuals with

VL-HIV [83]. Of the 141 participants, 92.9% (n=131) were male with 87% travelling from non-endemic to endemic areas, and of 109 participants with a known HIV status ten (9.2%) were PLHIV [83].

In a further study of 791 individuals in Tigray, the case fatality rate was four times higher among VL-HIV coinfected individuals (18.5%) [84]. High relapse rates have also been reported from Ethiopia, with 60% relapsing within one year in a study of 356 coinfected patients between 2003 and 2006 [85]. HAART partially protected participants from relapse, but participants with CD4 counts <100 cells/µl had a higher risk of relapse, and mortality [85].

1.6.4 VL-HIV coinfection on the Indian subcontinent

Most of what is known about VL-HIV coinfection comes from studies in the Mediterranean, East Africa, and Brazil. There are limited data on the burden of VL-HIV coinfection from the ISC. A study screening 2,077 VL patients ≥14 years of age between 2011 and 2013 in Bihar found 5.6% of individuals to be coinfected with HIV, with rates of relapse unknown [86]. Although screening for HIV in VL patients is currently recommended, it has been suggested that this procedure needs to be made mandatory [3,32]. Further data are needed to assess the burden of VL-HIV coinfection on the ISC where reservoirs of infection are anthroponotic and coinfected patients could act as reservoirs of infection.

1.6.5 Concurrent infection in VL-HIV

Concurrent OIs can further complicate VL-HIV coinfection. These include TB, which leads to immune suppression, with anecdotal evidence to suggest the rate of TB coinfection to be as high as 20% in VL-HIV patients treated by Médecins Sans Frontières (MSF) in Bihar. In northwest Ethiopia, concurrent TB infection was 27.2% in VL-HIV participants and 6% among those with VL only [82]. Sepsis is more common in VL-HIV patients [82], while the symptoms of malaria overlap with the symptoms of VL, and in areas where both diseases are endemic a diagnosis of malaria needs to be ruled out [15,82].

1.7 Diagnostics for VL

1.7.1 Rapid diagnostic tests (RDTs)

Rapid diagnostic tests (RDTs) are immunoassays which allow for the rapid diagnosis of disease, without requiring laboratory equipment and highly trained laboratory staff. RDTs typically use simple methods and includes samples of interest such as urine or capillary blood, sometimes followed by two to three drops of diluent to an absorbent sample pad. From the sample pad, the sample is released onto the conjugate pad which contains labelled antibodies or antigen e.g., conjugated to gold nanoparticles. If the analyte of interest is present in the sample, it will bind these labelled antibodies or antigen and the conjugate-analyte complex will continue to travel along the membrane. The conjugate-analyte complex then passes over the test line where a capture protein has been immobilised and a control line captures unbound conjugate.

The results of the test can usually be read between ten and 30 minutes. Results are generally easy to interpret. The presence of the control line indicates the test has worked correctly and a test line indicates the test is positive. Where a control line is not positive the test should be considered invalid and should be repeated. RDTs can have a lower sensitivity than an equivalent ELISA, a technique discussed in the following section. RDTs are qualitative rather than quantitative. However, RDTs are often less expensive, can be used in the field or at the point of care, and are of special value in resource poor settings.

1.7.2 ELISA

ELISAs are immunoassays for the detection and quantification of analytes such as peptides, carbohydrates, antibodies, and proteins. ELISAs can be direct and indirect (Figure 4). Antigens are directly adsorbed onto a 96- or 364-well polystyrene plate and a complementary primary antibody binds the antigen. A direct format uses primary antibodies directly conjugated to an enzyme, while an indirect format requires addition of a secondary antibody e.g., anti-IgG conjugated to an enzyme e.g., horse radish peroxidase (HRP) (Figure 4). The correct enzyme substrate e.g., 3,3',5,5'-Tetramethylbenzidine (TMB) can be added to give a measurable signal proportional to the concentration of analyte. This signal can then be measured e.g., on a spectrophotometer and the concentration of analyte can be calculated from a standard curve.

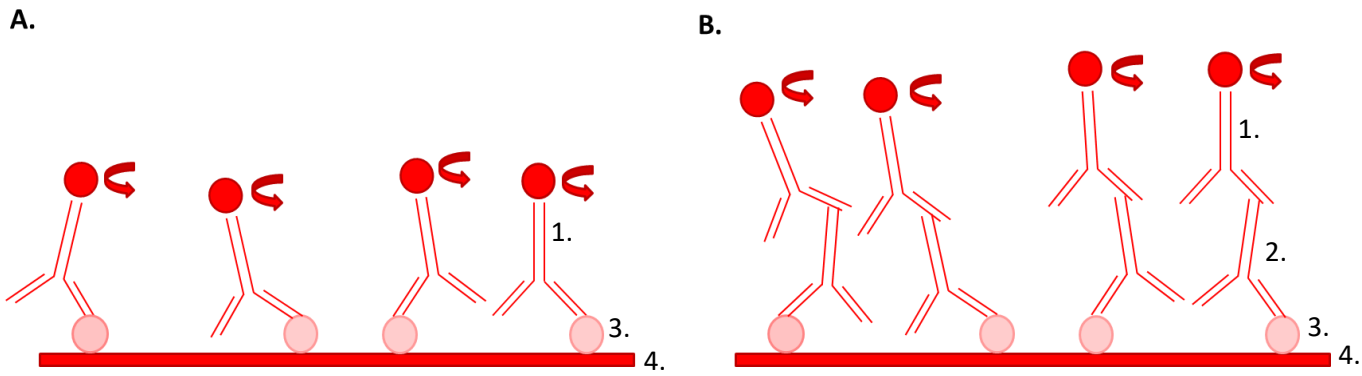


Figure 4. (A.) Direct and (B.) indirect enzyme-linked immunosorbent assay (ELISA). Each diagram is representative of one well of an ELISA plate. 1. Antibody conjugated to an enzyme. 2. Antibody. 3. Antigen directly adsorbed onto the polystyrene plate. 4. One well of a 96- or 364-well polystyrene plate.

Sandwich ELISAs use a capture antibody and a detection antibody to sandwich an analyte, so it is immobilised on the plate (Figure 5). Briefly, antibodies specific to the target are bound to a 96- or 364-well polystyrene plate through adsorption. The sample is added, and any target antigen present will bind its complementary antibody. As before, addition of an antibody conjugated to an enzyme and subsequent addition of the enzyme substrate leads to a measurable signal change. ELISAs are relatively high throughput devices, particularly if they are run on automated or robot machines, however automation can be costly and require trained laboratory staff.

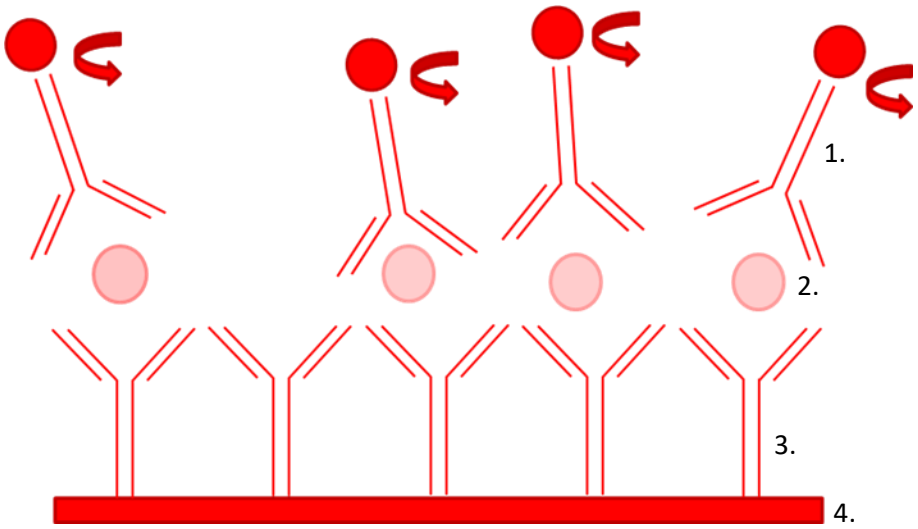


Figure 5. Sandwich enzyme-linked immunosorbent assay (ELISA). Antibodies bound to a polystyrene plate capture the target antigen. In this diagram a detection antibody directly bound to a fluorophore is added, followed by addition of an enzyme substrate which leads to a detectable colour change. This diagram is representative of one well of an ELISA plate. 1. Antibody conjugated to an enzyme. 2. Antigen. 3. Antibody directly adsorbed onto the polystyrene plate. 4. One well of a 96- or 364-well polystyrene plate.

1.7.3 Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR) is a highly sensitive technique which allows the detection and quantification of specific DNA or RNA in a sample. Due to its high sensitivity, it is often used as a reference standard in diagnostic research. A well-equipped laboratory with constant electrical supply and trained staff are required for qPCR. Briefly, qPCR uses complementary primers and other reagents to amplify a target, along with fluorescent dyes binding RNA, cDNA or DNA, to quantify PCR products as amplification occurs. A standard curve can be used to calculate the quantity of the unknown sample based on the threshold cycle (C_t) value. The C_t value is determined at the point at which the fluorescence of the amplification curve crosses a set threshold. This threshold is typically calculated as $1/10^{th}$ of the overall florescence detected during the qPCR run.

1.8 Tissue based diagnostics for VL

1.8.1 Parasitological diagnosis

Microscopic confirmation of *L. donovani* in splenic biopsies remains the reference standard for diagnosis of VL. Splenic biopsies are painful and carry a 1 in 1,000 risk of fatal bleed, making them incompatible with limited resources in field settings [87]. Patients are excluded from splenic aspiration if they have unpalpable spleens, platelet counts less than 40,000/µL, severe anaemia with haemoglobin (Hb) level <6g/dL, active bleeding, jaundice, and advanced pregnancy [88]. If splenic biopsy is contraindicated, parasites can be biopsied from other organs in the reticuloendothelial system, such as the bone marrow or lymph nodes. Bone marrow biopsies are painful but are safer than splenic aspiration [87].

Parasites are visualised under the microscope. Biopsied material is Giemsa stained and free amastigotes are counted. *L. donovani* bodies (LD bodies) contain a nucleus and kinetoplast and are 2-4µm in diameter. Slides are graded based on parasite counts, where a grade of 1+ is equivalent to 1-10 parasites per 1000 fields and 6+ to >100 parasites per field [1,89]. Microscopic diagnosis requires skilled technicians and well-maintained microscopes. Splenic aspiration has a sensitivity of approximately 95%, while bone marrow aspiration has a lower sensitivity of 70-80% [87].

1.8.2 Buffy coat smear microscopy

Buffy coat smear microscopy involves the preparation of blood films by smearing the fraction of a blood sample that contains mostly white blood cells - separated from whole blood by centrifugation - onto a slide for visualisation under a light microscope. The use of peripheral buffy coated blood smear has been shown to be of value in immunocompetent patients with VL in Bangladesh, where 92% were positive for LD bodies in buffy coat smear microscopy compared to splenic aspiration [90].

1.9 Immunological diagnostics for VL

Immunological assays, often in ELISA or RDT format, can detect pathogen-specific antibodies and therefore identify immune responses to the pathogen. Serological assays are widely used, with the direct agglutination test (DAT) (section 1.9.4) and the rK39 RDT (section 1.9.1) being part of the diagnostic algorithm used in East Africa and the rK39 RDT (section 1.9.1) used on the ISC [86,91]. However, these assays are unable to distinguish past from current infections, which has implications for

653 the elimination campaign, particularly in detecting asymptomatic individuals and diagnosing treatment
654 failure or relapsed VL which occurs with high frequency in PLHIV (section 1.6).

655

656 *1.9.1 RK39 serology*

657 RK39 is a recombinant protein of 39 amino acids, encoded by kinesin-like gene of *Leishmania* parasites.
658 The antigen was first described in 1993 in *Leishmania chagasi*, with the recombinant product still used in
659 current RDTs to detect circulating anti-*Leishmania* antibodies [92]. A finger prick of blood can be taken
660 and added to the strip along with diluent buffer. The result is read after ten minutes with a valid
661 negative test reading one bar and a valid positive test reading two bars. Limitations to the RDT include
662 the differences in sensitivities between manufacturers and lower sensitivity in VL-HIV patients and in
663 East Africa compared to South Asia [88,93]. Similarly, to the rK39 RDT, the ELISA uses the rK39
664 *Leishmania* protein to detect anti-*Leishmania* antibodies. The rK39 ELISA is yet to be standardised into a
665 commercially available kit and requires researchers to procure the required components, potentially
666 creating problems for standardisation.

667

668 *1.9.2 IgG1*

669 Immunoglobulin (Ig) IgG1 is generally the most abundant of the four subclasses of IgG [94]. Significantly
670 higher levels of IgG1 specific to an antigen from *L. donovani* (strain MHOM/IN/80/DD8) were found in
671 sera collected from patients in India with untreated and relapsed VL, compared to unpaired sera from
672 patients considered to be parasitologically cured [95]. The ELISA was developed into two prototype
673 RDTs, suitable for use at the point of care to detect *Leishmania* specific IgG1 [95]. One of these
674 prototypes, the VL Sero K-SeT using Sudanese *L. donovani* strain MHOM/SD/97/ LEM3458 has a
675 sensitivity of 77.9% (n=81) on patients from India [96]. Of 81 VL cases with a positive VL Sero K-SeT, 68
676 (84.0%) had a reduced test line intensity or negative RDT results at 6 months post-treatment [96]. The
677 RDT was capable of detecting 84.8% (n=28) of relapsed cases [96]. The test also showed promise for
678 predicting progression from asymptomatic (seropositive by DAT and/or rK39 ELISA) to symptomatic
679 infection, albeit in a small sample size (n=8) [96].

680

681

1.9.3 The indirect fluorescence antibody test (IFAT)

The indirect fluorescence antibody test (IFAT) uses parasite culture and an anti-IgG fluorescent secondary antibody to measure the presence of *Leishmania* specific IgG in serum [97]. Sensitivity is low (28.4%) compared to parasitology in Nepal [97] and has limited effectiveness for diagnosis in PLHIV [98,99]. The assay requires a fluorescence microscope.

1.9.4 The DAT

The DAT detects anti-*Leishmania* antibodies via addition of serum to a trypsinised, fixed and Coomassie blue-stained, freeze-dried preparation of *L. donovani* antigen [100]. The presence of antibodies in the sample which has been diluted two-fold, leads to visible agglutination by eye [100]. The procedure requires overnight incubation before the results are read [100]. The end titer recorded corresponds to the dilution at which the agglutination becomes visible, but still remains larger than the dots seen in the negative control [101]. Some inter-reader variability has been found between laboratories [101,102]. The DAT has enabled organisations such as MSF in East Africa, to reduce the number of tissue aspirations by an estimated 80% [88]. A meta-analysis of 30 studies found that the combined sensitivity and specificity of the DAT was 94.8% and 85.9% respectively [103]. The sensitivity of the test was lower in PLHIV [88]. The DAT is useful in monitoring transmission of *L. donovani* in an elimination setting [38].

1.9.5 Leishmanin skin test (LST)

Injection of *Leishmania* extract intradermally leads to a measurable delayed type hypersensitivity response to the *Leishmania* parasites [3]. The Leishmanin skin test (LST) is used to detect *Leishmania* infections via cellular immune responses, which develop in 80% of those treated for infection, but cannot be used to measure whether an infection is still active as LST responses can be elicited in asymptomatic individuals with immunity [104]. The test is useful for large scale epidemiological studies to measure prevalence, but not necessarily for disease diagnosis [105,106].

1.9.6 rK28, rK9 and rK26

Efforts to improve the sensitivity and specificity of RDTs. led to the use of one derivative of the rK39, the rK28, which detects IgG antibodies to the *Leishmania* parasite present in the blood against recombinant rK28 antigen [107]. The rK28 is made from two repeats of a Sudanese *L. donovani* kinesin flanked by proteins HASPB1 and two gene repeats (GenBank accession no. HM594686) [107]. The test in Sudan and Bangladesh has sensitivities of 95.9% and 98.1% respectively, higher than that seen with the rK39, and an improved response in sera that had low reactivity to rK39 [107]. Other derivatives (rK9 and rK26) have slightly lower sensitivities [107]. Whereas the rK9 has a lower specificity, the rK26 has a high specificity at 97.1% [107]. The rK26 however has a low sensitivity of 21.3% when tested on patients in India [108]. Future *in silico* searches may help identify new peptide epitopes [109].

1.10 Antigen tests for VL

Antigen tests, often in ELISA and RDT format, can be used to detect pathogen-specific antigens in clinical samples, therefore detecting active infection. These tests may be of particular benefit to the elimination campaigns. Uses may include, but are not limited to, populations where relapse rates are high, such as in PLHIV, and where it may be important to distinguish between active or former infections in the absence of symptoms. The most frequently used sample type for antigen based VL diagnostics is urine, which has the benefit of being non-invasive, but may require preparation steps such as boiling or freezing before testing (sections 1.10.1 and 1.10.2) and may not be acceptable for female patients in some settings.

1.10.1 KAtex (Clin-Tech, Guildford, UK, formerly Kalon Biological, Guildford, UK)

The KAtex (Clin-Tech, Guildford, UK) is a commercial test, which uses latex agglutination to detect the *Leishmania* carbohydrate antigen in urine (antigenuria) [110]. The test requires boiling the urine in water for five minutes, to avoid false-positive results [111,112]. Results are semi-quantitative with results scored strongly positive (+++), moderate (++), or weak (+) based on the level of agglutination [110,113]. Sensitivity ranged from 36% to 74% in Nepal and Ethiopia, respectively and an overall sensitivity of 63.6%, with 92.9% specificity [93]. Another study showed varied and low sensitivities, with

sensitivity in East Africa being higher than in the ISC [111], which resulted in the test not being widely adopted [93].

The KAtex has potential for monitoring VL treatment. In Ethiopia, a study of 42 participants reported a decline in antigen post-treatment, albeit with a low pre-treatment sensitivity of 61% (95% CI: 45.4 – 74.9) [112]. A study in India found that at the end of treatment (n=273), only 3% of participants remained positive by the KAtex, compared to a pre-treatment sensitivity of 87% (95% CI: 83.3 – 90.3%) (n=382) [114]. Similarly, in Bangladesh the test had a pre-treatment sensitivity of 75% (95% CI: 57 – 87%) (n=36) and a specificity of 100% (95% CI: 89 – 100%) (n=40), and 94.4% of patients became KAtex negative post-treatment [115]. Furthermore, KAtex correlates with treatment failure and relapse in PLHIV [113], which is discussed further in section 1.14.4.

1.10.2 Leishmania antigen ELISA (Clin-Tech, Guildford, UK, formerly Kalon Biological, Guildford, UK)

Kalon Biological further developed the KAtex into an ELISA format which also detects *Leishmania* carbohydrate in urine. The assay uses sheep anti-*Leishmania* antibodies against whole *L. donovani* (Sudanese strain LV9 and Nepalese strain BPK282) [112]. Approximately 10ml of urine is collected and stored, but small volumes are required to run the test. Samples need to be aliquoted and frozen at -20°C before testing to avoid false positive results.

A case-control evaluation of the *Leishmania* antigen ELISA by Kalon Biological found sensitivities of 95.2% (n=105, Bangladesh) and 100% (n=18, Kenya). All 48 healthy negative controls from Bangladesh and 17 healthy negative controls from Kenya had negative tests. Further external validation found variation in diagnostic accuracy between regions, with sensitivities of 87% (95% CI: 73.7 – 95.1), 78.1% (95% CI: 66.0 – 87.5), 76.9%, (95% CI: 46.2 – 95.0), and 81.4%, (95% CI: 66.6 – 91.6) in Ethiopia (n=46), Sudan (n=64), Bangladesh, (n=13), and Brazil (n=43), respectively [112]. The specificity was 100% (95% CI: 90.3-100), 90% (95% CI: 55.5-99.7), 100% (95% CI: 69.2-100), 70% (95% CI: 34.8-93.3), and 80% (95% CI: 44.4-97.5) in non-endemic (n=49), healthy endemic (n=10), human African trypanosomiasis positive (n=10), *Plasmodium falciparum* malaria positive (n=10), and TB positive (n=10) controls, respectively [112]. Therefore, specificity seems to be lower in endemic populations, those with malarial infection and people with TB. However, sample sizes are too small to demonstrate statistically significant differences.

1.10.3 Leishmania antigen detect™ (InBios International Inc., Seattle, USA)

Similarly, the *Leishmania* antigen detect™ (InBios International Inc., Seattle, USA) is an ELISA to detect *L. donovani* antigens in urine [112]. Anti-*Leishmania* IgG was affinity purified using soluble lysate antigen (SLA) from rabbits injected with whole cell lysate (WCL) made from *L. donovani* promastigotes (Sudanese strain MHOM/SD/00/1S-2D) [112]. The test had sensitivities of 93.5% (82.1-98.6), 96.9% (89.2-99.6), 100% (73.5 – 100), 88.4% (77.8 – 96.0) in cohorts of patients in Ethiopia (n=46), Sudan (n=64), Bangladesh (n=13) and Brazil (n=43), with 100% specificity in all control groups [112]. The test was evaluated in 42 patients receiving VL treatment in Ethiopia [112]. At day 0, 95.2% of patients were positive [112]. By day 30 post-treatment, 21.4% remained positive, all of whom had high parasite gradings of 4+ or 5+ at diagnosis [112].

1.10.4 Kala-azar antigen detection test (DetectoGen Inc. Westborough, MA, USA)

The kala-azar antigen detection test developed by DetectoGen Inc. (Westborough, MA, USA) detects *Leishmania* protein excreted in urine [116,117]. Reverse-phase high-performance liquid chromatography and mass spectrometry were used to identify *L. infantum* peptides excreted in the urine of a patient with VL in Brazil [118]. Three purified recombinant proteins - *L. infantum* iron superoxide dismutase (*Li-isd1*), *L. infantum* tryparedoxin (*Li-txn1*), and *L. infantum* nuclear transport factor (*Li-ntf2*) – were subsequently used to produce and select anti-*Leishmania* IgG from rabbits and IgY from chickens [118]. The three ELISAs developed - anti-*Li-isd1*, anti-*Li-txn1*, and anti-*Li-ntf2* - detected 17 (89.5%) of 19 individuals with VL when data from all three assays were combined, with 100% specificity in urine from individuals with Chagas (n=8), schistosomiasis (n=14), cutaneous leishmaniasis (n=10), TB (n=10), and healthy controls (n=16) [118].

The three assays were combined into a single assay with a reported 100% sensitivity (n=20) in Brazil and 100% specificity (cutaneous leishmaniasis (n=10), Chagas disease (n=8), schistosomiasis (n=14), and TB (n=10)) [116]. The ELISA was further evaluated in seven patients receiving treatment for VL in India [119]. Antigen was detected in urine of all patients prior to treatment and none had antigen detected 30-days post treatment [119]. However, further evaluation showed that the sensitivity of the ELISA was only 45-50% (n=15) and 30-40% (n=10) in patients in Kenya and India, respectively, despite the homology of these proteins between *L. donovani* and *L. infantum* being highly conserved [117].

To improve the sensitivity against Old World *Leishmania* infections, rabbit IgG and chicken IgY specific for *L. donovani* peptides from maoc family dehydratase (*Ld-mao1*) and peptidyl-prolyl isomerase (*Ld-ppi1*) found in the urine of VL patients in Kenya and India, were added to the ELISA [117]. This addition improved sensitivity to 82.2% (n=45) in VL patients in Kenya, with 100% specificity [117]. Finally, antibodies against the *L. donovani* protein malate dehydrogenase (*Ld-mad1*) were added, peptides of which were discovered in the urine of VL patients in India and Kenya [120]. This, along with production of monoclonal antibodies (mAbs) against the six *Leishmania* biomarkers, improved assay sensitivity to 91.7% in Brazil (n=24) and 93.3% in Kenya (n=45) [120]. Data on assay performance on the ISC is yet to be published.

1.10.5 Kala-azar antigen immunochromatographic test (ICT) (Xinjier Biotechnology Co., Ltd, Shanghai, China)

An RDT to detect *Leishmania* antigen in serum developed by Xinjier Biotechnology Co., Ltd (Shanghai, China) was evaluated in VL and VL-HIV patients in Ethiopia with no history of VL or treatment for VL prior to diagnosis [91]. The sensitivity was 88.2% (95% CI: 76.6-94.5) in PLHIV (n=51) and 90.0% (95% CI: 76.9-96.0) in those without HIV (n=40) compared to a combined reference standard of visualisation or detection of parasites by qPCR from a tissue aspirate [91]. However, specificity was only 20% (95% CI: 8-42), with eight of ten non-endemic controls and eight of ten malaria controls positive [91].

1.11 Molecular diagnostics for VL

1.11.1 QPCR

Leishmania DNA can be detected and quantified using qPCR. Various assays have been developed for *Leishmania* based on different genome targets, chemistries, DNA extraction techniques, and sample types. The technique is generally highly sensitive, and the detection of genetic material is often equated to active infection. An assay based on Taqman chemistry had 100% sensitivity (91.19–100) compared to a nested PCR [121]. Furthermore, no participants (n=20) were positive following treatment [121]. Parasite load measured by qPCR was positively correlated with the score of the splenic biopsy at diagnosis [122].

Of 246 healthy individuals screened by qPCR in West Bengal, India, 21.5% were positive, with poor agreement between the qPCR and serological tests [123]. A positive qPCR result was a significant risk factor for disease progression with odds of 20.9 in a cohort of 1,606 asymptomatic participants in Bihar, India [124]. qPCR is largely restricted to research purposes or as a reference standard in well-equipped facilities, as it is not compatible with field use and has a higher cost than serological techniques [125], limiting its use in monitoring and surveillance efforts post-elimination. However, qPCR has potential for use in PLHIV [125].

1.11.2 Loop-mediated isothermal amplification (*LAMP)*

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification technique (NAAT) that amplifies DNA and RNA of *Leishmania* species and is suitable for resource poor settings [126]. The assay requires one enzyme and target-specific primers which can be kept at room temperature [126]. The amplification cycle occurs at one temperature and therefore does not require a thermocycler [126]. Amplification occurs rapidly in under one hour [126]. If the target nucleic acid sequence is present, amplification is detected by visualisation of fluorescence in the tubes [126]. High copy numbers are seen in target regions such as the kinetoplast DNA (kDNA) and 18S ribosomal DNA [127]. LAMP still requires DNA to be extracted, with sensitivity and specificity varying by the extraction method and sample type, with the more costly Qiagen kits often providing the highest diagnostic accuracy [128]. A LAMP assay, the Loopamp *Leishmania* Detection Kit, had 100% sensitivity (n=84) and 99% specificity (n=101) with the Qiagen extraction technique on whole blood in Sudan compared to lymph node aspirate microscopy as the reference standard [128].

846 **Table 1. Summary of diagnostics for VL.**

Assay name	Technique	Advantages	Disadvantages
Parasitological diagnosis	Tissue-based diagnosis	Splenic and bone marrow aspirates offer relatively high sensitivity and specificity. The technique can be used in cases of VL relapse.	Lymph node aspirates have relatively poor sensitivity. Tissue aspiration is invasive and requires well-resourced facilities and highly trained staff.
Buffy Coat Smear	Tissue-based diagnosis	Relatively high sensitivity and less invasive than tissue aspiration. The technique can be used in cases of VL relapse.	The technique requires a well-maintained microscope and trained microscopist.
rK39 serology	Immunological diagnostics	The rK39 RDT has relatively high sensitivity in combination with clinical presentation. The rK39 RDT can be used with a capillary blood sample and is simple to conduct. The rK39 RDT is suitable for use at the point of care.	The technique cannot be used in cases of VL relapse. Has reduced sensitivity in PLHIV. The rK39 RDT shows variation in performance between manufacturers. The rK39 ELISA is prepared in-house and is yet to be made into a commercial kit. The rK39 ELISA requires relatively well-resourced facilities and trained staff.
IgG1	Immunological diagnostics	Has shown promise for use in VL relapse. Suitable for use at the point of care.	Requires further clinical evaluation.
IFAT	Immunological diagnostics		Poor performance including in PLHIV. Requires a fluorescence microscope.
DAT	Immunological diagnostics	Relatively high sensitivity. Can be used to reduce the number of splenic aspirates conducted in PLHIV. Requires minimal laboratory equipment.	Requires an overnight incubation step. The technique cannot be used in cases of VL relapse. Has reduced sensitivity in PLHIV.

847

848

849 **Table 1. Continued.**

Assay name	Technique	Advantages	Disadvantages
LST	Immunological diagnostics	Detects cell-mediated immunity and is useful for large-scale epidemiological studies.	Not necessarily suitable for VL diagnosis.
rK28, rK9 and rK26	Immunological diagnostics	Some promising performances in clinical evaluations.	Require further clinical evaluations. RK9 showed poor specificity and the rK26 showed poor sensitivity.
KAtex	Antigen tests	The technique can be used in cases of VL relapse.	Requires urine to be boiled. Poor specificity.
Leishmania antigen ELISAs	Antigen tests	The technique can be used in cases of VL relapse.	Requires further clinical evaluation Requires relatively well-resourced facilities and trained staff.
Leishmania antigen RDTs	Antigen tests	The technique can be used in cases of VL relapse. Has the potential to be used at the point of care.	The only commercially available antigen RDT showed poor performance.
qPCR	Molecular diagnostics	High sensitivity. There are ongoing developments in this technique to bring an assay to the point of care.	Requires well-resourced facilities and trained staff. Performance can vary with extraction technique, with more expensive kits generally showing better performance.
LAMP	Molecular diagnostics	Relatively simple assay in comparison to standard qPCR.	Does not negate DNA extraction. Performance can vary with extraction technique, with more expensive kits generally showing better performance.

850

851

852

853

1.12 Diagnostic algorithms for VL

Due to the low specificity of most tests and the overlap of symptoms with other diseases, diagnostic algorithms are often utilised to combine clinical syndromes with one or more tests [88]. In the initial diagnostic pathway, a case definition is based on clinical examination before testing. Individuals with prolonged fever, splenomegaly and/or wasting are then tested by rK39 RDT on the ISC, where the sensitivity of the test is higher than in East Africa [129,130]. Individuals who fulfil the WHO definition of a case (positive by rK39 RDT with clinical symptoms) receive treatment [130].

In East Africa, rK39 RDT negative individuals are further tested with the DAT and, if positive receive treatment, whereas individuals with borderline antibody titers have further confirmatory splenic or lymph node aspirates when not contraindicated [88]. Individuals with negative DAT assays are considered negative [88]. Using this algorithm in a scenario of 200 patients presenting with symptoms of VL in which 100 individuals have VL, the rK39 RDT was estimated to have a sensitivity of 84.3% and a specificity of 91.5% [88]. In this scenario, 108 individuals would be further assessed using the DAT [88]. In these individuals, the DAT was estimated to have a sensitivity of 95% and a specificity of 95% [88]. This scenario left two patients with an indeterminate DAT titer requiring a splenic aspiration of whom, one was positive [88]. In this scenario, prevalence of VL was 50%. As the prevalence of VL decreases the positive predictive value of the diagnostic tests decreases, meaning that the percentage of individuals testing positive whom have VL decreases. Additional or replacement tests within this diagnostic pathway may improve diagnostic accuracy.

1.13 Current diagnostics for VL-HIV

1.13.1 Reference standards for diagnostics

VL is diagnosed by a combination of clinical presentation, rK39 RDT, and tissue aspiration in HIV patients in India [86]. In patients found to be rK39 RDT positive in India and East Africa, parasitological confirmation is frequently carried out due to the high rates of treatment failure and relapse, however use of the DAT in East Africa has been reducing the numbers of splenic aspirations required as mentioned previously [88,131]. Furthermore, parasitological confirmation by splenic or bone marrow aspiration is carried out in India in rK39 RDT negative patients with high clinical suspicion of VL [131]. Splenic aspirates remain the reference standard for diagnosis of VL in PLHIV due to the high sensitivity and the ability to detect active infections [15]. The rK39 and DAT are less sensitive in PLHIV in Ethiopia

[88]. The effectiveness of most diagnostic tests for VL-HIV are yet to be evaluated on the ISC, with most available data on diagnostic accuracy based on studies in East Africa, the Mediterranean, and Brazil.

A series of interviews with VL-HIV patients found that within the private healthcare system in Bihar, VL was often misdiagnosed for other fever causing illnesses such as typhoid and malaria, with more complex presentations seen in VL-HIV making the diagnosis even more complex [132]. Patients were found to present first to private informal providers, delaying attendance at government hospitals where diagnosis and treatment are provided free of charge [132]. Further barriers to accessing diagnosis and treatment included severe stigmatisation PLHIV by the community and healthcare providers [132].

1.14 Treatment and test of cure

1.14.1 Treatment

An accurate diagnosis is essential due to the toxicity associated with treatments [133]. Pentavalent antimonials such as sodium stibogluconate (SSG) are more commonly used in East Africa and Latin America than in India due to a decline in the effectiveness of this drug class, with only between 36% and 69% of cases cured after a 30-day high dose regimen in the region [133,134]. Pentavalent antimonials can be administered intravenously or intramuscularly with varying regimens given [133,135,136]. Side effects reported include cardiotoxicity in a small percentage of cases, with a subset being fatal in one trial, pancreatitis, nephrotoxicity, and hepatotoxicity [133,135,136]. Paromomycin can be administered via intramuscular injection over 21 days, is generally well tolerated with rare reports of hearing disturbances (ototoxicity), nephrotoxicity, hepatotoxicity, and is more effective than pentavalent antimonials [133,136–138].

Miltefosine can be administered orally over 28 days and has a high cure rate in India, with as high as 97% of participants cured in one study, with generally mild gastrointestinal (GI) side effects reported in 20-55% of patients, some nephrotoxicity, and hepatotoxicity [136,139]. However, the long 28-day treatment resulted in poor adherence, along with a potential for teratogenic side effects, and the potential for resistance due to a long half-life [140,141]. Amphotericin B, primarily an anti-fungal drug, has anti-*Leishmania* properties against the promastigote and amastigote stages. Amphotericin B is effective, with a clinical efficacy of over 97% reported in all regions, although with moderate toxicity including nephrotoxicity that requires inpatient care [133,135,136]. Liposomal amphotericin B (AmBisome, LAMB), a lipid formulation of amphotericin B, has low toxicity with only limited

914 nephrotoxicity and is effective with a clinical efficacy of over 97% reported in India [136,142]. AmBisome
915 can be administered by intravenous infusion over several days (up to 21 days in immunocompetent
916 patients) or as a single dose in the case of first line treatment on the ISC [143,144]. AmBisome is
917 predominantly used on the ISC, but is less effective in East Africa and Brazil, where higher doses are
918 needed [143].

919

920 *1.14.2 Treatment Regimes for VL-HIV in India*

921 Treatment with AmBisome monotherapy in a cohort of 55 VL-HIV patients in India led to comparably
922 good survival rates, albeit much lower than in an immunocompetent population, and was well tolerated
923 with no treatment interruption [145]. However, the probability of VL relapse following treatment in VL-
924 HIV patients is high (26.5%) two years post-treatment with AmBisome monotherapy in India [131,145].
925 Therefore, a combination therapy of AmBisome and Miltefosine was trialled on a compassionate basis
926 by MSF [131,145,146]. Data from formal clinical trials of the combination therapy confirmed improved
927 treatment outcomes in individuals established or initiated on antiretroviral therapy (ART), with the risk
928 of relapse estimated to be 13.9% at 18 months, but with a slightly higher mortality than lower dose
929 AmBisome monotherapy [131,145,146]. The use of ART in this patient population has been associated
930 with improved outcomes, with initiation of ART following VL treatment reducing the risk of mortality in
931 one study by 64-66% compared to participants that did not start ART [131,145,146].

932

933 *1.14.3 Secondary prophylaxis for relapse VL in HIV patients*

934 Secondary prophylaxis can be administered to individuals following treatment for VL to help prevent the
935 relapse of disease. In areas such as the ISC and East the use of secondary prophylaxis in PLHIV is
936 cautioned by the WHO, as is the use of second-line drugs used to treat relapse VL being used as
937 secondary prophylaxis, due to the risk of emerging parasite resistance which could limit treatment
938 efficacy [1,15,143,147]. The limited data on the use of LAMB and pentamidine secondary prophylaxis is
939 based on studies in the Mediterranean and East Africa [3,148–150]. Pentamidine is not currently used in
940 the treatment of VL in East Africa [150]. A study to evaluate the use of a 12-month course of low dose
941 pentamidine secondary prophylaxis to prevent VL relapse reported the drug was effective in PLHIV in
942 Ethiopia for up to two and a half years after treatment initiation in patients with CD4 counts
943 >200cells/µL [150]. However, this remains to be examined on the ISC.

1.14.4 Test of cure in VL-HIV

Patients with VL-HIV coinfection are recommended to undergo a test of cure due to the high risk of treatment failure and relapse to administer second line therapeutics if treatment is not successful [75]. However, the rK39 RDT and DAT detect anti-*Leishmania* antibodies and cannot be used as tests of cure [151]. Tests of cure for VL in HIV patients are currently based on splenic aspiration due its high sensitivity [15]. Splenic aspiration however is invasive, requires a significant skill set and carries a small risk of fatal haemorrhage [87]. Similarly, although bone marrow and lymph node aspirates also have high sensitivity, they are also invasive [87].

A limited number of studies have assessed the detection of *Leishmania* antigenuria as an alternative and non-invasive test of cure. The KAtex has potential for monitoring treatment responses [112,114,115]. and predicting relapse in VL-HIV patients [110,113]. Pre-treatment sensitivity in a cohort of 49 VL-HIV patients in Spain was 85.7% [110]. The probability of relapse at 6 months among KAtex positive participants was 16% (95% CI: 15 – 17%), compared to 5% (95% CI: 2 – 8%) among KAtex negative participants [110]. A strongly positive KAtex score was associated with treatment failure, with an odds ratio of 11.9 (95% CI: 1.4 – 103) in 63 VL-HIV patients in Ethiopia, and a one year relapse rate of 6% among KAtex negative participants compared to 42% among participants that were strongly-positive by KAtex [113].

The *Leishmania* antigen ELISA (Clin-Tech, UK) has potential to monitor treatment responses, with a decline in antigen excreted in urine post-treatment in a cohort of 42 patients in Ethiopia [112]. A significant decline in urinary antigens was seen at 30 days post treatment initiation, with 35.7% of patients remaining positive compared to 90.5% at day zero [112]. The *Leishmania* antigen ELISA (Clin-Tech, UK) has not yet been evaluated in VL-HIV coinfection.

1.15 Background information on study areas

The studies presented in this thesis were largely performed in endemic settings of India and Bangladesh, where VL is the target of an elimination campaign. Some laboratory work was carried out in the UK.

1.15.1 India

Much of the laboratory work was conducted at a laboratory set up by the Liverpool School of Tropical Medicine (LSTM) in the Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Patna, Bihar state. RMRIMS is an Indian Council of Medical Research (ICMR) facility under the Ministry of Health and Family Welfare, of the Government of India. The RMRIMS' campus includes a 150-bed tropical disease hospital and a research institute, providing tertiary care for individuals with VL. All laboratory work on clinical samples collected in India were performed at the RMRIMS.

All work on VL-HIV was conducted as part of a collaboration with MSF who have run a specialist HIV ward in Patna for over ten years and are highly experienced in providing high-quality care for VL-HIV patients. MSF conducts clinical trials with VL-HIV patients and translate findings into clinical practice. Furthermore, they run local educational outreach campaigns to improve knowledge and awareness of VL and HIV. MSF have a long-standing relationship with the RMRIMS where their ward was based for several years before a move to Guru Gobind Singh Hospital (GGSH) in 2019, with a continued presence at the RMRIMS.

1.15.2 Bangladesh

All work in Bangladesh was conducted in partnership with the International Centre for Diarrhoeal Disease Research, Bangladesh (iccdr,b) in Dhaka, the Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland, and the WHO's Special Programme for Research and Training in Tropical Diseases (WHO-TDR). Iccdr,b has expertise in VL elimination on the ISC, is active in community engagement, and has a long standing relationship with LSTM.

1.16 Thesis outline

Accurate and appropriate implementation of diagnostics are a crucial part of the elimination campaign of VL on the ISC. Early diagnosis and treatment of active VL will help prevent resurgence from anthroponotic reservoirs. Individuals with asymptomatic *Leishmania* infection (ALI) and HIV-*Leishmania* coinfection are of significance on the ISC due to the anthroponotic nature of *Leishmania* reservoirs. Current diagnostics are limited to serological tests which cannot distinguish between past and current infections and have reduced sensitivity in PLHIV, while invasive procedures require significant

infrastructure and specialist skills. Molecular diagnostics such as qPCR can detect current infection and are highly sensitive but require well-equipped laboratories and can require lengthy sample preparation.

Should a suitable assay be identified, antigen tests could be incorporated into diagnostic algorithms, monitoring treatment efficacy, stratification of individuals by risk of relapse, and/or post-elimination surveillance, including among asymptomatic populations. Current antigen-based diagnostics require a urine sample, making them a more appropriate test for these applications. Antigen-based diagnostics are showing promise, however further evaluations are needed, along with further development of antigen tests suitable for resource-limited settings.

The overall aim of this thesis is to develop and evaluate diagnostic tools, capable of detecting current *Leishmania* infection, with a focus on their use in asymptomatic infections and PLHIV in India and Bangladesh.

The objectives of this thesis are:

1. To determine the prevalence and determinants of ALI in an elimination setting and to determine the clinical utility and diagnostic accuracy of antigen, molecular, and serological tests for ALI in an elimination setting (Chapter 2).
2. To determine the prevalence and determinants of ALI in PLHIV in an elimination setting and to determine the clinical utility of the antigen, molecular, and serological tests in this population (Chapter 3).
3. To determine the rate and risk factors for progression to VL and the utility of the antigen, molecular, and serological tests as markers for progression to VL in a cohort of PLHIV with ALI in an elimination setting (Chapter 4).
4. To establish a protocol to determine the clinical utility of the *Leishmania* antigen ELISA, blood smear microscopy and qPCR in blood and skin microbiopsies for diagnosis and test of cure for VL in PLHIV (Chapter 5).
5. To evaluate a panel of thirteen monoclonal antibodies for use in an alternative antigen detection test for VL and to evaluate other currently available antigen tests for VL (Chapter 6).

The thesis describes the prevalence and determinants of ALI, and the utility and diagnostic accuracy of the *Leishmania* antigen ELISA, DAT, LAMP, and qPCR for detecting ALI in a cohort of 720 contacts of VL and PKDL cases in Bangladesh (chapter 2). We then go on to evaluate the prevalence and determinants of ALI, and rate and risk factors for progression to VL in a cohort of 1,300 PLHIV in India, and examine

the use of the *Leishmania* antigen ELISA, qPCR, rK39 RDT, and rK39 ELISA for detection of ALI and their utility as prognostic markers (chapters 3 and 4). This is followed by a protocol to explore the potential use of minimally invasive alternatives for diagnosis and test of cure for VL, such as the *Leishmania* antigen ELISA, blood smear microscopy, and qPCR in a cohort of 91 HIV co-infected patients in India (chapter 5). Finally, we present a screening of thirteen monoclonal antibodies raised against *Leishmania* for their potential utility in an alternative antigen detection test and evaluate the kala-azar antigen detection test (DetectoGen Inc., USA) (chapter 6). All studies presented in this thesis are run in collaboration with, or form part of larger studies run by MSF, RMRIMS, iccdr,b, WHO-TDR, and FIND.

Chapter 2. Detection of asymptomatic *Leishmania* infection in Bangladesh by antibody and antigen diagnostic tools shows association with post kala-azar dermal leishmaniasis patients

2.1 Acknowledgement of work done

Isra Cruz, Albert Picado, Dinesh Mondal and Emily R. Adams were responsible for clinical enrolment, study conception, and study design. Faria Hossain, Prakash Ghosh, Rajashree Chowdhury, Sakhawat Hossain carried out sample collection, DNA extraction and LAMP assays presented in this paper. Sophie I. Owen was responsible for sample management in the UK including logging and keeping accurate records of human tissue in storage on Procuro, carrying out the *Leishmania* antigen ELISA, data analysis, writing and publication. Louise Ford conducted the qPCR assays presented in this paper. Christopher Jewell provided statistical support to the data analysis. Sophie I. Owen supported laboratory testing, data analysis and writing of other publications relating to this study presented in Appendix 1.

2.2 Introduction

Bangladesh is currently awaiting validation to confirm the elimination of VL as a public health concern to <1 case per 10,000 people at upazila level in line with WHO guidelines [33]. Given the anthroponotic nature of disease reservoirs on the ISC, testing will form a major part of the post-elimination strategy. Post-elimination testing could detect a potential resurgence in cases in a timely manner in combination with entomological surveillance to guide appropriate control measures and reduce transmission. A low prevalence setting such as Bangladesh presents diagnostic challenges, including the detection of asymptomatic *Leishmania* infection (ALI).

Definitions of ALI vary by study and involve serological tests, such as the LST and DAT, and/or molecular tests, such as LAMP and qPCR [55,152]. A systematic review of the literature found that the prevalence of asymptomatic infection was 4-17 times higher than the prevalence of VL on the ISC, with progression to symptomatic disease in 1.5% to 23% of cases [153]. Progression to acute infection was found to correlate with high anti-*Leishmania* antibody titres [153]. Furthermore, individuals with ALI and PKDL are estimated to contribute towards transmission in addition to VL cases [153]. The role of asymptomatic individuals in transmission was not supported by a xenodiagnoses study which found that 184 individuals with serologically confirmed ALI did not infect sand flies when the flies were allowed to feed on these individuals [154]. It remains to be determined whether contacts of cases and asymptomatic individuals are infectious to sand flies, but with a proportion of asymptomatic individuals going on to develop VL, it would concur that these individuals may act as a reservoir of infection.

Individuals with PKDL may act as a reservoir of infection. A small-scale screening of laboratory bred *P. argentipes* found that *L. donovani* DNA was present in a pool of ten flies following feeding on a patient with PKDL compared to newly emerged laboratory flies [155]. Similarly, a case of PKDL was suggested to be the source of an outbreak in a village in West Bengal in 1980. This case, along with three other PKDL cases blood-fed laboratory-bred *P. argentipes* (n=104), of which 32 flies became infected [156]. A total of 42 (55%) and 11 (42%) individuals with VL and PKDL, respectively were found to be infective to sand flies when sand flies were examined for the presence of promastigotes by microscopy following blood-feeding [154].

In the study presented in this chapter, we conducted serological, antigen, and molecular tests to identify individuals with ALI in contacts of individuals with VL and PKDL in Bangladesh. The *Leishmania* antigen ELISA utilises a non-invasive urine sample in a format compatible with large-scale testing, and therefore suitable for mass screening and surveillance. To our knowledge, no studies have yet included the

Leishmania antigen ELISA in evaluations of ALI at the time of publication. The DAT, also presented in this chapter, has previously been used for large-scale studies of long-term seroprevalence on the ISC [38,157]. At the secondary and tertiary level, the use of peripheral blood buffy coat smear microscopy may be able to replace splenic and bone marrow smear microscopy in symptomatic individuals, however this technique is not suitable for high throughput surveillance in an asymptomatic population [158]. The use of an antigen tests would complement serology for the detection of active cases.

Chapter two summary

In total, 720 contacts were recruited with ALI detected in 69 (9.6%) individuals. PKDL cases were found to act as a source of transmission for *L. donovani* on the ISC, complementing what has been seen in other studies [159]. Therefore, it is critical to identify PKDL and VL cases in order that acute disease is recognised and treated early. One (0.1%) participant was positive by all four of the diagnostic tests used, however the *Leishmania* antigen ELISA and the DAT in combination captured all individuals who were positive for more than one test. Therefore, we recommend the follow-up of contacts of PKDL cases as well as VL cases using the *Leishmania* antigen ELISA in combination with serology and a clinical history to detect ALI in contacts. How testing could be used in combination with other disease control strategies is yet to be determined on the ISC. An antigen test of high diagnostic accuracy in an RDT format would allow use at the primary healthcare level.

2.3 Detection of asymptomatic *Leishmania* infection in Bangladesh by antibody and antigen diagnostic tools shows association with post kala-azar dermal leishmaniasis (PKDL) patients

Sophie I Owen[1], Faria Hossain[2], Prakash Ghosh[2], Rajashree Chowdhury[2], Md. Sakhawat Hossain[2], Christopher Jewell[3], Isra Cruz[4,#a], Albert Picado[4], Dinesh Mondal[2,¶], Emily R Adams[1,¶,*].

[1]Department of Tropical Disease Biology, Liverpool School of Tropical Medicine (LSTM), Liverpool, UK

[2]Nutrition and Clinical Services Division, International Centre for Diarrhoeal Diseases Research (icddr,b), Dhaka, Bangladesh

[3]Lancaster Medical School, Lancaster University, Lancaster, UK

[4]Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland

[#a]Current address: National School of Public Health, Instituto de Salud Carlos III, Madrid, Spain

* Corresponding author

E-mail: Emily.Adams@lstmed.ac.uk (ERA)

¶DM and ERA are Joint Senior Authors

Owen S. I. *et al.* Parasites & Vectors (2021) 14:111

Abstract

Background: Asymptomatic *Leishmania* infections outnumber clinical infections on the Indian sub-continent (ISC), where disease reservoirs are anthroponotic. Diagnostics which detect active asymptomatic infection, which are suitable for monitoring and surveillance, may be of benefit to the visceral leishmaniasis (VL) elimination campaign on the ISC.

Methods: Quantitative polymerase chain reaction (qPCR), loop mediated isothermal amplification (LAMP), and the direct agglutination test (DAT), were carried out on blood samples, and the *Leishmania* antigen ELISA was carried out on urine samples collected from 720 household and neighbouring contacts of 276 VL and post-kala-azar dermal leishmaniasis (PKDL) index cases, with no symptoms or history of VL or PKDL, in endemic regions of Bangladesh between September 2016 and March 2018.

Results: Of the 720 contacts of index cases, asymptomatic infection was detected in 69 (9.6%) participants by a combination of qPCR (1.0%), LAMP (2.1%), DAT (3.9%), and *Leishmania* antigen ELISA (3.3%). Only one (0.1%) participant was detected positive by all four diagnostic tests. Poor agreement between tests was calculated using Cohen's kappa (κ) statistics; however, the *Leishmania* antigen ELISA and DAT in combination captured all participants as positive by more than one test. We find evidence for a moderately strong association between the index case being a PKDL case (OR 1.94, p = 0.009), specifically macular PKDL (OR 2.12, p = 0.004), and being positive for at least one of the four tests.

Conclusions: *Leishmania* antigen ELISA on urine detects active asymptomatic infection, requires a non-invasive sample, and therefore may be of benefit for monitoring transmission and surveillance in an elimination setting in combination with serology. Development of an antigen detection test in rapid diagnostic test (RDT) format would be of benefit to the elimination campaign.

Keywords

Visceral leishmaniasis, elimination, asymptomatic *Leishmania* infection, diagnostics, *Leishmania* antigen ELISA, qPCR

Background

Infection with the parasite *Leishmania donovani* (*L. donovani*) usually manifests as asymptomatic infection with a small risk of progression to visceral leishmaniasis (VL), which in the absence of treatment is considered fatal [1]. Progression from asymptomatic infection to symptomatic disease was estimated to be between 5.6 and 15.2% in individuals with high anti-*Leishmania* antibody titers, as measured by the direct agglutination test (DAT), in India and Nepal [2]. Globally, the ratio of asymptomatic to symptomatic VL varies [3]. In Bangladesh, the number of asymptomatic cases were found to outnumber symptomatic cases by 4 to 1 [4].

Asymptomatic infection is of importance to VL endemic regions of the Indian sub-continent (ISC - India, Nepal, and Bangladesh), where the disease has been the target of an elimination campaign since 2005 [5,6]. The epidemiology of VL is cyclical, and outbreaks occur approximately every 15 years on the ISC [7]. Asymptomatic carriers may represent a potential source of transmission in a region where parasite reservoirs are anthroponotic [8]. However, it is yet to be determined whether asymptomatically infected humans are infective to sand flies. A study in a small number of asymptomatically infected dogs showed that *L. infantum* parasites were transmittable to sand flies [9]; however, no human data with *L. donovani* have yet been recorded. Sixteen (8.2%) asymptomatic individuals who converted to VL within 2 years in a study in Bangladesh were found to have significantly higher anti-rK39 antibody titers compared to their counterparts who did not progress [10].

The rK39 enzyme-linked immunosorbent assay (ELISA), rK39 rapid diagnostic test (RDT) and the DAT measure the presence of anti-*Leishmania* antibodies [11–14]. These antibodies have been found to persist for months or years after infection, with patients in the VL endemic region of Muzaffarpur, India found positive by rK39 RDT (39.0%) and DAT (53.0%) ≥ 15 years post-treatment [15]. Therefore, a clinical history is required to determine whether a positive result is due to active or previous infection, or a previous asymptomatic infection that will not progress to disease. Tests which detect active infection, such as quantitative real-time polymerase chain reaction (qPCR), loop-mediated isothermal amplification (LAMP), or *Leishmania* antigen ELISA, could be used as tools to monitor active asymptomatic infection and quickly identify areas with increasing active transmission.

Highly sensitive qPCR was shown to be an effective technique for diagnosis of VL and monitoring of treatment response and could be of value in an elimination setting [16]. LAMP enables the robust, fast, simple, and highly specific amplification of nucleic acids and does not require a thermocycler or cold

chain; the Loopamp™ *Leishmania* Detection Kit (Eiken Chemical Co., Japan) targets both the 18S rDNA and kinetoplast DNA (kDNA) and was previously demonstrated to have a sensitivity of 92% in patients with suspected VL in Ethiopia [17]. Similarly high sensitivity of 98% and 100% was seen in a study in Sudan with the Loopamp™ *Leishmania* Detection Kit when DNA was extracted from peripheral blood using boil-and-spin and QIAamp DNA mini kits (Qiagen, Hilden, Germany), respectively [18]. Finally, the *Leishmania* antigen ELISA (Clin-Tech, Guilford, UK) detects low-molecular-weight *Leishmania* carbohydrates excreted in the urine and therefore detects active infection and uses a non-invasive sample type. A study found sensitivity to range from 77% (n=13) in Bangladesh to 87% (n=46) in Ethiopia, although more data are needed to evaluate this assay [19]. The clinical utility of the *Leishmania* antigen ELISA is yet to be determined in an asymptomatic population.

To determine the utility of the DAT, kDNA qPCR, Eiken LAMP, and the *Leishmania* antigen ELISA for monitoring and surveillance of asymptomatic *Leishmania* infection in an elimination setting, we tested samples collected from household or neighbouring contacts of index cases from endemic regions of Bangladesh. In this study, asymptomatic infection is defined as being positive for at least one of the aforementioned tests. Risk factors for asymptomatic infection were also investigated. We then compare the measure of prevalence with that obtained from a latent class analysis, in which the test characteristics of our four tests are formally synthesised through the use of a probability model.

Methods

Asymptomatic visceral leishmaniasis clinical samples

Blood and urine samples from 720 clinically healthy household and neighbouring contacts in adjacent households of 276 VL or PKDL index cases (between 1 and 8 contacts per index case), aged 5 to 60 years, with no symptoms or history of VL and PKDL, were collected between September 2016 and March 2018. The study was conducted in the VL endemic districts of Mymensingh, Gazipur, Tangail, Narail, Jamalpur, Pabna, and Brahmanbaria in Bangladesh. Symptoms considered included presence of fever, rash, loss of appetite, weight loss, lymph node enlargement, abdominal enlargement and pain. Demographic and clinical parameters were recorded. Blood and urine samples were transported to Dhaka using a cold chain for processing and laboratory analysis using DAT and LAMP. Urine and DNA samples for *Leishmania* antigen ELISA and qPCR, respectively, were transported on ice to the UK from Bangladesh and stored at -20˚C until testing.

DNA extraction

DNA was extracted in three different ways: (1) DNA was extracted from 100µl whole blood and eluted in 200µl buffer using DNeasy blood and tissue DNA extraction kits (Qiagen, Hilden, Germany) as per the manufacturer's instructions. (2) Boil-and-spin extractions were carried out by pretreating whole blood samples with sodium dodecyl sulphate (SDS). Briefly, 10% SDS solution was mixed with blood to a final concentration of 5% and stored at -20˚C. Once defrosted, samples were inverted 10 times and allowed to stand at room temperature for 10 minutes. Samples were further inverted, and 400µl of distilled water was added before incubation at 90˚C for 10 minutes. Tubes were then centrifuged at maximum speed for 3 minutes, and the supernatants stored for testing. (3) DNA was extracted from dried blood spots (DBS). Whole blood was air-dried onto Whatman filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK) for 30 minutes at room temperature and stored in individual bags. Discs of 7mm were punched out of the paper and added to an Eppendorf tube with 50µl of double-distilled water. Tubes were incubated at 90˚C for 10 minutes followed by centrifugation for 3 minutes at maximum speed. Supernatants were stored at -20˚C for testing.

qPCR

Real-time PCR (qPCR) was performed on DNA extracted from whole blood using Qiagen DNeasy kits (Qiagen, Germany) [17]. An aliquot of 1.25µL DNA was added to 11.25µL amplification mixture containing 2.5µL QuantiFast master mix (Qiagen, Germany), 0.4µM kDNA forward primer, 0.4µM kDNA reverse primer, and 0.2µM kDNA FAM probe. Amplification was performed on a Qiagen Rotor-Gene Q system with following reaction conditions: 5 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. Data were analysed using the Rotor-Gene Q series software (Qiagen, Germany). Standard curve analysis was performed using *Leishmania donovani* DNA (positive control), and the data were used to set a qPCR threshold. Samples with cycle threshold (Ct) <34 were considered positive to reduce detection of non-specific amplification.

Loop mediated isothermal amplification (LAMP)

LAMP was run on DNA extracted from whole blood using the DNeasy blood and tissue DNA extraction kits (Qiagen, Germany), boil-and-spin extraction, and extraction from dried blood spots as described above. Loopamp™ *Leishmania* Detection kits (Eiken Chemical Co., Ltd, Tokyo, Japan) were used. Samples to be tested were made up to total volume of 30µl by adding 3µl DNA sample to 27µl of water. The lids of the tubes were then closed, and the sample mixed with the master mix contained in the tube cap by inverting the tubes and leaving them to stand for 2 minutes cap-side down. The tubes were inverted 5

times, spun down and incubated at 65˚C for 40 minutes, then 80˚C for 5 minutes. Results were visualised under blue LED light illumination, using the fluorescence visual check unit of the HumaLoop M incubator (HUMAN, Wiesbaden, Germany). Results were read by two technicians blinded to each other. A third technician was consulted in the event of disagreement, and the majority decision used.

Direct agglutination test (DAT)

The DAT was carried out in Bangladesh and performed as previously described [20]. Following a dilution of sera 1:100, the samples were further diluted in eight twofold serial dilutions. Where samples did not react in the first dilution, the end titer was read as <1:200. Where samples still reacted at the final dilution, the end titer was read as >1:25,600. The threshold for a positive DAT result was set at ≥1:1600 as previously used by Hasker *et al.* for detection of asymptomatic infection [20].

Leishmania antigen ELISA

The *Leishmania* antigen ELISA (Clin-Tech, Guilford, UK) was performed on urine samples as per the manufacturer's instruction. Briefly, samples were diluted 1 in 20 with assay diluent. One hundred microlitres of antigen calibrators and diluted samples were added to a pre-coated 96-well plate and incubated at 37˚C for 30 minutes. Following four washes, 100µl of working strength tracer was added to the wells and incubated at 37˚C for 30 minutes. Following a further four washes, 100µl of TMB substrate was added to each well and incubated uncovered between 18˚ and 25˚C for 30 minutes. One hundred microlitres of stop solution was then added to each well. A standard curve was included on each plate. The optical densities (OD) were read at 450nm and blanked on air or with the 620nm reading within 30 minutes of addition of stop solution. Four-parameter curve fitting software was used to calculate the concentration (UAU/ml) of each sample. IBM SPSS Statistics version 24 software was used to generate receiver-operating characteristic (ROC) curves using 720 asymptomatic cases and 80 VL cases to determine the threshold in UAU/ml that gave a sensitivity of 98.8% and a specificity of 96.7%. The area under the curve (AUC) was calculated.

Statistical analysis

Data were analysed in R Studio Version 1.1.456. Discrete variables were summarised as counts and percentages. Continuous variables were summarised as the median and interquartile range (IQR). The software package 'Venny' was used to create Venn diagrams for comparison of diagnostic tests [21].

Percentage agreement between diagnostic tests and Cohen's kappa (κ) statistics with *p* values to measure agreement between diagnostic tests were calculated with the irr package version 0.84.1 in R. Logistic regression was used to regress asymptomatic *L. donovani* infection (defined as positive for at least one of the four tests) outcome variable onto potential risk factor variables identified in the literature. Latent class analysis was used to estimate diagnostic accuracy and prevalence [22]. Test results were assumed to be conditionally dependent, with Bayesian prior distributions on sensitivity, specificity, and prevalence set using Betabuster 1.0 (https://betabuster.software.informer.com/). The analysis was implemented in R Studio Version 1.1.456 using the 'lcaR' model written by Jonathan Marshall (version 2bc8ca6, 13th November 2015) [23].

Results

Study population

A total of 720 individuals were sampled, with a median age of 27 years (IQR = 25 years), of whom 280 (38.9%) were male (Table 1). The most common occupations were student (34.4%) and housewife (41.9%) (Table 1). A total of 505 (70.1%) contacts lived within the household of an index case, and 215 (29.9%) lived within a neighbouring household (Table 1).

A total of 69 individuals were positive for at least one diagnostic test, with a median age of 30 (IQR = 25) (Table 1). Of those, 31 (44.9%) were male (Table 1). The most common occupations within the 69 individuals were student (33.3%) and housewife (37.7%), and 50 (72.5%) lived within the household of an index case (Table 1). The 69 asymptomatic cases were spread across 59 (21.4%) of the 276 index cases. Of those 59 index cases, the median percentage positivity of the contacts was 33.3% (IQR = 25).

The 720 contacts were associated with VL cases (66.1%) – made up of new VL cases (90.1%), relapsed VL (9.5%), VL treatment failure (0.4%) - or PKDL cases (33.9%) (Table 1). Of the 242 PKDL index cases with known rash type, 230 (95.0%) presented with macular rash, four (1.7%) with macular and papular rash, six (2.5%) with nodular and macular rash, and two (0.8%) with macular, nodular, and papular rash (Table 1). The 69 asymptomatic cases were associated with new VL cases (49.3%) or PKDL cases (50.7%), with the majority of such PKDL cases presenting with macular rash (94.3%) (Table 1).

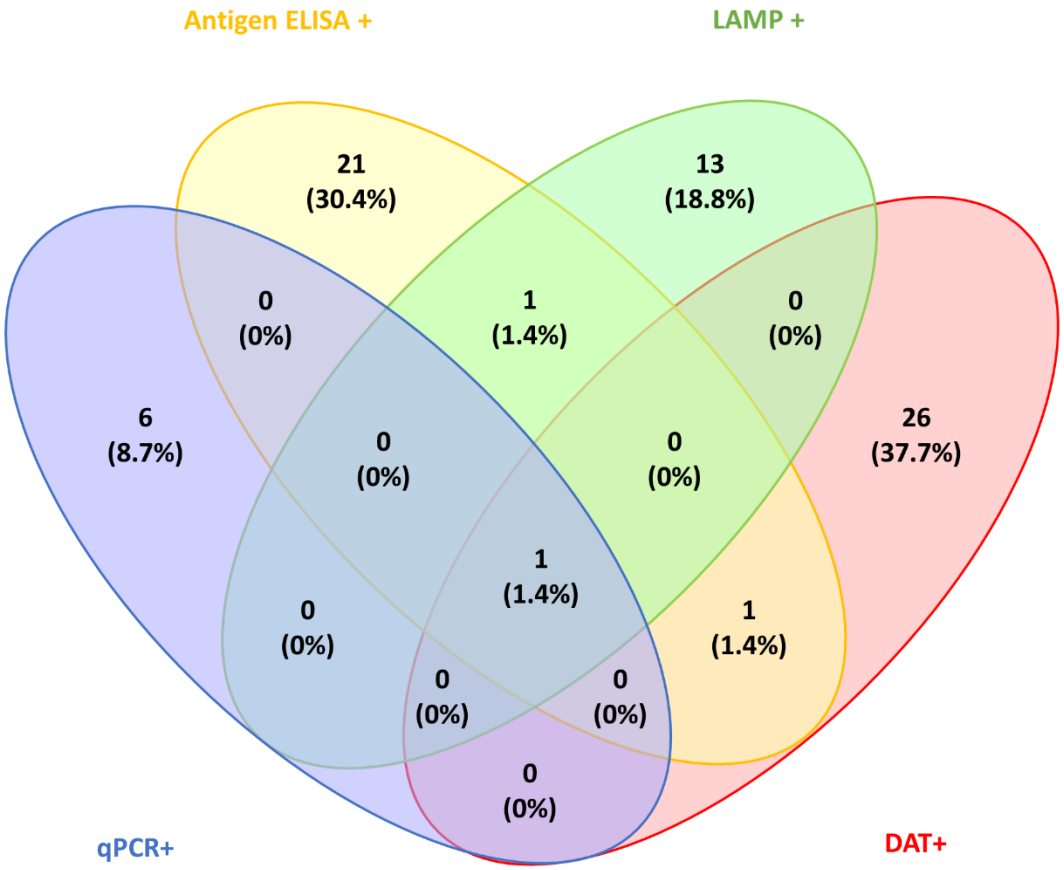
Table 1. Index cases were classified as new VL cases, relapsed VL cases, VL treatment failure or PKDL. Of the 720 contacts, 476 (66.1%) were associated with VL cases and 244 (33.9%) were associated with PKDL cases. Of the 69 participants positive for at least one test, 34 (49.3%) were VL cases and 35 (50.7%) were PKDL cases.

	720 contacts	**69 asymptomatics**
	N (%)	**N (%)**
Median Age (IQR)	27 (25)	30 (25)
Male	280 (38.9)	31 (44.9)
Occupation		
Students	248 (34.4)	23 (33.3)
Housewives	301.68 (41.9)	26 (37.7)
Lives within the household of an index case	505 (70.1)	50 (72.5)
VL	**476 (66.1)**	**34 (49.3)**
New VL case	429/476 (90.1)	34/34 (100.0)
Relapsed VL case	45/476 (9.5)	0
VL treatment failure	2/476 (0.4)	0
Post kala-azar dermal leishmaniasis (PKDL)	**244 (33.9)**	**35 (50.7)**
Macular rash	230/244 (95.0)	33/35 (94.3)
Macular and papular rash	4/244 (1.7)	0
Nodular and macular rash	6/244 (2.5)	0
Macular, nodular and papular rash	2/244 (0.8)	1/35 (2.9)
Rash type unknown	2/244 (0.8)	1/35 (2.9)

Estimates of asymptomatic infection in contacts of index cases using tests to detect active infection

Of the 720 participants screened, 69 (9.6%) were positive by at least one test. Of the 720 asymptomatic DNA samples screened, seven (1.0%) were positive by kDNA qPCR, with a mean Ct value of 31.9 (range 26.7 - 33.9). Urine samples were screened with the *Leishmania* antigen ELISA, of which 24 (3.3%) were found to be positive. Samples screened by DAT were considered positive at a titer of ≥1:1600. A total of 28 (3.9%) samples were found to be DAT positive, 11 (39.3%) of which had a titer ≥1:12,800. LAMP detected six (0.8%), eight (1.1%), and three (0.4%) asymptomatic infections when DNA was extracted using Qiagen kits, boil-and-spin, and from DBS, respectively. For the purposes of further analysis, a participant with a positive LAMP result from any one of the three extraction techniques was considered LAMP-positive, of which there were 15 (2.1%).

Leishmania antigen ELISA and the DAT identified the highest proportion of positive subjects. Only one (0.1%) subject was identified as positive by all four diagnostic methods, two (0.3%) were identified by two diagnostic methods, and 66 (9.2%) were identified by one diagnostic method only. In the 69 asymptomatic participants, 26 (37.7%) were positive by DAT only, and six (8.7%) were positive by qPCR only. Of the 24 (34.8%) participants positive by ELISA, three (4.3%) were positive by at least one other test (Fig. 1). Generally, poor agreement was found between tests. However, antigen and molecular tests showed better agreement in combination compared to the same tests in combination with serology (Table 2). In combination, the DAT and *Leishmania* antigen ELISA capture all participants positive by more than one of the four tests.



1380

1381 **Fig 1. Asymptomatic infection was detected in 69 (9.6%) contacts by a combination of four diagnostic**
1382 **tests.** DAT was positive in 28 (40.6%) participants, 26 (37.7%) of whom were positive for DAT alone,
1383 11/28 (39.3%) of whom had a titre greater than 1:12,800. qPCR was positive in seven (10.1%)
1384 participants, six (8.7%) of whom were positive for qPCR alone. LAMP was positive in 15 (21.7%)
1385 participants, 13 (18.8%) of whom were positive for LAMP alone. *Leishmania* antigen ELISA was positive
1386 in 24 (34.8%) participants, of whom 21 (30.4%) were positive for ELISA alone and three (4.3%) were
1387 positive by ELISA and at least one other test.

1388

Table 2. Kappa scores and agreement for four diagnostic tests in 69 asymptomatic participants

Test combination	Agreement (%)	Kappa score	p-value
DAT and *Leishmania* ELISA	30.4	-0.476	$6.8x10^{-5}$
DAT and qPCR	52.2	-0.126	0.135
DAT and LAMP	40.6	-0.330	0.003
Leishmania ELISA and qPCR	58.0	-0.110	0.230
Leishmania ELISA and LAMP	49.3	-0.225	0.049
LAMP and qPCR	71.0	-0.055	0.614

Risk factors for asymptomatic VL

Logistic regression was used to confirm risk factors associated with being positive for at least one of the four diagnostic tests. Age, gender, occupation, and living within the index household compared to neighbouring household were not found to be associated with asymptomatic infection. The index case being a PKDL case (OR 1.94, p = 0.009), specifically macular PKDL (OR 2.12, p = 0.004), was found to be significantly associated with being positive by at least one of the four tests.

Latent class analysis to estimate infection status and diagnostic accuracy in the absence of a gold standard

In the absence of a single reference standard or a composite reference standard, latent class analysis (LCA) was used to estimate infection status. LCA estimated qPCR, LAMP, DAT, and *Leishmania* antigen ELISA to have sensitivities (2.5 - 97.5 percentiles) of 85.6% (55.1 - 99.5), 99.8% (99.2 - 99.9), 97.5% (90.5 - 99.9), and 98.9% (96.2 - 99.9) and specificities of 96.1% (94.7 - 97.5), 96.7% (95.3 – 97.8), 99.0% (98.1 - 99.6), and 97.9% (96.7 - 98.9), respectively. The prevalence of *L. donovani* asymptomatic infection in VL and PKDL contacts in Bangladesh was estimated to be 0.3% (0.03 - 0.7).

Discussion

In this study, we assessed the utility of the DAT, qPCR, LAMP, and *Leishmania* antigen ELISA for detection of asymptomatic *Leishmania* infection in household or neighbouring contacts of VL and PKDL index cases in endemic regions of Bangladesh. Both the DAT and *Leishmania* antigen ELISA capture all samples which are positive by more than one test, and both utilise sample types that have a relatively non-invasive sample collection, which can be transported back to a central laboratory for testing.

The DAT detected the highest proportion of positive individuals. The DAT detects anti-*Leishmania* antibodies that could be circulating from a previously cleared asymptomatic infection. It is not possible to ascertain the time of infection in this cohort as it may be in a symptomatic cohort. However, a recent study found that DAT titers could be a useful tool to monitor transmission in an elimination setting during repeat surveys [14]. Plate-to-plate variation with the DAT in manufacturing and reading, and the relatively low-throughput nature have been previously suggested to be a limitation of this assay for monitoring and surveillance purposes [24]. Our findings are in concordance with previous studies in Bangladesh and India which used serological methods to detect asymptomatic infection [25,26]. Where qPCR requires more laboratory infrastructure, the *Leishmania* antigen ELISA and LAMP are relatively simple techniques suitable for use in resource poor settings. Furthermore, the *Leishmania* antigen ELISA

requires a non-invasive urine sample and is relatively high throughput, which may aid in screening of high numbers of asymptomatic contacts.

PKDL cases are a potential reservoir of *Leishmania* infection, with experimental infectivity to sand flies estimated to be between 32 and 53% [27]. Here, we demonstrate a risk factor for asymptomatic infection is living close to a PKDL case, specifically macular PKDL. This follows the launch of the World Health Organization's Road Map for Neglected Tropical Diseases 2021-2030, which identifies early detection through methods such as active case detection and development of treatments and diagnostics for both VL and PKDL, as critical actions for the elimination of VL as a public health concern [28]. Our data and the road map highlight the importance of diagnosis and follow-up of PKDL cases, in recognition of their potential role in transmission.

Previous studies have identified risk factors for VL broadly linked to poverty, such as mud walls, with sleeping off the floor found to reduce the risk [29]. Proximity to a previous VL case was identified as a risk factor for VL in Bangladesh [30]. No difference based on sex, occupation, or income, among others, was seen in an analysis of risk factors in the same study [30]. Age trends associated with VL infection were found to vary between studies; however, the prevalence of seropositivity was generally found to increase with age [31].

The specificity of all diagnostics falls below 100% for identification of *L. donovani* asymptomatic infection, according to the LCA conducted, and thus we may expect some false positives on a cohort of this size. This is more probable for the antibody detection test DAT rather than for the direct detection tests LAMP and antigen ELISA. Therefore, we have looked for overlap in tests which were positive. We acknowledge that sample size may have limited our analysis of risk factors. Additionally, we use latent class analysis to estimate the probability that a participant is tests positive at a population level; however, we do not apply this at the individual level for further analysis, given the potential for LCA to be unstable. A further limitation of the study is the lack of follow-up data, therefore the accuracy of the tests as predictors of progression to clinical disease is unknown.

Conclusions

In an elimination setting such as Bangladesh, where disease reservoirs are anthroponotic, a relatively simple test such as the *Leishmania* antigen ELISA, which requires a non-invasive urine sample and detects active infection, may be of benefit in combination with serology for surveillance and monitoring of *Leishmania* transmission. Since living with or close to a macular PKDL patient is a risk-factor for

asymptomatic infection, we propose the follow-up of contacts with PKDL patients as an operational priority. Development of an antigen detection test in RDT format would be of benefit to identify those contacts in the field.

Declarations

Acknowledgements

We would like to thank the field teams and the patients. FIND is grateful to its donors, public and private, who have helped bring innovative new diagnostics for diseases of poverty. A full list of FIND's donors can be found at: https://www.finddx.org/donors/.

Authors' contributions

SIO, FH, and PG contributed to data acquisition, data curation, analysis of data, and writing the manuscript, RC contributed to data acquisition, SH contributed to data curation, CJ contributed to analysis of data, IC contributed to conception and design, AP contributed to conception and design, DM contributed to conception and design, ERA contributed to conception and design. All authors contributed to manuscript revisions, read and approved the final manuscript.

Funding

Funding was received for this study from the German Federal Ministry of Education and Research (BMBF) through the KfW Entwicklungs bank, MRC-DTP (MR/N013514/1), and Wellcome Seed fund (108080/Z/15/Z).

Availability of data and materials

Data available upon request.

Ethics approval and consent to participate

This study was approved by the Ethical Review Committee (ERC) of the ICDDR,B (PR-14093). Adult participants provided written informed consent, and in the case of any participants under 18 years of age, a parent or guardian provided informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sengupta PC. History of kala-azar in India. Ind Med Gaz. 1947;82:281–6.

2. Hasker E, Malaviya P, Gidwani K, Picado A, Ostyn B, Kansal S, et al. Strong association between serological status and probability of progression to clinical visceral leishmaniasis in prospective cohort studies in India and Nepal. PLoS Negl Trop Dis. 2014;8:e2657.

3. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S. Asymptomatic *Leishmania* infection: a new challenge for *Leishmania* control. Clin Infect Dis. 2014;58:1424–9.

4. Bern C, Haque R, Chowdhury R, Ali M, Kurkjian KM, Vaz L, et al. The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. Am J Trop Med Hyg. 2007;76:909-14.

5. Das VNR, Siddiqui NA, Verma RB, Topno RK, Singh D, Das S, et al. Asymptomatic infection of visceral leishmaniasis in hyperendemic areas of Vaishali district, Bihar, India: a challenge to kala-azar elimination programmes. Trans R Soc Trop Med Hyg. 2011;105:661–6.

6. WHO. Process of validation of elimination of kala-azar as a public health problem in South-East Asia. 2016. https://img1.wsimg.com/blobby/go/c5156b45-48df-4ba4-ab15-be2bb6261d20/downloads/1bu1begqv_714167.pdf

7. Muniaraj M. The lost hope of elimination of kala-azar (visceral leishmaniasis) by 2010 and cyclic occurrence of its outbreak in India, blame falls on vector control practices or co-infection with human immunodeficiency virus or therapeutic modalities? Trop Parasitol. 2014;4:10–9.

8. Ready PD. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 2014;6:147–54.

9. Guarga JL, Lucientes J, Peribáñez MA, Molina R, Gracia MJ, Castillo JA. Experimental infection of *Phlebotomus perniciosus* and determination of the natural infection rates of *Leishmania infantum* in dogs. Acta Trop. 2000;77:203–7.

10. Mondal D, Ghosh P, Chowdhury R, Halleux C, Ruiz-Postigo JA, Alim A, et al. Relationship of serum antileishmanial antibody with development of visceral leishmaniasis, post-kala-azar dermal leishmaniasis and visceral leishmaniasis relapse. Front Microbiol. 2019; doi:10.3389/fmicb.2019.02268

11. Zijlstra EE, Daifalla NS, Kager † P A, Khalil EAG, El-Hassan AM, Reed SG, et al. RK39 enzyme-linked immunosorbent assay for diagnosis of *Leishmania donovani* infection. Clin. Diagn. Lab. Immunol. 1998;5:717-20.

12. Chappuis F, Rijal S, Soto A, Menten J, Boelaert M. A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. BMJ. 2006;333:723.

13. Meredith SEO, Kroon NCM, Sondorp E, Seaman J, Goris MGA, Van Ingen CW, et al. Leish-KIT, a stable direct agglutination test based on freeze-dried antigen for serodiagnosis of visceral leishmaniasis. J. Clin. Microbiol. 1995;33:1742-5.

14. Cloots K, Uranw S, Ostyn B, Bhattarai NR, Le Rutte E, Khanal B, et al. Impact of the visceral leishmaniasis elimination initiative on *Leishmania donovani* transmission in Nepal: a 10-year repeat survey. Lancet Glob Heal. 2020;8:e237–43.

15. Gidwani K, Picado A, Ostyn B, Singh SP, Kumar R, Khanal B, et al. Persistence of *Leishmania donovani* antibodies in past visceral leishmaniasis cases in India. Clin Vaccine Immunol. 2011;18:346–8.

16. Hossain F, Ghosh P, Khan MAA, Duthie MS, Vallur AC, Picone A, 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.

17. Adams ER, Schoone G, Versteeg I, Gomez MA, Diro E, Mori Y, 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.

18. Mukhtar M, Ali SS, Boshara SA, Albertini A, Monnerat S, Bessell P, et al. Sensitive and less invasive confirmatory diagnosis of visceral leishmaniasis in Sudan using loop-mediated isothermal amplification (LAMP). PLoS Negl Trop Dis. 2018;12:e0006264.

19. Vallur AC, Tutterrow YL, Mohamath R, Pattabhi S, Hailu A, Abdoun AO, et al. Development and comparative evaluation of two antigen detection tests for visceral leishmaniasis. BMC Infect Dis. 2015;15:1–10.

20. Hasker E, Kansal S, Malaviya P, Gidwani K, Picado A, Singh RP, et al. Latent Infection with *Leishmania donovani* in highly endemic villages in Bihar, India. PLoS Negl Trop Dis. 2013;7:e2053.

21. Oliveros JC. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html

22. van Smeden M, Naaktgeboren CA, Reitsma JB, Moons KGM, de Groot JAH. Latent class models in diagnostic studies when there is no reference standard--a systematic review. Am J Epidemiol. 2014;179:423–31.

23. Marshall J. Latent class analysis in R. 2015. https://raw.githubusercontent.com/jmarshallnz/lcar/master/lca.R

24. Vallur AC, Reinhart C, Mohamath R, Goto Y, Ghosh P, Mondal D, et al. Accurate serodetection of asymptomatic *Leishmania donovani* infection by use of defined antigens. J Clin Microbiol. 2016;54:1025-30.

25. Banu SS, Meyer W, Ahmed B-N, Kim R, Lee R. Detection of *Leishmania donovani* in peripheral blood of asymptomatic individuals in contact with patients with visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2016;110:286–93.

26. Rabi Das VN, Bimal S, Siddiqui NA, Kumar A, Pandey K, Sinha SK, et al. Conversion of asymptomatic infection to symptomatic visceral leishmaniasis: a study of possible immunological markers. PLoS Negl Trop Dis. 2020;14:e0008272.

27. Hirve S, Boelaert M, Matlashewski G, Mondal D, Arana B, Kroeger A, et al. Transmission dynamics of visceral leishmaniasis in the Indian Subcontinent – a systematic literature review. PLoS Negl Trop Dis.2016;10:e0004896.

28. World Health Organization. Ending the neglect to attain the sustainable development goals: a road map for neglected tropical diseases 2021–2030. 2020. http://apps.who.int/bookorders.

29. Bern C, Courtenay O, Alvar J. Of cattle, sand flies and men: a systematic review of risk factor analyses for South Asian visceral leishmaniasis and implications for elimination. PLoS Negl Trop Dis. 2010;4:e599.

30. Bern C, Hightower AW, Chowdhury R, Ali M, Amann J, Wagatsuma Y, et al. Risk factors for kala-azar in Bangladesh. Emerg Infect Dis. 2005;11:655–62.

31. Chapman Id LAC, Morgan Id ALK, Adams ER, Bern C, Medley GF, Dé Irdre Hollingsworth T. Age trends in asymptomatic and symptomatic *Leishmania donovani* infection in the Indian subcontinent: a review and analysis of data from diagnostic and epidemiological studies. PLoS Negl Trop Dis. 2018;12:e0006803.

Chapter 3. Prevalence and determinants of asymptomatic *Leishmania* infection in HIV-infected individuals living within visceral leishmaniasis endemic areas of Bihar, India

3.1 Acknowledgement of work done

Sakib Burza, Amit Harshana and Shahwar Kazmi were responsible for conception and design of the study, and study oversight. Sophie I. Owen was responsible for ethics applications in the UK. Emily R. Adams and Sophie I. Owen were responsible for setting up the LSTM laboratory within the RMRIMS, Patna which can run highly sensitive molecular tests and for providing training opportunities to staff at the RMRIMS. Vikash Kumar, Louise Ford, and Sophie I. Owen were responsible for the procurement needs of the study. Shiril Kumar conducted the DNA extraction, qPCR and rK39 ELISA. Sophie I. Owen conducted the *Leishmania* antigen ELISA and monitoring visits. Sophie I. Owen was responsible for data analysis supported by Raman Mahajan. Sophie I. Owen wrote the full draft for publication supported by Raman Mahajan. Sakib Burza had overall responsibility of the study including ethics and sponsorship.

3.2 Introduction

Following on from work conducted in chapter 2, looking at the prevalence and methods to detect ALI in Bangladesh, I now focus on ALI in PLHIV in India. Literature shows that the HIV populations have increased likelihood of VL, poor outcomes associated with VL, and higher parasite loads in some instances, than an immunocompetent population [3,15,17,99]. HIV infection was previously detected in 5.6% (n=116) of individuals ≥14 years of age with VL, screened in Bihar between 2011 and 2013, the same setting as the study presented in this chapter [86]. However, the number of PLHIV with ALI in India remains to be determined.

Much of the data on the prevalence of ALI in PLHIV come from studies conducted in the Mediterranean, East Africa, and Brazil. A study in Brazil found that the prevalence of ALI was higher in HIV coinfected patients than comparative studies in the general population [160]. A longitudinal study of 511 individuals in Ethiopia, using similar serological, molecular, and antigenuria detection methods to those presented in this chapter, found that the prevalence of ALI in PLHIV was 12.8% and 4.2% in male and female participants respectively [161]. Most infections were identified by rK39 RDT (7.4%, n=38), and DAT (4.3%, n=22), with one (0.2%) participant detected by PCR and KAtex [161]. The same study found that being male, and a concurrent malaria infection, were independent risk factors for ALI with odds ratios of 3.2 and 6.1, respectively [161].

As mentioned in chapter 2, the number of asymptomatic infections are found to outnumber VL cases with the ratio of asymptomatic to symptomatic infections in immunocompetent individuals estimated to be nine to one in a study in the highly endemic regions of Bihar, India, and Nepal between 2006 and 2009 [57]. However, to our knowledge there are no data on the prevalence of and risk factors for ALI in PLHIV in India, and the best methods to identify this population. Given previous studies of the prevalence of ALI and pathogenesis of *Leishmania*-HIV coinfection, we could therefore expect the prevalence of asymptomatic infections in PLHIV in India to be higher than an immunocompetent population, however this is yet to be determined.

Methods to detect ALI in PLHIV are yet to be established in India. Current antibody-based diagnostics are unable to distinguish between past and current infection and therefore would need to be used in combination with a clinical history of VL or PKDL when screening individuals who may be asymptomatically infected. Little is known about how long anti-*Leishmania* antibodies from an asymptomatic infection may persist in PLHIV. Vogt *et al*. longitudinal study in Ethiopia found the probability of losing markers (serological, antigen, and molecular) of ALI in HIV-infected individuals was

40.1% at one year of follow-up, which did not change when restricted to serological markers only [161]. The KAtex antigen detection assay was evaluated in a cohort with symptomatic VL-HIV coinfection in Ethiopia with a sensitivity of 84%, and in a cohort of PLHIV with no symptoms or history of VL in Spain, where no asymptomatic infections were detected by the KAtex, despite 34% being positive by qPCR [162,163]. In the latter study, only 3.5% of participants had detectable anti-*Leishmania* antibodies [163]. The KAtex was further developed by Kalon Biological (Guilford, UK) into a sandwich ELISA which detects low molecular weight carbohydrate excreted in the urine of those infected with *Leishmania*.

In the cross-sectional study presented in this chapter 3, we determine the prevalence and determinants of asymptomatic *L. donovani* infection in a cohort of PLHIV presenting consecutively at ART centres across the endemic districts of Bihar, India. We use rK39 serology (rK39 RDT and rK39 ELISA) and qPCR to detect ALI. Additionally, antigenuria was detected by the *Leishmania* antigen ELISA (Clin-Tech, UK).

Chapter three summary

The prevalence of ALI was found to be 7.4% (n=96), with an additional 20 participants identified as having antigenuria by *Leishmania* antigen ELISA in a cohort of 1,296 PLHIV. All positive participants were identified by rK39 ELISA and *Leishmania* antigen ELISA when used in combination. Similarly, low CD4 counts, and a larger household size were found to be a risk factor for ALI. This cross-sectional cohort was monitored longitudinally to better understand progression to VL and is presented in a chapter 4.

3.3 Prevalence and determinants of asymptomatic *Leishmania* infection in HIV-infected individuals living within visceral leishmaniasis endemic areas of Bihar, India

Sophie I Owen[2, ¶], Raman Mahajan[1, ¶], Shiril Kumar[3], Krishna Pandey[3], Shahwar Kazmi[1], Vikash Kumar[1], Emily R Adams[2], Amit Harshana[1], Sakib Burza[1, *]

[1] Médecins Sans Frontières (MSF), New Delhi, India

[2] Department of Tropical Disease Biology, Liverpool School of Tropical Medicine (LSTM), Liverpool, UK

[3] Rajendra Memorial Research Institute of Medical Sciences, Patna, Bihar, India

* Corresponding author

E-mail: Sakib.Burza@barcelona.msf.org (SB)

[¶] RM and SIO are joint first authors

Mahajan R &Owen S. I. *et al.* PLOS NTDs (2022)

Abstract

People living with HIV (PLHIV) have an increased risk of developing visceral leishmaniasis (VL) and poor outcomes compared to HIV negative individuals. Here, we aim to establish the prevalence and determinants of asymptomatic *Leishmania* infection (ALI) in a cohort of PLHIV in Bihar, India. We hoped to evaluate optimal diagnostic algorithms to detect ALI in PLHIV. We conducted a cross-sectional survey of PLHIV ≥18 years of age with no history or current diagnosis of VL or post kala-azar dermal leishmaniasis (PKDL) at anti-retroviral therapy centres within VL endemic districts of Bihar. ALI was defined as a positive rK39 enzyme-linked immunosorbent assay (ELISA), rK39 rapid diagnostic test (RDT) and/or quantitative polymerase chain reaction (qPCR). Additionally, the urinary *Leishmania* antigen ELISA was evaluated. Determinants for ALI were established using logistic regression and agreement between diagnostic tests calculated using Cohen's Kappa. A total of 1,296 PLHIV enrolled in HIV care, 694 (53.6%) of whom were female and a median age of 39 years (interquartile range 33–46), were included in the analysis. Baseline prevalence of ALI was 7.4% (n=96). All 96 individuals were positive by rK39 ELISA, while 0.5% (n=6) and 0.4% (n=5) were positive by qPCR and rK39 RDT, respectively. Negligible or weak agreement was seen between assays. Independent risk factors for ALI were CD4 counts <100 (OR 3.1; 95% CI 1.2–7.6) and CD4 counts 100-199 (OR=2.1; 95% CI: 1.1-4.0) compared to CD4 counts ≥300, and a household size ≥5 (OR=1.9; 95% CI: 1.1-3.1). A total of 2.2% (n=28) participants were positive by *Leishmania* antigen ELISA, detecting 20 additional participants to the asymptomatic cohort. Prevalence of ALI in PLHIV in VL endemic villages in Bihar was relatively high. Using the *Leishmania* antigen ELISA, prevalence increased to 9.0%. Patients with low CD4 counts and a larger household size were found to have significantly higher risk of ALI.

Author Summary

People living with HIV (PLHIV) are more likely to develop visceral leishmaniasis (VL) and are more likely to have poor outcomes associated with VL-HIV coinfection than HIV negative individuals. While an estimated 3-14% of immunocompetent individuals living in endemic areas show serological evidence of asymptomatic infection with VL, such data on *Leishmania* infection in PLHIV in India are lacking. As such we sought to establish the prevalence and determinants of asymptomatic *Leishmania* infection (ALI) in PLHIV in VL endemic districts of Bihar, India using a combination of molecular and serological assays. We further expand our definition of ALI to include the urinary *Leishmania* antigen ELISA. The prevalence of ALI in a population of PLHIV residing in VL endemic districts of Bihar, India was found to be as high as 9.0% (n=116). The majority of individuals were detected by the rK39 enzyme-linked immunosorbent assay (ELISA) (7.4%, n=96), and very few individuals were detected by quantitative polymerase chain reaction (qPCR) (0.5%, n=6) or the rK39 rapid diagnostic test (RDT) (0.4%, n=5). In total, 2.2% (n=28) were positive by urinary *Leishmania* antigen ELISA. Low CD4 counts and larger household size were found to be risk factors for ALI. Here, we add to the evidence base for *Leishmania*-HIV coinfection on the Indian subcontinent.

Introduction

Human immunodeficiency virus (HIV) and leishmaniasis are found to be co-endemic in several regions (1). Infection with the protozoa *Leishmania donovani* (*L. donovani*) can remain asymptomatic or can lead to visceral leishmaniasis (VL), with asymptomatic infections outnumbering clinical infections by an estimated nine times on the Indian subcontinent (ISC) (2). The risk of developing VL in people living with HIV (PLHIV) is estimated to be far higher compared to HIV negative individuals (3). Furthermore, risk of poor clinical outcomes from VL-HIV such as treatment failure and relapse are increased, with the virus and parasite mutually accelerating disease progression (3–6).

In 2019, within the state of Bihar, India, there were estimated to be 134,000 PLHIV representing 0.18% of the state population and the second highest number of new infections behind the state of Maharashtra (7). There are limited data on VL-HIV coinfection in India. In 2014, a consecutive HIV screening of 2,077 people over 13 years of age with VL infection in Bihar, found a 5.6% coinfection rate (8). The Indian National AIDS Control Organisation (NACO) subsequently recommended screening all patients presenting with VL for HIV; and inversely recommended screening all HIV patients living in VL

endemic areas for VL (9). However, there are no optimal screening methods for the latter cohort in this setting (10).

Furthermore, where asymptomatic *Leishmania* infection (ALI) may represent an anthroponotic reservoir on the ISC (11), data on prevalence and determinants for ALI and guidelines on optimal screening algorithms in PLHIV are absent. This evidence gap is potentially important; the majority of patients diagnosed with VL-HIV present at a late stage with advanced HIV; assuming a reasonably high progression from asymptomatic to symptomatic VL infection in PLHIV, the utility of a tool that could potentially be used to identify the subclinical form earlier could be of major benefit in the early detection and management of this co-infection. Such screen-and-treat strategies in East Africa have been conceptionally described elsewhere (12).

As the effort to eliminate VL as a public health problem has progressed substantially in the ISC, the proportion of patients with VL-HIV has increased both in absolute numbers and as a proportion of all VL cases. Indeed, more recent analysis of the epidemiological spread and impact of VL-HIV has suggested that the presence of VL-HIV cases was associated with a greater than two-fold increase in VL incidence at the village level, with an incidence risk ratio similar to that of post kala-azar dermal leishmaniasis (PKDL) (13). As such, establishing the scale of asymptomatic infections in HIV patients may contribute significantly to improved programmatic policy in sustaining elimination targets.

Molecular methods to detect *Leishmania* kinetoplast DNA (kDNA) such as quantitative polymerase chain reaction (qPCR) are highly sensitive techniques but require a good laboratory set-up and expertise. The rK39 enzyme-linked immunosorbent assay (ELISA) and rapid diagnostic test (RDT) detect anti-*Leishmania* antibodies and are used in the diagnosis of VL, however they may detect convalescence and have shown a reduced sensitivity in HIV infection in a study in Ethiopia (14). The *Leishmania* antigen ELISA detects *Leishmania* carbohydrate in a urine sample, making it a non-invasive test which detects active infection (15); however, the assay remains research use only and there are few data to support use in an asymptomatic population (16).

The primary objective of this cross-sectional study was to determine the prevalence of ALI in PLHIV residing in VL endemic areas in Bihar. In doing so, we seek to evaluate and correlate results of different diagnostic tools to detect ALI in PLHIV on the ISC. Finally, we determined risk factors for asymptomatic infection in this cohort.

Methods

Study design, population, and recruitment

Over a period of 12 months commencing in May 2018, PLHIV residing in VL endemic villages presenting to three anti-retroviral therapy (ART) centres in one of four VL endemic districts (Saran, Siwan, Muzaffarpur, and Gopalganj) in the state of Bihar, India were screened. Enrolment was open to PLHIV aged ≥ 18 years at any stage of illness, on the condition that they resided in a list of pre-specified villages which had reported at least one VL infection in 2017-18 as per the government kala-azar management information system (KA-MIS). PLHIV with a history of previous treatment for or current diagnosis of symptomatic VL or PKDL were excluded, as was any patient presenting in critical condition or with a severe underlying medical condition whose participation in the study may interfere with immediate medical intervention.

Sample Size

At the time of design, there were few reliable estimate data available on the prevalence of ALI in PLHIV, and none from the Indian context. As such, evidence of ALI in non-immunocompromised individuals living in endemic areas was taken as a point estimate – this ranged from 3.16% to 14% (17–20). Assuming that PLHIV living within endemic areas would be more likely *a priori* to have ALI due to the degree of lessened immunity, we used an upper threshold of 15% as a likely estimate in PLHIV, in keeping with similar studies in East Africa (21). A total of 784 and 1352 participants were required to allow for a precision of 2.5% at a confidence level of 95% and 99% respectively. As a lower number of participants would preclude further planned studies on monitoring progression of ALI patients, we targeted the higher number.

Recruitment

Over the 12-month recruitment period, the study team rotated between the four ART centres. All patients presenting to the ART centre on the recruitment day were consecutively screened, with a daily maximum target of 20 eligible consenting participants to ensure manageable workload and allow adequate time to transport samples back to the state capital under cold chain. A screening log was maintained to prevent re-enrolment, and to ensure patients who had previously declined to enrol were not reapproached. Sociodemographic data were collected from all enrolled patients, followed by a comprehensive clinical examination.

Blood and urine were then collected for serological and molecular testing, while an immediate rK39 RDT (Kala-azar Detect Rapid Test, Inbios International Inc., WA, USA) was performed on all patients. Any patient meeting the clinical case definition of VL (fever, splenomegaly and a positive rK39 RDT) were immediately referred to a specialist VL-HIV treatment centre in Patna for further assessment and excluded from the study.

HIV-related information including duration of diagnosis, World Health Organization (WHO) clinical staging, and presence of opportunistic infections were collected, as was information on current and past medical conditions. Routine clinical parameters were documented, while nutritional status was determined based on the body mass index (BMI).

Blood and urine were used for determining ALI through serological methods (rK39 RDT and ELISA), molecular methods (qPCR), and the urinary *Leishmania* antigen ELISA (detailed below). Blood was also used for CD4 counts, full blood counts, and HIV viral load. Urine was used for those with CD4 counts <200 cells/mm^3 to test for lipoarabinomannan (LAM) using the Determine TB-LAM point-of-care tuberculosis assay (Abbott Diagnostics, Lake Bluff, IL, USA). All samples were stored at -80°C and run in batches over the course of the study, with all remaining samples retained in the biobank repository for future research.

ALI was defined as a positive rK39 RDT, rK39 ELISA, and/or qPCR in the absence of clinical symptoms and history of VL or PKDL. A positive urinary *Leishmania* antigen ELISA was not considered ALI in the primary analysis as there were no performance data on the *Leishmania* antigen ELISA in an asymptomatic population and few data in a symptomatic population at the time of study design but was included as ALI in a secondary analysis (detailed extensively in supplementary materials).

rK39 RDT and ELISA

rK39 RDTs (Kala-azar Detect Rapid Test, Inbios International Inc., USA) were performed using finger-prick capillary blood. Venous blood collected in ethylenediaminetetraacetic acid (EDTA)-treated vacutainers was transported to Patna on ice and stored at 4˚C until centrifugation at 3,000rpm for 15 minutes to separate plasma. For the rK39 ELISA, plates were coated overnight at 4˚C with rK39 antigen and blocked the next day for two hours at 25˚C in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Plates were washed five times with wash buffer (0.1% TWEEN-20 in PBS). Following washing, 100µl of each plasma sample, and positive and negative controls diluted 1:400 were incubated on the plate for 30 minutes at 25˚C. Plates were washed as before, followed by addition of 100µl of HRP-

conjugated secondary antibody for 30 minutes at 25˚C. Plates were washed as before and 100µl 3,3',5,5'-tetramethylbenzidine (TMB) substrate added for five minutes in the dark. The reaction was stopped with 1N sulfuric acid. The optical density (OD) was read at 450nm. Results were expressed as the percentage positivity of the positive control.

QPCR

DNA was extracted from 100µl peripheral blood using DNeasy Blood and Tissue Kits (Qiagen, Germany) as per the manufacturer's instructions. Following extraction, appropriate volumes of Quantifast mastermix from the Qiagen QuantiFast PCR Kit, nuclease-free water, kDNA forward and reverse primers and probe were prepared (22). A total of 1.25µl of extracted DNA was added to each well containing mastermix to a total volume of 12.5µl per well. Samples were loaded onto the BioRad Real-Time PCR Detection System: C1000 Touch and CFX-96 and run at: 1. 95˚C for five minutes for the initial denaturation; 2. 95˚C for 15 seconds at following denaturation steps; 3. 60˚C for 30 seconds for annealing and elongation; 4. repetition of steps 2-3, followed by a further 34 cycles. A pre-specified Ct value of <35 was considered positive. Although not run in duplicate, PCR was run with negative, positive and extraction controls.

Leishmania antigen ELISA in urine samples

The *Leishmania* antigen ELISA (Clin-Tech, Guilford, UK) was carried out on urine transported to the Rajendra Memorial Research Institute of Medical Science (RMRIMS) in 15ml falcon tubes on ice and stored at -20°C until testing according to manufacturer's instructions. Briefly, 10µl of urine was diluted 1 in 20 in assay diluent, applied to a pre-coated plate and incubated at 37˚C for 30 minutes. Plates were then washed three times before addition of a secondary detection antibody conjugated to horseradish peroxidase. Following incubation at 37˚C for 30 minutes plates were washed as before and TMB substrate added for 30 minutes at room temperature. The reaction was then stopped with addition of a weak acid solution, the plates read within 30 minutes at 450nm and 620nm and the OD values recorded. Any samples with an OD value less than or equal to that of the 2UAU/ml calibrator were considered negative. Concentration of urinary antigen (UAU/ml) was calculated using a standard curve of calibrators and four-parameter curve fitting software, and dilution factor corrected for.

Statistical analysis

Anonymised data were entered into the database from case report forms by double data entry. The primary outcome measure was the prevalence of ALI. Numerical variables such as age, household size,

time to ART, and baseline CD4 counts were grouped into categories as seen in other studies of ALI. A household was defined as a "group of persons who commonly live together and would take their meals from a common kitchen unless the exigencies of work prevented any of them from doing so" as per the Indian census definition of a household. At the time of study design, the latest consensus had found the average household size in Bihar to be 5.5 people per house, and as such, household size was split into <5 or ≥5. Socioeconomic status was divided into five categories based on the BG Prasad Scale (23). Individuals were classified as severely underweight (BMI<16.5 kg/m^2), underweight (BMI 16.5-18.5 kg/m^2), normal (BMI 18.5-25 kg/m^2), and overweight (>25 kg/m^2). All continuous variables were summarised as mean (standard deviation) and median (inter-quartile range). Categorical data were presented as counts and percentages. The difference in proportion was analysed by the chi-square or Fisher's exact test. Student's t and Wilcoxon rank sum (Mann-Whitney) tests were done to assess differences in mean and median parameters, respectively. The Association of all covariates and the outcome were assessed one by one in univariate analyses. Odds ratio calculations and 95% confidence intervals around proportions in a second step were carried out, and covariates with p-value < 0.2 in the univariate model were included in a logistic regression model. A backward stepwise selection method was applied to determine the independent risk factors for asymptomatic *Leishmania* infection. A p-value ≤ 0.05 was considered a statistically significant difference. Agreement between two tests was calculated using Cohen's Kappa and the level of agreement was interpreted according to Landis and Koch scale (24). Data analysis was carried out in R Studio (version 1.3.1056) and SPSS (version 23 Armonk, NY: IBM Corp).

Ethics

Informed written consent was obtained from all participants. Ethical approval for this study was granted by Médecins Sans Frontières (MSF) (Ref: 1763). Rajendra Memorial Research Institute of Medical Sciences (Ref: 02/RMRI/EC/2017) and the Liverpool School of Tropical Medicine (LSTM) (Ref: 18-087). The study was prospectively registered at the Clinical Trial Registry India: CTRI/2017/03/008120.

Results

Prevalence and determinants of asymptomatic Leishmania infection in PLHIV

A total of 2,993 individuals were screened, of those, 1,697 individuals did not meet the inclusion criteria and were excluded from the study (Fig 1).

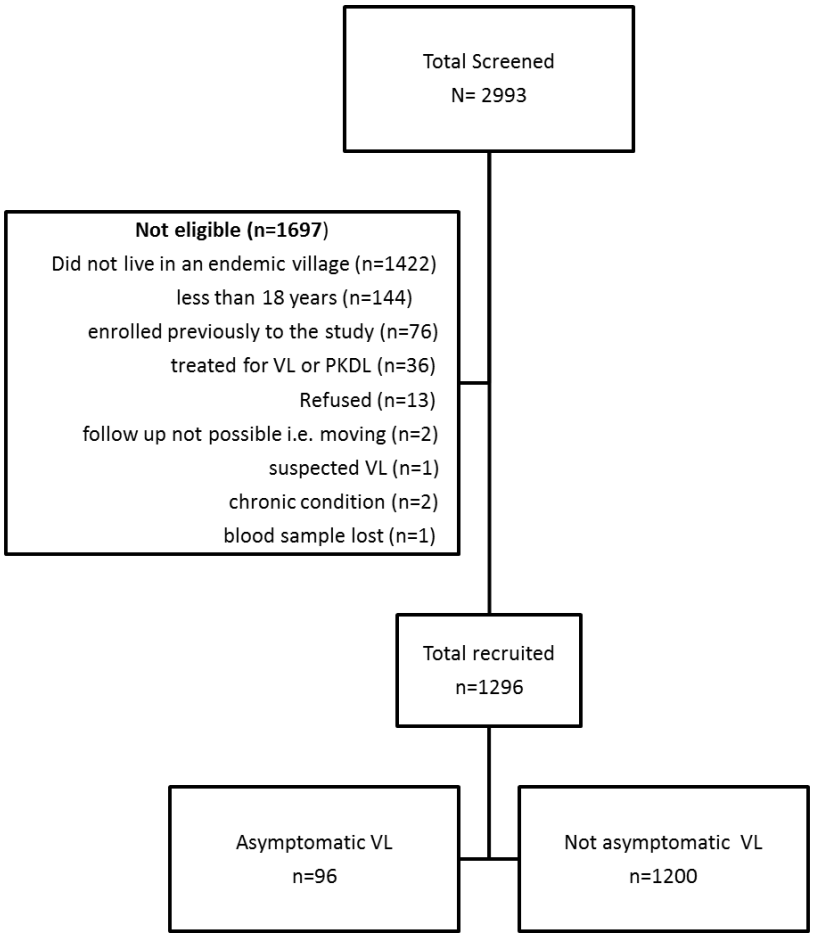


Fig 1. Flow diagram showing recruitment of study participants in Bihar, India between May 2018 and June 2019.

Of the 1,296 PLHIV enrolled in the study, 7.4% (n=96) met the primary study definition of ALI, detected by rK39 ELISA, rK39 RDT, and/or qPCR. Of the 96 with ALI, the median age was 41 (interquartile range (IQR): 33-50), and 46 (47.9%) were female. Of the ALI and non-ALI (PLHIV negative to all three tests) cohort, 95.8% and 97.8% of patients were on ART respectively, with a median of 32 months on treatment (IQR 12-63). On enrolment, the median CD4 count was 443 cells/mm^3 (IQR: 303-595) with counts significantly lower in the ALI cohort. Baseline temperature was significantly lower in the non-ALI cohort, although the mean body temperature was within a normal range in both the ALI and non-ALI groups. No significant difference on all other baseline vitals and haematology was seen between the non-asymptomatic and asymptomatic cohort (Table 1).

Table 1. Comparison of baseline clinical parameters and haematology results in 1,296 people living with HIV (PLHIV) with and without asymptomatic *Leishmania* infection (ALI) in Bihar, India.

	All Mean (SD)	ALI (SD) (n=96)	Non-ALI (SD) (n=1200)	Mean difference (95% CI)	Sig. (2-tailed)
CD4 (cells/mm^3)	466 (230)	400 (227)	471 (229)	-71 (-119, -24)	**.003**
Total white cell (count $x10^3/\mu L$)	7.3 (2.4)	7.2 (2.7)	7.3 (2.4)	-0.1 (-0.6, 0.4)	.689
Total red cell (count $x10^6/\mu L$)	3.9 (0.7)	3.9 (0.9)	3.9 (0.7)	0 (-0.1, 0.2)	.938
Haemoglobin (g/dL)	12.2 (2.1)	12 (2.1)	12.2 (2.1)	-0.2 (-0.6, 0.2)	.396
Hematocrit (%)	36.2 (5.6)	35.9 (5.5)	36.2 (5.6)	-0.3 (-1.5, 0.8)	.583
Platelet ($x10^3/\mu L$)	209 (89.1)	213 (101.7)	209 (88.1)	4.2 (-14.3, 22.8)	.654
Lymphocyte (%)	28.1 (8.8)	27.4 (9.7)	28.1 (8.7)	-0.7 (-2.5, 1.2)	.465
Neutrophil (%)	56.5 (12.2)	55.6 (12.9)	56.5 (12.1)	-0.9 (-3.5, 1.6)	.480
Axillary body temperature (°F)	97.5 (1.1)	97.8 (1)	97.5 (1.1)	0.3 (0.1, 0.5)	**.007**
Pulse/minute	90.8 (12.2)	92.1 (14.2)	90.7 (12)	1.4 (-1.1, 3.9)	.274
Systolic blood pressure (mmHg)	113.3 (16.1)	113.6 (18.1)	113.3 (15.9)	0.3 (-3, 3.7)	.849
Diastolic blood pressure (mmHg)	72.2 (10.4)	71.9 (9.7)	72.2 (10.5)	-0.4 (-2.5, 1.8)	.738
Oxygen saturation (SpO2) (%)	98.3 (1.5)	98.5 (1.5)	98.3 (1.5)	0.2 (-0.2, 0.5)	.337

Median HIV viral load at baseline in individuals with ALI was found to be 20.0 copies/ml (IQR: 1.0-218.2) (Table 2). HIV viral load was not run in the non-ALI cohort. Of the 139 PLHIV with CD4 count <200 cells/mm^3, 18 (13%) were positive to TB-LAM test.

Table 2. Comparison of baseline clinical parameters and haematology results in 1,296 people living with HIV (PLHIV) with and without asymptomatic *Leishmania* infection (ALI) in Bihar, India.

Viral Load (copies/ml)	N (%)
Undetectable	39 (44.3)
<150	26 (29.5)
150 to 999	6 (6.8)
1,000 to 9,999	1 (1.1)
10,000 to 99,999	9 (10.2)
100,000 to 1,000,000	6 (6.8)
≥1,000,000	1 (1.1)
Missing	8 (8.3)
Median (IQR)	20 (1.0-218)

In a univariate analysis, sex, age, socioeconomic status, type of house, proximity to a pond or livestock, time since last indoor residual spraying (IRS), number of IRS rounds in the last 18 months, contact with a presumptive VL case, contact with a presumptive PKDL case, contact with a cured VL/PKDL case, and the use of bed nets were not significant determinants for ALI (Table 3). Having a household size ≥5 people was found to be a risk factor for ALI compared to a smaller household OR=1.9 (95% CI: 1.1-3.1).

Table 3. Household-related risk factors for asymptomatic *Leishmania* infection (ALI) among 1,296 people living with HIV (PLHIV) in Bihar, India.

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95% CI)	P value
Sex					
Female	694 (53.5)	648 (54)	46 (47.9)	Ref	
Male	602 (46.5)	552 (46)	50 (52.1)	1.3 (0.8, 1.9)	0.250
Age (in years)					
18-29	174 (13.4)	159 (13.3)	15 (15.6)	Ref	
30-44	731 (56.4)	686 (57.2)	45 (46.9)	0.7 (0.4, 1.3)	0.240
45-59	329 (25.4)	296 (24.7)	33 (34.4)	1.2 (0.6, 2.2)	0.610
≥ 60	62 (4.8)	59 (4.9)	3 (3.1)	0.5 (0.1, 2)	0.420
Median (IQR)	39 (33-46)	39 (33-46)	41 (33-50)		0.150
Household size					
< 5	395 (30.5)	376 (31.3)	19 (19.8)	Ref	
≥ 5	901 (69.5)	824 (68.7)	77 (80.2)	1.9 (1.1, 3.1)	**0.020**
Median (IQR)	6 (4-7)	6 (4-7)	6 (5-7)		0.271
Socioeconomic status classification					
1 or 2	130 (10.1)	122 (10.2)	8 (8.3)	Ref	
3	333 (25.7)	297 (24.8)	36 (37.5)	1.9 (0.8, 4.1)	0.125
4	513 (39.6)	480 (40.1)	33 (34.4)	1.1 (0.5, 2.3)	0.908
5	318 (24.6)	299 (25.0)	19 (19.8)	1.0 (0.4, 2.3)	0.942

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95% CI)	P value
Type of house					
Brick	619 (47.8)	571 (47.6)	48 (50.0)	Ref	
Thatched	281 (21.7)	261 (21.8)	20 (20.8)	0.9 (0.5, 1.6)	0.738
Mud	396 (30.6)	368 (30.7)	28 (29.2)	0.9 (0.6, 1.5)	0.686
Proximity to a pond					
No	1,028 (79.3)	945 (78.8)	83 (86.5)	Ref	
Yes	268 (20.7)	255 (21.3)	13 (13.5)	0.6 (0.3, 1.1)	0.066
Proximity to livestock					
No	534 (41.2)	494 (41.2)	40 (41.7)	Ref	
Yes	762 (58.8)	706 (58.8)	56 (58.3)	1.0 (0.6, 1.5)	0.924
Time of last IRS (months)					
< 6	998 (77.0)	915 (76.3)	83 (86.5)	Ref	
Never	144 (11.1)	137 (11.4)	7 (7.3)	0.6 (0.3, 1.2)	0.150
6-12	124 (9.6)	118 (9.8)	6 (6.3)	0.6 (0.2, 1.3)	0.177
> 12	30 (2.3)	30 (2.5)	0 (0)	0 (0, 1.5)	0.154
Number of IRS rounds in the last 18 months					
0	140 (10.8)	133 (11.1)	7 (7.3)	Ref	
1	132 (10.2)	128 (10.7)	4 (4.2)	0.6 (0.1, 2.1)	0.540
2	642 (49.5)	593 (49.4)	49 (51.0)	1.6 (0.7, 3.8)	0.270
> 2	382 (29.5)	346 (28.8)	36 (37.5)	2.0 (0.9, 4.6)	0.100

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95% CI)	P value
Contact with people with presumptive VL 50 metres around the house					
No/Don't know	1,236 (95.4)	1,145 (95.4)	91 (94.8)	Ref	
Yes	60 (4.6)	55 (4.6)	5 (5.2)	1.0 (0.4, 2.8)	0.991
Contact with people with presumptive PKDL 50 metres around the house					
No/ Don't know	1,278 (98.6)	1,184 (98.7)	94 (97.9)	Ref	
Yes	18 (1.4)	16 (1.3)	2 (2.1)	1.6 (0.2, 6.1)	0.780
Contact with people with cured VL/ PKDL 50 metres around the house					
No/ Don't know	1,262 (97.4)	1,170 (97.5)	92 (95.8)	Ref	
Yes	34 (2.6)	30 (2.5)	4 (4.2)	1.7 (0.5, 4.6)	0.480
Use of bed nets while sleeping					
Mostly (>80%)	1,177 (90.8)	1,092 (91.0)	85 (88.5)	Ref	
Never (0%)	30 (2.3)	27 (2.3)	3 (3.1)	1.4 (0.3, 4.8)	0.480
Rarely (1-49%)	22 (1.7)	19 (1.6)	3 (3.1)	2.0 (0.4, 7.1)	0.220
Sometimes (50- 80%)	67 (5.2)	62 (5.2)	5 (5.2)	1.0 (0.4, 2.5)	0.941

ART status, WHO stage, concomitant TB infection, ATT status, time since HIV diagnosis, and BMI were not found to be determinants of ALI (Table 4). A CD4 count <100 (OR=3.1 (95% CI: 1.2-7.6) and a CD4 count between 100-199 (OR=2.1; 95% CI: 1.1-4.0) were found to be significant independent risk factors for ALI compared to a CD4 count ≥300 (Table 4).

Table 4. HIV-related risk factors for asymptomatic *Leishmania* infection (ALI) in 1,296 people living with HIV (PLHIV) in Bihar, India.

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95%CI)	P value
Time on ART					
≥12 months	974 (75.2)	904 (75.3)	70 (72.9)	Ref	
6-<12 months	139 (10.7)	127 (10.6)	12 (12.5)	1.2 (0.6, 2.3)	0.530
<6 months	151 (11.7)	141 (11.8)	10 (10.4)	0.9 (0.4, 1.8)	0.830
Pre-ART	32 (2.5)	28 (2.3)	4 (4.2)	1.8 (0.6, 2.3)	0.410
median (IQR) (excluding Pre-Art)	33 (14-60)	33 (14-60)	32 (12-63)		0.560
WHO clinical Stage					
I	1206 (93.1)	1113 (92.8)	93 (96.9)	Ref	
II	69 (5.3)	68 (5.7)	1 (1)	0.2 (0.004, 1.04)	0.060
III	19 (1.5)	17 (1.4)	2 (2.1)	1.4 (0.2, 5.4)	0.880
IV	2 (0.2)	2 (0.2)	0 (0)	0 (0, 64.8)	1.00
Tuberculosis treatment status (ATT)					
Not on ATT	1144 (88.3)	1057 (88.1)	87 (90.6)	Ref	
History of ATT	134 (10.3)	128 (10.7)	6 (6.3)	0.6 (0.2, 1.3)	0.190
Currently on ATT	18 (1.4)	15 (1.3)	3 (3.1)	2.4 (0.4, 8.8)	0.160
Time since HIV diagnosis (years)					
≥ 1	1059 (81.7)	984 (82.0)	75 (78.1)	Ref	

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95%CI)	P value
< 1	237 (18.3)	216 (18.0)	21 (21.9)	1.3 (0.8, 2.1)	0.345
BMI (kg/m^2)					
<16.5	123 (9.5)	110 (9.2)	13 (13.5)	1.5 (0.8, 2.8)	0.241
16.5-<18.5	292 (22.5)	272 (22.7)	20 (20.8)	0.9 (0.5, 1.5)	0.721
18.5-<25	761 (58.7)	704 (58.7)	57 (59.4)	Ref	
≥25	120 (9.3)	114 (9.5)	6 (6.3)	0.7 (0.3, 1.5)	0.325
Median (IQR)	19.8 (18-22.2)	19.8 (18-22.2)	20.3 (17.3-21.5)		0.538
CD4 (cells/µL)					
≥ 300	978 (75.5)	916 (76.3)	62 (64.6)	Ref	
< 100	35 (12.7)	29 (2.4)	6 (6.3)	3.1 (1.2, 7.6)	**0.012**
100 - 199	104 (8.0)	91 (7.6)	13 (13.5)	2.1 (1.1, 4.0)	**0.019**
200 - 299	179 (13.8)	164 (13.7)	15 (15.6)	1.4 (0.8, 2.4)	0.316
Median (IQR)	443 (303-595)	446 (309-598)	367 (223-544)		**0.002**

In a multivariate analysis, a lower CD4 count, and household size ≥5 family members were associated with a significantly higher risk of ALI. Living in proximity of a pond was the only protective factor for ALI. The final variables in which significance was retained in a multivariable model are shown in table 5.

Table 5. Multivariable risk factor analysis for asymptomatic *Leishmania* infection (ALI) in 1,296 people living with HIV (PLHIV) in Bihar, India.

Variable	aOR (95% CI)	P value
Household size		
< 5	Ref	
≥ 5	2.3 (1.3, 4)	**0.006**
CD4 group (cells/µL)		
≥ 300	Ref	
<100	3.4 (1.3, 8.8)	**0.012**
100 - 199	2.4 (1.2, 4.7)	**0.01**
200 - 299	1.3 (0.7, 2.3)	0.461
Proximity to a pond		
No	Ref	
Yes	0.5 (0.3, 1)	**0.042**

Effect of Urinary Antigen on prevalence and determinants of ALI

Prevalence increased to 9.0% (n=116) when the urinary *Leishmania* antigen ELISA was included in the definition of ALI. Again, having a household size ≥5 was found to be a risk factor for ALI compared to a smaller household OR=1.8 (95% CI: 1.1-2.8) when urinary *Leishmania* antigen ELISA was included (S1 Table). No other household-related risk factors were identified upon inclusion of the urinary *Leishmania* antigen ELISA (S1 Table).

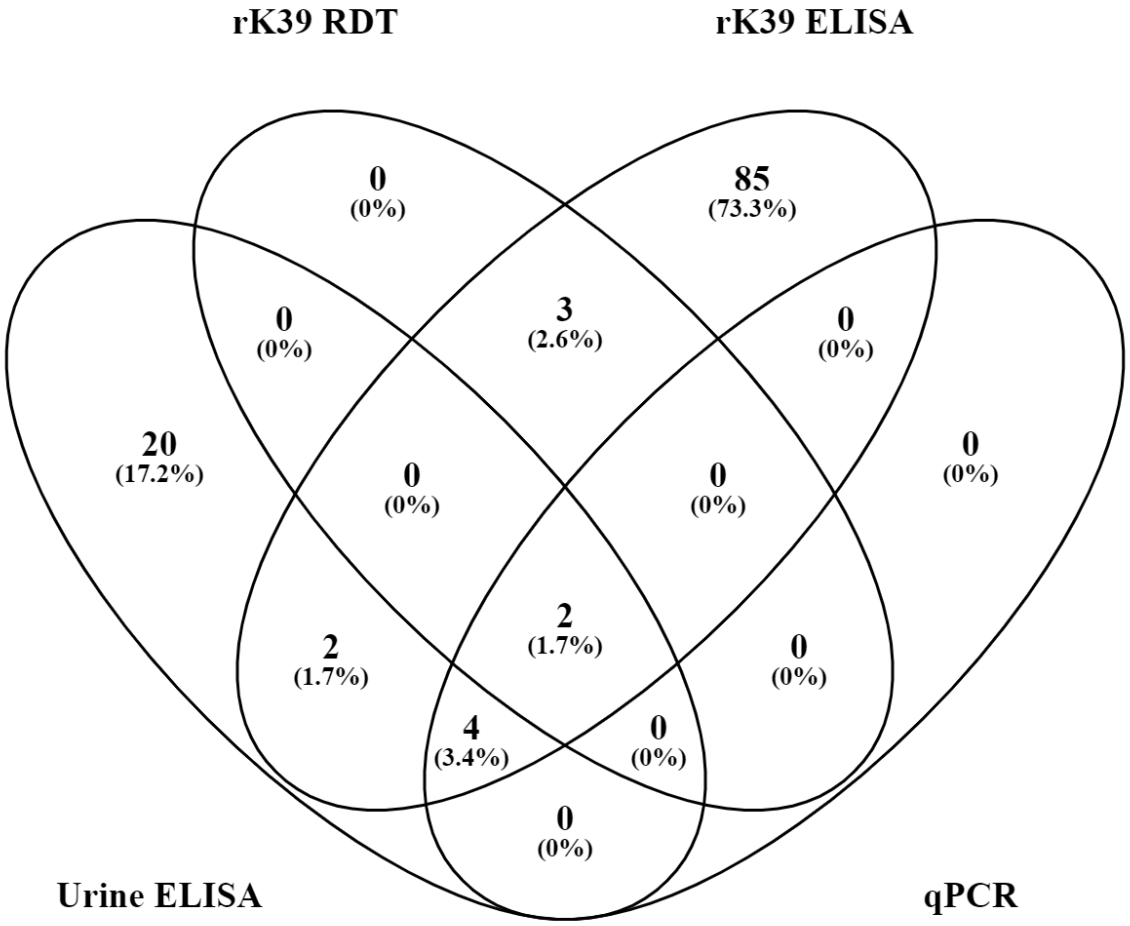
A CD4 count <100 was no longer significant when urinary *Leishmania* antigen ELISA was included (OR=2.3 (95% CI: 0.9-5.8, p=0.06) (S2 Table). A CD4 count between 100-199 (OR=1.9; 95% CI: 1.1-3.4) remained a significant independent risk factors for ALI compared to a CD4 count ≥300 (S2 Table). No other HIV-related risk factors were identified upon inclusion of the urinary *Leishmania* antigen ELISA (S2 Table). The final variables in which significance was retained in a multivariable model are shown in S3 Table. There were few differences in characteristics of individuals testing positive by urinary *Leishmania* antigen ELISA only in comparison to other individuals (S4 Table).

Diagnostic algorithm to detect asymptomatic Leishmania infection in PLHIV

Ninety-six (7.4%) participants were positive by the rK39 ELISA, five (0.4%) by rK39 RDT, and six (0.5%) by qPCR, making up the asymptomatic cohort (Table 6). Twenty-eight (2.2%) participants were positive by *Leishmania* antigen ELISA, 20 of which were in addition to the asymptomatic cohort (Table 6). Of the 96 participants, 85 (73.3%) were positive for rK39 ELISA only. Two (1.7%) of the 96 participants tested positive by all four tests. The rK39 ELISA and *Leishmania* antigen ELISA in combination capture all positive participants when the *Leishmania* antigen ELISA is included in the definition of ALI (Fig 2).

Table 6. Prevalence of asymptomatic *Leishmania* infection (ALI) in 1,296 people living with HIV (PLHIV) in Bihar, India by serological, molecular, and antigen detection methods.

	N	% (95% CI)
Total recruited	1296	
Total positive by *Leishmania* antigen ELISA	28	2.2 (1.5, 3.1)
Total ALI (positive by rK39 RDT or rK39 ELISA or qPCR)	96	7.4 (6.1, 9.0)
Total positive with rK39 RDT	5	0.4 (0.2, 0.9)
Total positive by rK39 ELISA	96	7.4 (6.1, 9.0)
Total positive by qPCR	6	0.5 (0.2, 1.0)



2106

2107 **Fig 2. Four assays to detect asymptomatic *Leishmania* infection (ALI) in 1,296 people living with HIV**

2108 **(PLHIV) in Bihar, India.**

2109

2110

2111

Negligible agreement was seen between the rK39 RDT and rK39 ELISA, rK39 RDT and *Leishmania* antigen ELISA, qPCR and rK39 ELISA, and rK39 ELISA and *Leishmania* antigen ELISA. Weak agreement was seen between rK39 RDT and qPCR, and qPCR and the *Leishmania* antigen ELISA (Table 7).

Table 7. Kappa scores and agreement for the rK39 RDT, rK39 ELISA, qPCR, and *Leishmania* antigen ELISA in 96 people living with HIV (PLHIV) with asymptomatic *Leishmania* infection (ALI) in Bihar, India.

Test combination	Agreement (%)	Kappa score	p-value
RK39 RDT and qPCR	99.5	0.361	<0.001
RK39 RDT and rK39 ELISA	93.0	0.092	<0.001
RK39 RDT and *Leishmania* antigen ELISA	90.6	0.115	<0.001
QPCR and rK39 ELISA	93.1	0.110	<0.001
QPCR and *Leishmania* antigen ELISA	98.5	0.348	<0.001
RK39 ELISA and *Leishmania* antigen ELISA	91.7	0.099	<0.001

Discussion

There has been considerable interest in the role and evolution of ALI over the last decade. No clear consensus exists on this population's role in transmission; one recent xenodiagnostic study from India showed that none of 184 non-HIV infected individuals with ALI were infectious to sand flies; whereas a recent Spanish study demonstrated that sandflies fed on the blood from one ALI-HIV patient, who had been under continuous secondary prophylaxis for leishmaniasis, demonstrated the presence of viable parasites post exposure (25,26). Similarly, there remains no clear consensus on the actual definition of asymptomatic infection, and as such caution should be taken when comparing results of different studies reporting prevalence and progression of ALI (27).

Prior to this study the prevalence of ALI in PLHIV residing in VL endemic areas in India was unknown. Prevalence of ALI in this population was 7.4% when detected by a combination of rK39 ELISA, rK39 RDT, and/or qPCR. All individuals with ALI were positive by the rK39 ELISA. A smaller proportion were positive by qPCR (0.5%), and the rK39 RDT (0.4%). All individuals positive by rk39 RDT were also positive by rk39 ELISA. As expected, the rK39 RDT detected a lower number of positive participants compared to the rK39 ELISA, in keeping with the reduced sensitivity of RDTs compared to their equivalent ELISA, and reduced performance in PLHIV as seen in studies in East Africa (14). Prevalence of ALI increased to 9.0% with the addition of *Leishmania* antigen ELISA, with 20 additional participants identified with the expanded definition of ALI. A low CD4 count and a household size of five individuals or more were found to be risk factors for ALI. Similar to our study findings, a larger household size was identified as a risk factor (OR=4.4) for *Leishmania* infection in a study by Schenkel *et al.* in Nepal (28). Household size may be associated with several other factors that may be linked with increased risk of ALI, such as low socioeconomic status. It may be that individuals with low CD4 counts are more susceptible to ALI, or conversely, ALI could lead to lower CD4 counts. A CD4 count <100 was no longer significant when urinary *Leishmania* antigen ELISA was included in the definition of ALI, likely due to a change in proportions of individuals falling within each CD4 count category. As per 2017 estimates of HIV infection in India, 41.2% of PLHIV in Bihar were female compared to 53.5% in this study (29).

To the best of our knowledge there has been one other study of ALI in PLHIV in an *L. donovani* endemic area (21). The study in Ethiopia used the lower sensitivity KAtex to detect antigenuria and the DAT to detect anti-*Leishmania* antibodies in addition to PCR and rK39 RDT (21), compared to the *Leishmania* antigen ELISA and the rK39 ELISA used in this study. In Ethiopia, prevalence was found to be 12.8% in males, with being male and a concurrent malaria infection found to be risk factors for ALI (21).

Furthermore, the population had relatively high median CD4 counts (377 cells/mm3 (IQR: 250-518)) with generally good overall ART adherence (21). In this study, median CD4 counts were 443 cells/mm3 (IQR: 303-595) with the majority of participants (75.2%) on ART for 12 months or more.

Much of the data on ALI in PLHIV has been collected in areas where *Leishmania infantum* is endemic (20,30,31). A study in Brazil used PCR, rK39 ELISA, indirect fluorescent antibody test, and an ELISA based on a crude *L. infantum* preparation and found the prevalence of ALI in PLHIV to be 20.2% (30). More recently, a study in Brazil used the rK39 ELISA, rK39 RDT, DAT, KAtex, and PCR to estimate prevalence of ALI in PLHIV, and found prevalence to be 9.1% (31). Further, a meta-analysis of studies in PLHIV in *L. infantum* endemic areas found the prevalence of ALI to be 11.8% (20). Together these studies provide vital data to inform programmatic policy in a population at risk of poor disease outcomes.

Two participants tested positive by all four tests in combination. As is seen in previous studies of ALI (16,30), weak to negligible agreement was seen between tests. Negligible agreement was seen between the rK39 RDT and *Leishmania* antigen ELISA, qPCR and rK39 ELISA, and rK39 ELISA and *Leishmania* antigen ELISA, which may be expected given the tests in these combinations detect anti-*Leishmania* antibodies in comparison to assays which detect active infection (32). Better, albeit weak agreement was seen between the rK39 RDT and qPCR possibly due a lower sensitivity of the rK39 RDT and a higher specificity of qPCR. There was similarly better, but weak agreement between qPCR and the *Leishmania* antigen ELISA, in keeping with them both detecting active infection. To the best of our knowledge, this is the first study to use the *Leishmania* antigen ELISA to detect ALI in PLHIV. We acknowledge that the specificity of the tests falls below 100% and as such there will be false positives within the data.

This study is limited by absence of follow-up data, although follow-up data is due to be presented in the next year. This study was further limited by the lack of data on the *Leishmania* antigen ELISA in an asymptomatic population at the time of study conception, and as such was not included in the primary definition of ALI. Further tests could have included the DAT given its wide use in other studies of ALI.

Here, we provide estimates of prevalence and determinants of ALI in PLHIV in a VL endemic region of India. Work is ongoing to determine rate and risk factors for progression to VL in this population. Further longitudinal data are required to estimate incidence of ALI in this population.

Acknowledgements

With thanks to the participants and the MSF field team. With thanks to Professor Steven Reed for providing the rK39 antigen.

Funding

This work was funded by Medecins Sans Frontiers, Spain, who fulfilled a sponsor-investigator role in the study. Salary support to SIO was provided through The Medical Research Council (MRC) Doctoral Training Partnership (DTP) (MR/N013514/1), and to EA through Wellcome Trust -108080/Z/15/Z.

References

1. Burza S, Croft SL, Boelaert M. Leishmaniasis. The Lancet. 2018. doi:10.1016/S0140-6736(18)31204-2

2. Ostyn B, Gidwani K, Khanal B, Picado A, Chappuis F, Singh SP, et al. Incidence of Symptomatic and Asymptomatic *Leishmania donovani* Infections in High-Endemic Foci in India and Nepal: A Prospective Study. Milon G, editor. PLoS Negl Trop Dis. 2011;5(10):e1284.

3. Alvar J, Aparicio P, Aseffa A, Boer M Den, Cañavate C, Dedet J-P, et al. The Relationship between Leishmaniasis and AIDS: the Second 10 Years. Clin Microbiol Rev. 2008;21(2):334–59.

4. Mock DJ, Hollenbaugh JA, Daddacha W, Overstreet MG, Lazarski CA, Fowell DJ, et al. *Leishmania* Induces Survival, Proliferation and Elevated Cellular dNTP Levels in Human Monocytes Promoting Acceleration of HIV Co-Infection. PLoS Pathog. 2012;8(4):e1002635

5. Barreto-de-Souza V, Pacheco GJ, Silva AR, Castro-Faria-Neto HC, Bozza PT, Saraiva EM, et al. Increased *Leishmania* Replication in HIV-1–Infected Macrophages Is Mediated by Tat Protein through Cyclooxygenase-2 Expression and Prostaglandin E2 Synthesis. J Infect Dis. 2006;194(6):846–54.

6. Wolday D, Akuffo H, Fessahaye G, Valantine A, Britton S. Live and Killed Human Immunodeficiency Virus Type-1 Increases the Intracellular Growth of *Leishmania donovani* in Monocyte-derived Cells. Scand J Infect Dis. 1998;30(1):171.

7. National AIDS Control Organization & ICMR National Institute of Medical Statistics. India HIV Estimates 2019: Report. 2020.

8. Burza S, Mahajan R, Gonzalez Sanz M, Sunyoto T, Kumar R, Mitra G, et al. HIV and Visceral Leishmaniasis Coinfection in Bihar, India: An Underrecognized and Underdiagnosed Threat Against Elimination. Clin Infect Dis. 2014;59(4):552–5.

9. 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.

10. Medrano FJ, Cañavate C, Leal M, Rey C, Lissen E, Alvar J. The role of serology in the diagnosis and prognosis of visceral leishmaniasis in patients coinfected with human immunodeficiency virus type-1. Am J Trop Med Hyg. 1998;59(1):155–62.

11. Stauch A, Sarkar RR, Picado A, Ostyn B, Sundar S, Rijal S, et al. Visceral leishmaniasis in the Indian subcontinent: Modelling epidemiology and control. PLoS Negl Trop Dis. 2011.doi:10.1371/journal.pntd.0001405

12.Van Griensven J, Diro E, Lopez-Velez R, Ritmeijer K, Boelaert M, Zijlstra EE, et al. A Screen-and-Treat Strategy Targeting Visceral Leishmaniasis in HIV-Infected Individuals in Endemic East African Countries: The Way Forward? PLoS Negl Trop Dis. 2014;8(8):e3011.

13. Cloots K, Marino P, Burza S, Gill N, Boelaert M, Hasker E. Visceral Leishmaniasis-HIV Coinfection as a Predictor of Increased *Leishmania* Transmission at the Village Level in Bihar, India. Front Cell Infect Microbiol. 2021;11:604117.

14. Horst R ter, Tefera T, Assefa G, Ebrahim AZ, Davidson RN, Ritmeijer K, 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;80:929-934.

15. Vallur AC, Tutterrow YL, Mohamath R, Pattabhi S, Hailu A, Abdoun AO, et al. Development and comparative evaluation of two antigen detection tests for visceral leishmaniasis. BMC Infect Dis. 2015;15(1):1–10.

16. Owen SI, Hossain F, Ghosh P, Chowdhury R, Sakhawat Hossain M, Jewell C, et al. Detection of asymptomatic *Leishmania* infection in Bangladesh by antibody and antigen diagnostic tools shows an association with post-kala-azar dermal leishmaniasis (PKDL) patients. Parasites Vectors.2021;14(1):1–7.

17. Picado A, Ostyn B, Singh SP, Uranw S, Hasker E, Rijal S, et al. Risk Factors for Visceral Leishmaniasis and Asymptomatic *Leishmania donovani* Infection in India and Nepal. PLoS One. 2014;9(1):e87641.

18. Topno RK, Das VNR, Ranjan A, Pandey K, Singh D, Kumar NN, et al. Asymptomatic infection with visceral leishmaniasis in a disease-endemic area in Bihar, India. Am J Trop Med Hyg. 2010;83(3):502–6.

19. Saha P, Ganguly S, Chatterjee M, Das SB, Kundu PK, Guha SK, et al. Asymptomatic leishmaniasis in kala-azar endemic areas of Malda district, West Bengal, India. PLoS Negl Trop Dis. 2017;11(2):e0005391.

20. Mannan S Bin, Elhadad H, Loc TTH, Sadik M, Mohamed MYF, Nam NH, et al. Prevalence and associated factors of asymptomatic leishmaniasis: a systematic review and meta-analysis. Parasitol Int. 2021;81:102229.

21. van Griensven J, van Henten S, Mengesha B, Kassa M, Adem E, Endris Seid M, et al. Longitudinal evaluation of asymptomatic *Leishmania* infection in HIV-infected individuals in North-West Ethiopia: A pilot study. PLoS Negl Trop Dis. 2019;13(10):e0007765.

22. Adams ER, Schoone G, Versteeg I, Gomez MA, Diro E, Mori Y, et al. Development and evaluation of a novel loop-mediated isothermal amplification assay for diagnosis of cutaneous and visceral leishmaniasis. J Clin Microbiol. 2018. doi:10.1128/JCM.00386-18

23. Mangal A, Kumar V, Panesar S, Talwar R, Raut D, Singh S. Updated BG Prasad socioeconomic classification, 2014: A commentary. Indian J Public Health. 2015;59(1):42.

24. Landis JR, Koch GG. The Measurement of Observer Agreement for Categorical Data. Biometrics. 1977 Mar;33(1):159.

25. Singh OP, Tiwary P, Kushwaha AK, Singh SK, Singh DK, Lawyer P, et al. Xenodiagnosis to evaluate the infectiousness of humans to sandflies in an area endemic for visceral leishmaniasis in Bihar, India: a transmission-dynamics study. The Lancet Microbe. 2021;2(1):e23–31.

26. Molina R, Jiménez M, García-Martínez J, Martín JVS, Carrillo E, Sánchez C, et al. Role of asymptomatic and symptomatic humans as reservoirs of visceral leishmaniasis in a Mediterranean context. PLoS Negl Trop Dis. 2020;14(4):e0008253.

27. Ibarra-Meneses AV, Corbeil A, Wagner V, Onwuchekwa C, Fernandez-Prada C. Identification of asymptomatic *Leishmania* infections: a scoping review. Parasites and Vectors. 2022;15(1):1–22.

28. Schenkel K, Rijal S, Koirala S, Koirala S, Vanlerberghe V, Van Der Stuyft P, et al. Visceral leishmaniasis in southeastern Nepal: A cross-sectional survey on Leishmania donovani infection and its risk factors. Trop Med Int Heal. 2006;11: 1792–1799. doi:10.1111/J.1365-3156.2006.01735.X

29. National AIDS Control Organisation. India HIV Estimates 2017, Technical Report. 2017.

30. Orsini M, Canela JR, Disch J, Maciel F, Greco D, Toledo A, et al. High frequency of asymptomatic *Leishmania spp*. infection among HIV-infected patients living in endemic areas for visceral leishmaniasis in Brazil. Trans R Soc Trop Med Hyg. 2012;106(5):283–8.

31. Guedes DL, Justo AM, Júnior WLB, da Silva ED, de Aquino SR, Junior MS da CL, et al. Asymptomatic *Leishmania* infection in HIV-positive outpatients on antiretroviral therapy in Pernambuco, Brazil. PLoS Negl Trop Dis. 2021;15(1):e0009067.

32. Sundar S, Singh OP. Molecular Diagnosis of Visceral Leishmaniasis. Mol Diagn Ther. 2018;22(4):443-57.

Supplementary Material

S1 Table. Household related risk factors for asymptomatic *Leishmania* infection (ALI) in PLHIV including the *Leishmania* antigen ELISA in addition to qPCR, rK39 ELISA and RDT in the definition of ALI.

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95% CI)	P value
Sex					
Female	694 (53.5)	637 (54)	57 (49.1)	Ref	
Male	602 (46.5)	543 (46)	59 (50.9)	1.2 (0.8, 1.8)	0.32
Age					
18-29	174 (13.4)	154 (13.1)	20 (17.2)	Ref	
30-44	731 (56.4)	679 (57.5)	52 (44.8)	0.6 (0.3, 1.02)	0.06
45-59	329 (25.4)	289 (24.5)	40 (34.5)	1.1 (0.6, 1.9)	0.83
≥ 60	62 (4.8)	58 (4.9)	4 (3.4)	0.5 (0.1, 1.7)	0.33
Household size					
< 5	395 (30.5)	371 (31.4)	24 (20.7)	Ref	
≥ 5	901 (69.5)	809 (68.6)	92 (79.3)	1.8 (1.1, 2.8)	**0.02**
Socioeconomic status					
1 or 2	130 (10.1)	118 (10.0)	12 (10.3)	Ref	
3	333 (25.7)	291 (24.7)	42 (36.2)	1.4 (0.7, 2.8)	0.31
4	513 (39.6)	473 (40.2)	40 (34.5)	0.8 (0.4, 1.6)	0.59
5	318 (24.6)	296 (25.1)	22 (19)	0.7 (0.4, 1.5)	0.4
Type of house					

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95% CI)	P value
Brick	619 (47.8)	560 (47.5)	59 (50.9)	Ref	
Mud	396 (30.6)	363 (30.8)	33 (28.4)	0.9 (0.6, 1.4)	0.52
Thatched	281 (21.7)	257 (21.8)	24 (20.7)	0.9 (0.5, 1.5)	0.63
Proximity to a pond					
No	1028 (79.3)	929 (78.7)	99 (85.3)	Ref	
Yes	268 (20.7)	251 (21.3)	17 (14.7)	0.6 (0.4, 1.1)	0.09
Proximity to livestock					
No	534 (41.2)	489 (41.4)	45 (38.8)	Ref	
Yes	762 (58.8)	691 (58.6)	71 (61.2)	1.1 (0.8, 1.7)	0.58
Time of last IRS (months)					
Never	144 (11.1)	133 (11.3)	11 (9.5)	0.8 (0.4, 1.5)	0.41
< 6	998 (77)	900 (76.3)	98 (84.5)	Ref	
6-12	124 (9.6)	118 (10)	6 (5.2)	0.5 (0.2, 1.1)	0.07
> 12	30 (2.3)	29 (2.5)	1 (0.9)	0.3 (0.01, 2.0)	0.35
Number of IRS in last 18 months					
0	140 (10.8)	129 (10.9)	11 (9.5)	Ref	
1	132 (10.2)	127 (10.8)	5 (4.3)	0.5 (0.2, 1.4)	0.15
2	642 (49.5)	588 (49.8)	54 (46.6)	1.1 (0.6, 2.1)	0.83
> 2	382 (29.5)	336 (28.5)	46 (39.7)	1.6 (0.8, 3.2)	0.17
Contact with people with presumptive VL 50 metres around the house					

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95% CI)	P value
No/Don't know	1236 (95.3)	1125 (95.4)	111 (95.7)	Ref	
Yes	60 (4.6)	55 (4.7)	5 (4.3)	0.9 (0.3, 2.2)	0.91
Contact with people with presumptive PKDL 50 metres around the house					
No/ Don't know	1278 (98.6)	1164 (98.7)	114 (98.2)	Ref	
Yes	18 (1.4)	16 (1.4)	2 (1.7)	1.3 (0.2, 4.9)	0.98
Contact with people with cured VL/ PKDL 50 metres around the house					
No/ Don't know	1262 (97.4)	1151 (97.5)	111 (95.7)	Ref	
Yes	34 (2.6)	29 (2.5)	5 (4.3)	1.8 (0.6, 4.5)	0.26
Use of bed nets while sleeping					
Mostly (>80%)	1177 (90.8)	1073 (90.9)	104 (89.7)	Ref	
Never (0%)	30 (2.3)	27 (2.3)	3 (2.6)	1.2 (0.2, 3.8)	0.74
Rarely (1-49%)	22 (1.7)	18 (1.5)	4 (3.4)	2.3 (0.6, 7.1)	0.13
Sometimes (50- 80%)	67 (5.2)	62 (5.3)	5 (4.3)	0.8 (0.3, 2.1)	0.7

S2 Table. HIV related risk factors for asymptomatic *Leishmania* infection (ALI) in PLHIV including the *Leishmania* antigen ELISA in addition to qPCR, rK39 ELISA and RDT in the definition of ALI.

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95%CI)	P value
Time on ART					
≥ 12 months	974 (75.2)	890 (75.4)	84 (72.4)	Ref	
6-<12 months	139 (10.7)	123 (10.4)	16 (13.8)	1.4 (0.8, 2.4)	0.27
<6 months	151 (11.7)	140 (11.9)	11 (9.5)	0.8 (0.4, 1.6)	0.60
Pre-ART	32 (2.5)	27 (2.3)	5 (4.3)	2.0 (0.7, 5.0)	0.2
WHO clinical Stage					
I	1206 (93.1)	1093 (92.6)	113 (97.4)	Ref	1
II	69 (5.3)	68 (5.8)	1 (0.9)	0.1 (.004, 0.8)	**0.021**
III	19 (1.5)	17 (1.4)	2 (1.7)	1.1 (0.1, 4.9)	1
IV	2 (0.2)	2 (0.2)	0 (0)	0 (0, 51.8)	1
Anti-tubercular treatment (ATT) status					
Not on ATT	1144 (88.3)	1038 (88)	106 (91.4)	Ref	
History of ATT	134 (10.3)	127 (10.8)	7 (6)	0.5 (0.3, 1.2)	0.12
Currently on ATT	18 (1.4)	15 (1.3)	3 (2.6)	2.0 (0.4, 7.1)	0.24
Time since HIV diagnosis (years)					
< 1	252 (19.4)	225 (19.1)	27 (23.3)	1.3 (0.8, 2.1)	0.25
≥ 1	1044 (80.6)	955 (80.9)	89 (76.7)	Ref	

	All N (%)	Non- ALI N (%)	ALI N (%)	Odds Ratio (95%CI)	P value
Body Mass Index (kg/m^2)					
<16.5	123 (9.5)	108 (9.2)	15 (12.9)	1.5 (0.8, 2.7)	0.21
16.5-<18.5	292 (22.5)	267 (22.6)	25 (21.6)	1 (0.6, 1.6)	0.95
18.5-<25	761 (58.7)	695 (58.9)	66 (56.9)	Ref	
≥25	120 (9.3)	110 (9.3)	10 (8.6)	1 (0.5, 1.9)	0.9
CD4 (cells/µL)					
< 100	35 (2.7)	29 (2.4)	6 (5.2)	2.3 (0.9, 5.8)	0.06
100 - 199	104 (8)	89 (7.5)	15 (12.9)	1.9 (1.1, 3.4)	**0.03**
200 - 299	179 (13.8)	164 (13.9)	15 (12.9)	1 (0.6, 1.8)	0.93
≥ 300	978 (75.5)	898 (76.1)	80 (69)	Ref	

S3 Table. Multivariable risk factor analysis for ALI in PLHIV including the *Leishmania* antigen ELISA in addition to qPCR, rK39 ELISA and RDT in the definition of ALI.

Variable	aOR (95% CI)	P value
Household size		
< 5	**Ref**	
≥ 5	1.7 (1.1, 2.8)	**0.025**
CD4 group (cells/µL)		
≥ 300	**Ref**	
<100	2.6 (1.0, 6.9)	**0.05**
100 - 199	2 (1.1, 3.7)	**0.032**
200 - 299	1 (0.6, 1.8)	0.933
Proximity to pond		
No	**Ref**	
Yes	0.6 (0.3, 1)	**0.038**

S4 Table. Differences in baseline characteristics of individuals testing positive by urinary Leishmania antigen ELISA only in comparison other individuals.

	All (n=1296)	ALI (n=96)	Non-ALI (n=1200)	Urine positive (n=28)	Urine positive only (n=20)
Median Age (IQR)	39 (33-46)	41 (33-50)	39 (33-46)	42 (34-46)	42 (30-47)
Female - N (%)	694 (53.5)	46 (47.9)	648 (54.0)	15 (53.6)	11 (55.0)
Median time in months on ART (IQR)	33 (14-60)	32 (12-63)	33 (14-60)	36 (14-59)	37 (13-53)
Mean CD4 counts in cells/mm^3 (SD)	466 (230)	400 (227)	471 (229)	442 (263)	524.5 (245)

Chapter 4. Progression from asymptomatic *Leishmania* infection to visceral leishmaniasis in HIV-infected individuals living within visceral leishmaniasis endemic areas of Bihar, India

4.1 Acknowledgement of work done

This study is a continuation of the work presented in the previous chapter for which Sakib Burza, Amit Harshana and Shahwar Kazmi were responsible for conception and design of the study, and study oversight. Sophie I. Owen was responsible for ethics applications in the UK. Emily R. Adams and Sophie I. Owen were responsible for laboratory set-up. Vikash Kumar and Sophie I. Owen were responsible for the procurement. The MSF field team were responsible for participant recruitment and sample collection. Shiril Kumar performed the DNA extraction, qPCR and rK39 ELISAs. Sophie I. Owen conducted half of the *Leishmania* antigen ELISAs but due to the COVID-19 pandemic was unable to finish testing the remaining samples. Shiril Kumar kindly tested the remaining samples by *Leishmania* antigen ELISA. Sophie I. Owen led the data analysis and publication supported by Raman Mahajan.

4.2 Introduction

In the cross-sectional study presented in chapter 3 we estimated the prevalence of and risk factors for ALI in PLHIV in India. In this chapter, we followed this cohort longitudinally over an 18-month period to assess progression of disease, risk factors for progression, and to look at the diagnostic profiles of those who progressed to VL. Given the lack of data on ALI in PLHIV on the ISC, little is known about the rate of progression to VL in PLHIV and the risk factors for progression of disease in this population. Similarly, there are little data for prognostic markers of progression from an asymptomatic infection to VL in PLHIV on the ISC. Given the reciprocal exacerbation of *Leishmania* and HIV coinfections, together with the high rates of treatment failure and relapse seen in patients with VL-HIV coinfection, a better understanding of disease progression in this population would improve clinical management where early intervention may improve patient outcomes [164].

A study of 1,606 participants ≥2 years of age living in endemic villages in Bihar, India looked at seroconversion using the rK39 ELISA and DAT, with some participants followed up at a six month interval and some participants followed up at a one year interval [124]. Participants were considered to have seroconverted if they were negative in the baseline survey but were positive by one or both of the assays on the second survey [124]. Of the 1,606 participants recruited to the initial two surveys, 17 participants went on to develop VL, with an average follow-up time of 52 months [124]. DAT titers over ≥1:25,600 (odds ratio (OR)=19.1; 95% CI: 4.4-57.1), a high titer rK39 ELISA result (OR=30.3; 95%CI: 9.6-95.2), and a qPCR result representing >1 parasite genomes/ml (OR=20.9; 95% CI: 6.5-66.8), were strongly associated with progression to VL from seroconversion [124]. Furthermore, recent seroconversion measured by the DAT was found to be a risk factor for progression of an asymptomatic infection to VL in a study of 9,034 participants with no history of VL in endemic regions of India and Nepal [57].

As mentioned previously, much of the data on VL-HIV coinfection comes from studies in the Mediterranean, East Africa and Brazil. The prevalence of asymptomatic *L. infantum* infection was found to be 20.2% (95% CI: 16.2-24.2) in a cohort of 381 PLHIV in Brazil screened by an *L. infantum* ELISA, rK39 ELISA, IFAT, and PCR [160]. No progression of disease was seen in this cohort and it was thought to be because of the high coverage of HAART [160]. However, there are limited data on ALI in PLHIV in areas where reservoirs of *Leishmania* are thought to be anthroponotic, including East Africa. A longitudinal study of 511 PLHIV in Ethiopia found a baseline prevalence of ALI of 12.8% (n=41) and 4.2% (n=8) in males and females respectively [161]. Over the 12-month follow-up period 36 new asymptomatic

infections were detected [161]. However, only one case of VL and no deaths occurred over the follow-up period [161].

In this study, we follow-up a cohort of 1,306 PLHIV living in *Leishmania* endemic districts of Bihar, India over an 18-month period. Participants identified as having an ALI by rK39 serology and qPCR in the study presented in chapter 3 were followed up every 3 months in-person for further samples. The remaining participants were followed up every 3 months by telephone. At the time of writing the *Leishmania* antigen ELISA (Clin-Tech, UK), qPCR, and rK39 ELISA and RDT were run on all follow-up samples, with the samples collected during the study stored in a biobank for evaluating other assays of interest. Other assays of interest include the kala-azar antigen detection test (DetectoGen Inc., USA) to detect *Leishmania* antigenuria and rK28 serology.

Chapter four summary

In this study, four (3.7%) of the 108 PLHIV with ALI developed VL over 18-months follow-up and no progression was observed in the non-asymptomatic cohort. Within the same follow-up period, mortality was found to be higher in PLHIV with ALI, highlighting the reciprocal and complicated nature of *Leishmania*-HIV coinfection. The four individuals who progressed to VL could be identified by at least three of the four tests used, with high levels of antigenuria and high anti-*Leishmania* antibody titres detected and could be used to help stratify risk of poor outcomes associated with asymptomatic *Leishmania* coinfection and progression to VL. Detection of *Leishmania* markers at 18-months in a large proportion of individuals suggests a persistent *Leishmania* infection that remains asymptomatic in most individuals.

4.3 Progression from asymptomatic *Leishmania* infection to visceral leishmaniasis in HIV-infected individuals living within visceral leishmaniasis endemic areas of Bihar, India

Sophie I Owen[2, ¶], Raman Mahajan[1, ¶], Shiril Kumar[3], Shahwar Kazmi[1], Vikash Kumar[1], Emily R Adams[2], Amit Harshana[1], Sakib Burza[1, *]

[1] Médecins Sans Frontières, New Delhi, India

[2] Department of Tropical Disease Biology, Liverpool School of Tropical Medicine (LSTM), Liverpool, UK

[3] Rajendra Memorial Research Institute of Medical Sciences, Patna, Bihar, India

* Corresponding author

E-mail: Sakib.Burza@barcelona.msf.org (SB)

[¶] RM and SIO are joint first authors

Submitted

Abstract

There are no data on rate and risk factors for progression from asymptomatic *Leishmania* infection (ALI) to visceral leishmaniasis (VL) in people living with HIV (PLHIV) on the Indian subcontinent (ISC). Here, we aim to establish the rate and risk factors for progression of ALI to VL in a cohort of PLHIV in Bihar, India. We conducted a prospective cohort study of 1,306 PLHIV, of whom 108 were identified as having ALI (rK39 enzyme-linked immunosorbent assay (ELISA) and/or rK39 rapid diagnostic test (RDT) and/or quantitative polymerase chain reaction (qPCR)) in a previous cross-sectional survey. Additionally, the urinary *Leishmania* antigen ELISA was evaluated. The ALI (n=108) and non-ALI (n=1,198) cohorts were followed up every three months for 18 months in person and by telephone, respectively. Within the ALI cohort, four (3.7%) participants developed VL, compared to no progression in the non-ALI cohort. All-cause mortality was higher in ALI compared to non-ALI (odds ratio (OR)=2.7; 95% confidence intervals (CI): 1.1-6.1). However, in a multivariate model only low CD4 counts, being clinical stage three for HIV infection according to the World Health Organization (WHO) system, and not being on anti-retroviral therapy (ART) at baseline were significantly associated with mortality. All four participants with VL were positive by a minimum of three of the four tests in combination and had high levels of antigenuria and anti-*Leishmania* antibody titers compared to asymptomatic non-progressors. The overall risk of developing VL in ALI diagnosed by rK39 ELISA, rK39 RDT, qPCR, and *Leishmania* antigen ELISA was 3.7% (4/108), 40% (2/5), 57% (4/7) and 50% (4/8), respectively. There is a low rate of progression from ALI to VL in PLHIV. Individuals with ALI have higher mortality than those without, however, ALI was not a statistically significant factor for mortality after adjusting for other factors in a multivariate model.

Author summary

People living with HIV (PLHIV) are at higher risk of developing visceral leishmaniasis (VL) and poor associated outcomes. We conducted a prospective cohort study of 1,306 PLHIV in India to establish the rate and risk factors for progression of asymptomatic *Leishmania* infection (ALI) to VL. A prior cross-sectional survey identified 108 individuals with ALI (defined as positive rK39 enzyme-linked immunosorbent assay (ELISA) and/or rK39 rapid diagnostic test (RDT) and/or quantitative polymerase chain reaction (qPCR)) Additionally, we evaluated the urinary *Leishmania* antigen ELISA. The ALI (n=108) and non-ALI (n=1,198) cohorts were followed up over 18 months in-person and by telephone, respectively. Mortality associated with any cause was higher in those with ALI compared to non-ALI. However, only low CD4 counts, being clinical stage three for HIV infection according to the World Health Organization (WHO) system (one to four), and not being on anti-retroviral therapy (ART) at baseline

were significantly associated with mortality. Four (3.7%) individuals with ALI developed VL, compared to no progression in the non-ALI cohort. The four participants with VL were positive by at least three of the four tests in combination and had high levels of antigen in the urine and anti-*Leishmania* antibody titers compared to asymptomatic non-progressors.

Introduction

People living with HIV (PLHIV) often present with symptoms of visceral leishmaniasis (VL) late in the course of disease, posing a challenge for clinical management (1). The prevalence of asymptomatic *Leishmania* infection (ALI) was found to be 7.4% in a cross-sectional survey of 1,296 PLHIV from endemic villages of Bihar, India (Mahajan & Owen et al., in press). To the best of our knowledge, there are no data on the rate and risk factors of progression from ALI to VL in PLHIV in India. Early identification of PLHIV with ALI, at risk of developing VL, would allow for monitoring and earlier clinical intervention. In VL-endemic areas, it is estimated that 13% (95% confidence intervals (CI): 10%-17%) of the general population may harbour ALI (2), the vast majority of whom will not progress to VL (3). However, similar data are lacking for PLHIV in South Asia.

A study of 1,606 seroconverters and controls in Bihar found a significant association between high anti-*Leishmania* antibody titers measured by the direct agglutination test (DAT) or rK39 enzyme-linked immunosorbent assay (ELISA), or a positive quantitative PCR (qPCR) result indicating parasitaemia, and progression from seroconversion to VL, with odds ratios of 19.1 (95% CI: 4.4-57.1), 30.3 (95% CI: 9.6-85.2), and 20.9 (95% CI: 6.5-66.8), respectively (4). Similarly, a review of 98 studies from the Indian subcontinent (ISC) found the proportion of those that progressed from ALI to VL was higher in those with high anti-*Leishmania* antibody titers (3). A screening of 2,603 individuals in West Bengal, India identified 79 individuals with ALI detected by the rK39 rapid diagnostic test (RDT), of whom two were lost to follow-up and eight (10.4%) developed VL within the three-year follow-up period (5). A meta-analysis of 111 studies conducted globally found being male was a risk factor for progression of ALI to VL (odds ratio (OR)=1.9; 95% CI: 1.2-3.0) (2).

A study in Brazil found the prevalence of ALI in 483 PLHIV to be 9.1% (n=44) using the rK39 ELISA (2.5%), rK39 RDT (1.1%), DAT (3.5%), PCR (2.3%), and the KAtex (0.4%) which measures *Leishmania* antigen excreted in the urine and is the predecessor to the *Leishmania* antigen ELISA used in this study (6). Higher HIV viral load (up to 100,000 copies/ml) was associated with a higher odds (2.0 (95% CI: 1.0-4.1)) of ALI in PLHIV, but the study lacked follow-up data to monitor progression (6). Data on progression of

asymptomatic *Leishmania donovani* infection to VL in PLHIV are limited to a single pilot study in East Africa (7). A longitudinal study of 511 PLHIV in Ethiopia found a baseline prevalence of 9.6% (n=49) using the rK39 RDT (7.4%), DAT (4.3%), PCR (0.2%), and KAtex (0.2%), with one participant developing VL within the median 12-month follow-up period (7).

Here, we follow on from a cross-sectional survey to determine the prevalence and determinants of ALI in PLHIV in Bihar (Mahajan & Owen et al., in press). In this prospective cohort study, we aim to determine the rate and risk factors for progression of ALI to VL in a cohort of 1,306 PHIV residing in VL-endemic areas in Bihar over 18 months of follow-up. Furthermore, we explore the diagnostic profiles of the asymptomatic participants over time.

Methods

Study-design and population

Participants were enrolled over a 12-month period from May 2018 and followed-up between July 2018 and November 2020. PLHIV were enrolled at anti-retroviral therapy (ART) centres within four districts (Saran, Siwan, Muzaffarpur, and Gopalganj) endemic for VL in Bihar, India. All participants were ≥18 years of age, with any stage of HIV infection, and had no current diagnosis or history of VL or post kala-azar dermal leishmaniasis (PKDL). Participants requiring immediate medical intervention were excluded from the study.

Sample Size

The sample size calculation has been described in detail elsewhere (Mahajan & Owen et al., in press). Briefly, it was estimated that the prevalence of ALI in PLHIV would be 15% based on studies of non-immunocompromised (2,5,8,9) and immunocompromised individuals (7). Given a precision of 2.5% and a confidence level of 99%, samples size was estimated to be 1,352 participants.

Recruitment

Individuals presenting at ART centres were screened consecutively as detailed previously (Mahajan & Owen et al., in press). Upon enrolment, all participants underwent a clinical examination and sociodemographic data were collected. An rK39 RDT (Kalazar Detect Rapid Test, Inbios International Inc., WA, USA) was conducted upon enrolment as per manufacturers' instructions. A peripheral blood sample was collected for qPCR and rK39 ELISA, and a urine sample was collected for *Leishmania* antigen ELISA and the Determine TB-LAM tuberculosis assay (Abbott Diagnostics, Lake Bluff, IL, USA) in individuals with

CD4 counts <200 cells/mm^3. At enrolment, any participant meeting the clinical case definition of VL (fever, splenomegaly, and a positive rK39 RDT) were referred for diagnosis and treatment at a VL-HIV treatment centre in Patna, Bihar and were excluded from the study if VL was confirmed.

Data relating to HIV diagnosis including World Health Organization (WHO) clinical staging (one to four) for HIV infection and routine clinical information were collected. Individuals were classified according to body mass index (BMI) as severely underweight (BMI<16.5 kg/m^2), underweight (BMI 16.5-18.5 kg/m^2), normal (BMI 18.5-25 kg/m^2), and overweight (>25 kg/m^2).

Participants testing positive at baseline by rK39 serology and qPCR were followed up for every three months for 18 months for further blood (rK39 serology, qPCR, full blood counts, CD4 counts, and HIV viral load) and urine (*Leishmania* antigen ELISA) samples. An rK39 RDT (Kalazar Detect Rapid Test, Inbios International Inc., USA) was conducted at each follow-up visit. Samples were batched and stored at -80°C until testing. Following testing, remaining samples were stored in a biobank repository intended for future research. Participants testing negative for rK39 serology and qPCR at baseline were followed up by telephone every three months for 18 months. At follow-up, participants were asked if they had received treatment for VL. At the time of study design, data on the urinary *Leishmania* antigen ELISA were limited and as such was not considered in the primary definition of ALI.

Any participant meeting the clinical case definition of VL at follow-up were referred for diagnosis and treatment and excluded from further follow-up if VL was confirmed. Where follow-up of participants was disrupted due to adverse weather conditions such as flooding events or restrictions related to the COVID-19 pandemic, a final follow-up telephone call or visit was sought at 18-months with the aim of reducing loss to follow-up.

Diagnostic assays

Low molecular weight *Leishmania* antigen was detected in the urine (antigenuria) of participants using the *Leishmania* antigen ELISA (Clin-tech, Guildford, UK) according to manufacturer's instructions. *Leishmania* kinetoplast DNA, extracted from whole blood (100µl) using the DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany), was detected in peripheral blood by qPCR as previously described (Mahajan & Owen et al., in press). Anti-*Leishmania* antibodies were detected using the rK39 ELISA on plasma separated from venous blood as previously described (Mahajan & Owen et al., in press) and using the rK39 RDT (Kalazar Detect Rapid Test, Inbios International Inc., USA) on finger-prick capillary blood samples according to manufacturer's instructions.

Statistical analysis

R Studio (version 1.3.1056) and SPSS (version 23) were used to conduct data analysis. Continuous variables were summarised as mean (standard deviation) and median (inter-quartile range), and categorical data were presented as counts and percentages. The software package 'Venny' was used to create Venn diagrams for comparison of diagnostic tests (10). Chi-square or Fisher's exact test were used to analyse difference in proportion. Bivariate analysis was used to individually assess the association of all covariates. Odds ratios with 95% confidence intervals were calculated. Covariates in the bivariate model with a p-value <0.2 were included in a logistic regression model to determine independent risk factors for mortality. The cumulative incidence of treatment outcome was estimated using the Kaplan–Meier method. Comparisons between groups were based on the log-rank test. Statistical significance was considered with a p-value ≤0.05.

Ethics

All participants gave written informed consent before participating in the study. Ethical approval for this study was given by Médecins Sans Frontières (MSF) (Ref: 1763), the RMRIMS (Ref: 02/RMRI/EC/2017), and the Liverpool School of Tropical Medicine (LSTM) (Ref: 18-087). This study was registered prospectively with the Clinical Trial Registry-India (CTRI/2017/03/008120).

Results

Progression of ALI to VL in PLHIV

In total, 1,589 PLHIV were screened, of whom 1,296 were enrolled in a cross-sectional survey which found baseline prevalence of ALI to be 7.4% (n=96) (Mahajan & Owen et al., in press). An additional 13 participants were identified as having ALI and followed-up in-person but were excluded from calculations of baseline prevalence of ALI in the cross-sectional survey as their villages of residence were later found to have not reported at least one case of VL in 2017-18, as per the criteria for the cross-sectional survey. Of the 1,309 participants enrolled in the study, three participants withdrew and were excluded from the analysis.

The 1,306 participants included in the analysis had a median age of 30 years (IQR:33-46) and 697 (53.4%) were female. Within the follow-up period, 37 participants died and four developed VL. Of the remaining 1,265 participants, 1,245 (98.4%) were available for assessment at 18-months follow-up and the remaining 20 participants were followed up for a median period of 13 (IQR: 9-15) months.

Of the 1,306 PLHIV, 108 were identified as having ALI (defined as positive by rK39 ELISA, and/or rK39 RDT, and/or qPCR). All four (3.7%) participants who developed VL were in the ALI cohort (n=108). There was no development of VL in the non-ALI cohort (n=1,198).

Mortality over 18-months of follow-up in PLHIV with and without ALI

Over the 18-month follow-up period, seven (6.5%) participants with ALI died. In the non-ALI cohort, thirty (2.5%) individuals died over the same period. In a univariate analysis, being male, not being on antiretroviral therapy (ART) at baseline, being on anti-tubercular treatment (ATT) at baseline, low CD4 counts, having a WHO clinical stage three HIV infection, and ALI were significantly associated with high mortality (S1 Table). However, in multivariate model only low CD4 counts, being pre-ART at baseline, and having a WHO clinical stage three HIV infection were significantly associated with mortality (Table 1).

Table 1. Multivariate analysis of risk factors for mortality in PLHIV.

	aOR (95% CI)	p-value
Sex		
Female	Ref	
Male	1.2 (0.6, 2.6)	0.573
Age (in years)	1.03 (0.997, 1.063)	0.075
BMI (kg/m^2)	0.897 (0.792, 1.016)	0.088
CD4 count (cells/mm^3)	0.997 (0.994, 0.999)	**0.003**
ART status		
On ART	Ref	
Pre-art	3.9 (1.2, 12.7)	**0.026**
ATT status		
Never on ATT	Ref	
History of ATT	1.4 (0.2, 8.9)	0.743
Currently on ATT	1.3 (0.5, 3.6)	0.614
WHO clinical stage		
I	Ref	
II	0.3 (0, 2.4)	0.259
III	6.7 (1.3, 33.8)	**0.022**
IV	0	0.999
ALI		
No	Ref	
Yes	1.8 (0.7, 4.7)	0.206

The cumulative hazard of mortality at 3-, 6-, 9-, 12-, 15-, and 18-months following recruitment was 0.3%, 0.8%, 1.3%, 1.6%, 2.2% and 2.4%, respectively in the non-ALI cohort. Whereas the cumulative hazard of mortality at 3-, 6-, 9-, 12-, 15-, and 18-months following recruitment was 1.9%, 2.8%, 4.7%, 5.7%, 6.6% and 6.6%, respectively in the ALI cohort. The survival distribution of patients with ALI was significantly different from patents without ALI (p=0.02) (Fig 1).

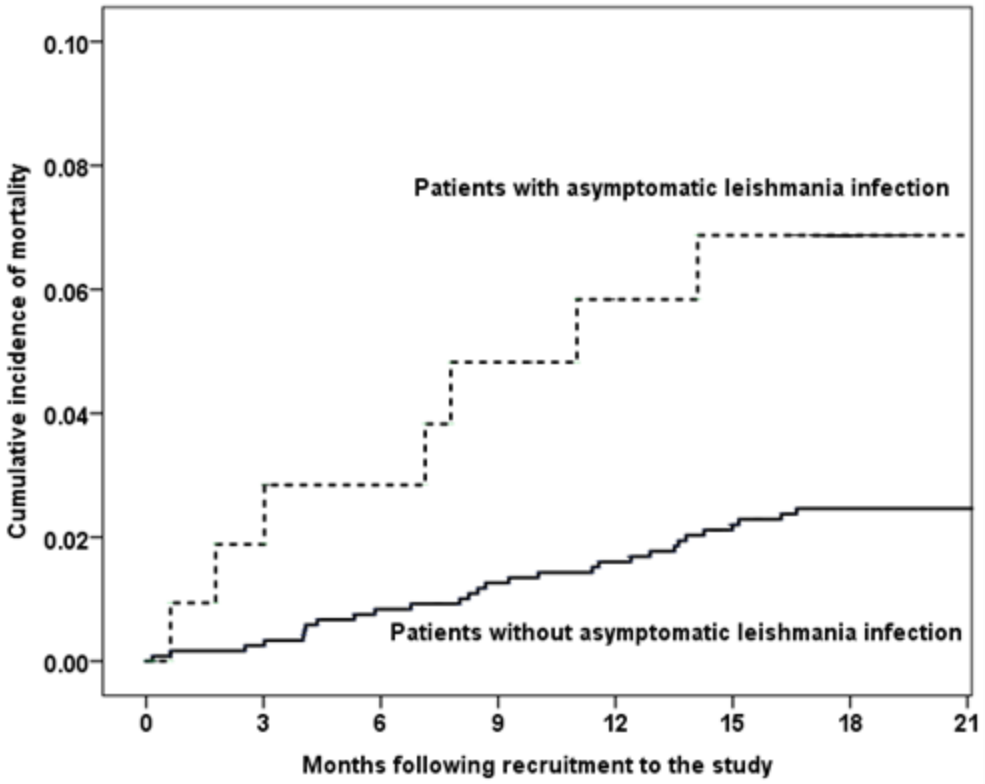


Figure 1. Kaplan–Meier estimates of cumulative incidence of mortality at different time points following enrolment.

Diagnostic profiles of individuals with ALI

All four participants who progressed from ALI to VL were positive by three or more tests (*Leishmania* antigen ELISA, qPCR, rK39 RDT, and/or rK39 ELISA) in combination (Fig 2). Two progressors were positive by three tests in combination, the rK39 ELISA, qPCR, and the *Leishmania* antigen ELISA (Fig 2). The only two individuals positive by all four tests developed VL within the follow-up period (Fig 2). Median antigenuria at baseline was 1,932.0 UAU/ml in the four participants who progressed to VL, compared to 12.4 UAU/ml in the asymptomatic non-progressors (n=104). The mean (SD) percentage positivity by rK39 ELISA at baseline was 65.4% (42.3) in the four participants who progressed to VL compared to 17.4% (17.5) in asymptomatic non-progressors (n=104). The overall risk of developing VL in ALI diagnosed by rK39 ELISA, rK39 RDT, qPCR, and *Leishmania* antigen ELISA was 3.7% (4/108), 40% (2/5), 57% (4/7) and 50% (4/8), respectively (Fig 2).

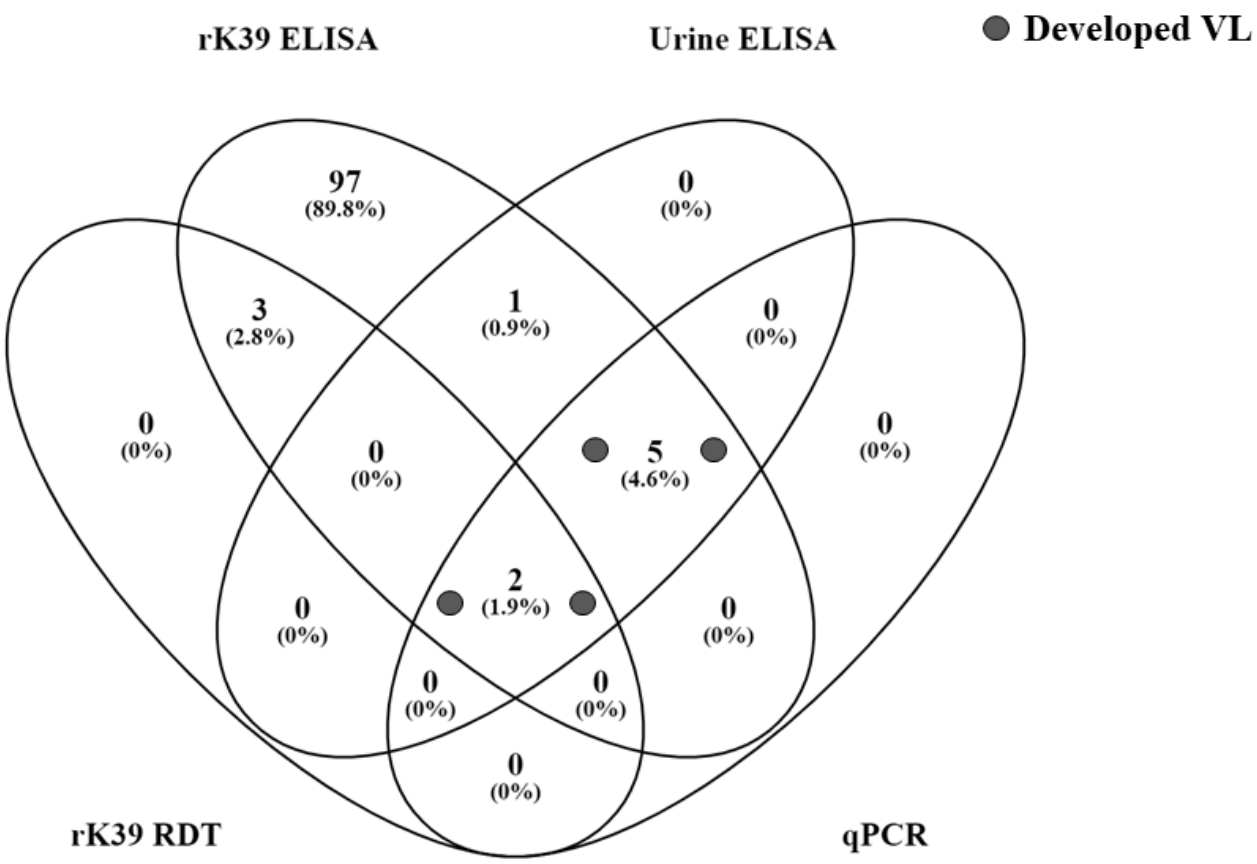


Figure 2. Four (3.7%) of the 108 participants found to have ALI (defined as a positive rK39 RDT, and/or rK39 ELISA, and/or qPCR) developed VL over the 18-month follow-up period. Of the four individuals who progressed, two were positive by the rK39 ELISA, qPCR, and the *Leishmania* antigen ELISA in combination, and two were positive by rK39 RDT, rK39 ELISA, qPCR, and the *Leishmania* antigen ELISA in combination.

Days from recruitment to parasitological conformation of VL in the four patients with ALI was 6, 93, 110, and 362 days, respectively. One of the four participants presenting with symptoms of VL at follow-up and subsequently diagnosed with VL was treated prior study sample collection and another participant was diagnosed and treated with VL elsewhere prior to a study follow-up visit (Table 2).

All asymptomatic individuals were positive by rK39 ELISA at baseline. Of the 95 participants positive by rK39 ELISA at baseline with a matched sample at 18-months, 28 (29.5%) remained positive for anti-*Leishmania* antibodies. Of the four asymptomatic participants positive by *Leishmania* antigen ELISA at baseline with a matched sample at 18-months, three (75.0%) remained positive for the *Leishmania* antigen ELISA, with a median antigenuria of 1543.0 UAU/ml and 1568.0 UAU/ml at baseline and 18 months, respectively. Of the three asymptomatic participants positive by qPCR at baseline with a matched sample at 18-months, two (66.6%) remained positive by qPCR. Of the three asymptomatic participants positive by rK39 RDT at baseline with a matched sample at 18-months, all three (100%) remained positive for by rK39 RDT.

No asymptomatic participants negative by *Leishmania* antigen ELISA or qPCR at baseline and with a matched sample at 18-months subsequently became positive by *Leishmania* antigen ELISA or qPCR. Five asymptomatic participants who were negative by rK39 RDT at baseline subsequently became positive by rK39 RDT at 18-months. Overall, 32 (34.0%) of the 94 asymptomatic participants with matched samples at baseline and 18-months remained positive for at least one of the four markers.

2738 **Table 2. Diagnostic characteristics of participants progressing from ALI to VL over the 18-month follow-up period.**

Participant no.	Month of follow-up	qPCR	*Leishmania* antigen ELISA	rK39 RDT	rK39 ELISA	Splenic aspiration grading	Days of illness at presentation	Symptoms at presentation
1	0	Positive	Positive	Positive	Positive	NA	NA	NA
	3	Positive	Positive	Negative	Positive	6+	7	Fever, weight loss, cough, hepatosplenomegaly, decreased appetite
2*	0	Positive	Positive	Positive	Positive	NA	NA	NA
	3	NA	NA	Positive	NA	5+	28	Fever, weight loss, cough, hepatosplenomegaly
3¶	0	Positive	Positive	Positive	Positive	NA	NA	NA

Participant no.	Month of follow-up	qPCR	*Leishmania* antigen ELISA	rK39 RDT	rK39 ELISA	Splenic aspiration grading	Days of illness at presentation	Symptoms at presentation
3	3	NA	NA	NA	NA	5+	NA	NA
4	0	Positive	Positive	Negative	Positive	NA	NA	NA
	3	Positive	Positive	Negative	Negative	NA	NA	NA
	6	Positive	Positive	Negative	Negative	NA	NA	NA
	9	Positive	Positive	Negative	Negative	NA	NA	NA
	12	NA	NA	Negative	Negative	NA	7	Fever and left facial palsy

* Patient treated before sampling.

¶ Patient diagnosed and treated elsewhere.

Discussion

PLHIV in *Leishmania* endemic areas are estimated to be at 100-2,320 times greater risk of developing VL and have poor outcomes (11). To our knowledge there are no data on rate and risk factors for progression of ALI to VL in PLHIV in India. Data which improve the clinical management of patients with VL-HIV coinfection on the Indian subcontinent (ISC) are needed to improve patient outcomes in a population where early intervention could reduce treatment failure and mortality. A better understanding of the scale of progression and markers to identify those most at risk of developing VL would aid clinical decision making.

In this prospective cohort study, we followed up 1,306 PLHIV with and without ALI to monitor for progression to VL over an 18-month period. We identified four individuals who presented with VL within the follow-up period, all of whom had ALI at baseline. The low rate of disease progression would indicate administration of prophylaxis would likely not be required to prevent development VL in most individuals. High viral load was found to be a risk factor for ALI in Brazil (6). Conversely, HAART was suggested to have a protective role in preventing progression of ALI in another study in Brazil (12). HIV viral load is low in most participants and adherence to HAART is high in this patient population, including the four participants who progressed from ALI to VL. Our aim was to assess risk factors for progression to VL, however, the low number of progressors in this study limits the statistical analysis of risk factors for progression.

Mortality was higher in individuals with ALI than the non-ALI cohort over the 18-month follow-up period. Multivariate analysis indicated that there were more severe and complicated HIV infections in the ALI cohort. The reciprocal acceleration of *Leishmania* and HIV infections is well documented (13,14). A high percentage of asymptomatic participants still had markers of *Leishmania* infection at 18-months, including parasitaemia detected by qPCR and antigenuria, suggesting persistent asymptomatic infection. Of the 511 participants identified as having asymptomatic *Leishmania*-HIV infection at baseline in a study in Ethiopia, there was estimated to be a reversion to negative markers of 40.1% over one year of follow-up and an incidence of 36 asymptomatic infections (7). In this study, we are unable to calculate the incidence of ALI as those testing negative at baseline were not followed up for further tests.

All individuals who progressed were positive by three or more tests (rK39 RDT, rK39 ELISA, qPCR, and/or *Leishmania* antigen ELISA). In the study in Ethiopia, the asymptomatic participant who developed VL was positive by all four of the diagnostic assays used (rK39 RDT, DAT, PCR and KAtex) at baseline, three

months, six months, and at nine months where fever and hepatosplenomegaly were detected (7). This individual was the only study participant that tested positive for all four tests used (7). DAT titer and KAtex score remained stable at each follow-up visit, however PCR Ct value declined from 28.2 at baseline to 17.2 at nine months indicating an increasing parasite load (7). In this study, asymptomatic progressors had higher anti-*Leishmania* antibody titers than asymptomatic non-progressors, in keeping with what was seen in studies in immunocompetent individuals (3,4). Similarly, we found high antigenuria in asymptomatic progressors compared to non-progressors, with the *Leishmania* antigen ELISA allowing quantification of antigenuria over the KAtex, it's semi-quantitative predecessor.

All but one of the individuals that progressed developed VL by the three-month follow-up visit, the remaining participant developed VL by nine-months. One participant who developed VL in a study of 511 participants in Ethiopia was asymptomatically infected at baseline and had developed VL between the six- and nine-month follow-up visits (7). A study in immunocompetent participants in Bihar found the median time to progression to VL was five months (4). Of the 79 asymptomatic individuals identified in a study in West Bengal, seven (9.1%) remained sero-positive after three years and eight (10.4%) progressed from asymptomatic to symptomatic disease, three doing so after 30 months of follow-up (5).

Here we address the need for data on ALI in PLHIV on the ISC and identify a population at risk of poor disease outcomes regardless of progression to VL. Further work to calculate the incidence of ALI in PLHIV in India would provide a more complete picture of ALI-HIV coinfection on the ISC. Furthermore, an investigation of biomarkers for progression of ALI in PLHIV, such as ADA and IL-10 which have been found to be high in individuals with ALI and remained elevated in those that progressed to VL, could add to the spectrum of tools available to identify those most at risk of progression (15).

Acknowledgements

With thanks to the participants and the MSF field team. With thanks to Professor Steven Reed for providing the rK39 antigen.

Funding

This work was funded by Médecins Sans Frontiers, Spain, who fulfilled a sponsor-investigator role in the study. Additional funding in kind was provided by the Medical Research Council (MRC) Doctoral Training Partnership (DTP) (MR/N013514/1).

References

1. Pasquau F, Ena J, Sanchez R, Cuadrado JM, Amador C, Flores J, et al. Leishmaniasis as an opportunistic infection in HIV-infected patients: Determinants of relapse and mortality in a collaborative study of 228 episodes in a Mediterranean region. European Journal of Clinical Microbiology and Infectious Diseases. 2005;24:411-8.

2. Mannan S Bin, Elhadad H, Loc TTH, Sadik M, Mohamed MYF, Nam NH, et al. Prevalence and associated factors of asymptomatic leishmaniasis: a systematic review and meta-analysis. Parasitol Int. 2021;81:102229.

3. Hirve S, Boelaert M, Matlashewski G, Mondal D, Arana B, Kroeger A, et al. Transmission Dynamics of Visceral Leishmaniasis in the Indian Subcontinent – A Systematic Literature Review. Chatterjee M, editor. PLoS Negl Trop Dis. 2016;10(8):e0004896.

4. Chakravarty J, Hasker E, Kansal S, Singh OP, Malaviya P, Singh AK, et al. Determinants for progression from asymptomatic infection to symptomatic visceral leishmaniasis: A cohort study. PLoS Negl Trop Dis. 2018;13(3).

5. Saha P, Ganguly S, Chatterjee M, Das SB, Kundu PK, Guha SK, et al. Asymptomatic leishmaniasis in kala-azar endemic areas of Malda district, West Bengal, India. Louzir H, editor. PLoS Negl Trop Dis. 2017;11(2):e0005391.

6. Guedes DL, Justo AM, Júnior WLB, da Silva ED, de Aquino SR, Junior MS da CL, et al. Asymptomatic *Leishmania* infection in HIV-positive outpatients on antiretroviral therapy in Pernambuco, Brazil. PLoS Negl Trop Dis. 2021;15(1):e0009067.

7. van Griensven J, van Henten S, Mengesha B, Kassa M, Adem E, Endris Seid M, et al. Longitudinal evaluation of asymptomatic *Leishmania* infection in HIV-infected individuals in North-West Ethiopia: A pilot study. PLoS Negl Trop Dis. 2019;13(10):e0007765.

8. Picado A, Ostyn B, Singh SP, Uranw S, Hasker E, Rijal S, et al. Risk Factors for Visceral Leishmaniasis and Asymptomatic *Leishmania donovani* Infection in India and Nepal. PLoS One. 2014;9(1):e87641.

9. Topno RK, Das VNR, Ranjan A, Pandey K, Singh D, Kumar NN, et al. Asymptomatic infection with visceral leishmaniasis in a disease-endemic area in Bihar, India. Am J Trop Med Hyg. 2010;83(3):502–6.

10. Oliveros JC. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. [Internet]. Available from: https://bioinfogp.cnb.csic.es/tools/venny/index.html.

11. Alvar J, Aparicio P, Aseffa A, Boer M Den, Cañavate C, Dedet J-P, et al. The Relationship between Leishmaniasis and AIDS: the Second 10 Years. Clin Microbiol Rev. 2008;21(2):334–59.

12. Orsini M, Canela JR, Disch J, Maciel F, Greco D, Toledo A, et al. High frequency of asymptomatic *Leishmania spp.* infection among HIV-infected patients living in endemic areas for visceral leishmaniasis in Brazil. Trans R Soc Trop Med Hyg. 2012;106(5):283–8.

13. Bernier R, Turco SJ, Olivier M, Tremblay M. Activation of human immunodeficiency virus type 1 in monocytoid cells by the protozoan parasite *Leishmania donovani*. J Virol. 1995;69(11):7282–5.

14. Wolday D, Akuffo H, Britton S, Hathaway A, Sander B. HIV-1 inhibits *Leishmania*-induced cell proliferation but not production of interleukin-6 and tumour necrosis factor alpha. Scand J Immunol. 1994;39(4):380–6.

15. Das VNR, Bimal S, Siddiqui NA, Kumar A, Pandey K, Sinha SK, et al. Conversion of asymptomatic infection to symptomatic visceral leishmaniasis: A study of possible immunological markers. PLoS Negl Trop Dis. 2020;14(6):e0008272.

2849 **Table S1. Univariate analysis of risk factors for mortality in PLHIV.**

	Died (n=37)	Survived (n=1269)	Total (n=1306)	OR (95% CI)	p-value
Sex					
Female	13 (35.1)	684 (53.9)	697 (53.4)	Ref	
Male	24 (64.9)	585 (46.1)	609 (46.6)	2.2 (1.1, 4.4)	**0.026**
Age group (in years)					
18-29	4 (10.8)	171 (13.5)	175 (13.4)	Ref	1
30-44	16 (43.2)	719 (56.7)	735 (56.3)	1 (0.3, 3.4)	0.892
45-59	12 (32.4)	321 (25.3)	333 (25.5)	1.6 (0.5, 5.8)	0.442
≥ 60	5 (13.5)	58 (4.6)	63 (4.8)	3.7 (0.9, 15.8)	0.07
ART status					
On ART	32 (86.5)	1243 (98)	1275 (97.6)	Ref	
Pre-ART	5 (13.5)	26 (2.1)	29 (2.4)	7.4 (2.4, 19.8)	**0.002**
ATT status					
Never on ATT	28 (75.7)	1122 (88.4)	1150 (88.1)	Ref	
History of ATT	5 (13.5)	133 (10.5)	138 (10.6)	1.5 (0.5, 3.8)	0.407
Currently on ATT	4 (10.8)	14 (1.1)	18 (1.4)	11.4 (2.6, 39.4)	**0.002**
BMI (kg/m^2)					
<16.5	7 (18.9)	120 (9.5)	127 (9.7)	2.6 (1, 6.2)	0.056
16.5-<18.5	11 (29.7)	281 (22.1)	292 (22.4)	0.7 (0.8, 3.7)	0.178
18.5-<25	17 (45.9)	747 (58.9)	764 (58.5)	Ref	
≥25	2 (5.4)	121 (9.5)	123 (9.4)	0.7 (0.1, 3.1)	0.993
CD4 count (cells/mm^3)					
≥ 300	16 (43.2)	971 (76.5)	987 (75.6)	Ref	
200 - 299	2 (5.4)	178 (14)	180 (13.8)	0.7 (0.1, 2.6)	0.666
100 - 199	11 (29.7)	93 (7.3)	104 (8)	7.2 (3.1, 15.9)	**<0.0001**

50-99	6 (16.2)	20 (1.6)	26 (2)	18 (5.9, 50.3)	**<0.0001**
<50	2 (5.4)	7 (0.6)	9 (0.7)	17.1 (1.6, 100.1)	**0.021**
WHO clinical stage					
I	31 (83.8)	1185 (93.4)	1216 (93.1)	Ref	
II	1 (2.7)	68 (5.4)	69 (5.3)	0.6 (0, 3.5)	0.958
III	5 (13.5)	14 (1.1)	19 (1.5)	13.6 (4.1, 39.1)	**<0.0001**
IV	0 (0)	2 (0.2)	2 (0.2)		
ALI					
No	30 (81.1)	1168 (92)	1198 (91.7)	Ref	
Yes	7 (18.9)	101 (8)	108 (8.3)	2.7 (1.1, 6.1)	**0.037**

Chapter 5. 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

5.1 Acknowledgement of work done

Sophie I. Owen, Emily Adams and Sakib Burza were responsible for study design. Sophie I. Owen was responsible for writing all ethics and HMSC applications, submission of ethics and HMSC applications to LSTM and RMRIMS and publication. Kristien Cloots was responsible submission of ethics applications to the Institute of Tropical Medicine and the University of Antwerp. Sophie I. Owen was responsible for budgeting and procurement. Shiril Kumar, Neena Verma, Raman Mahajan, and Amit Harshana provided feedback to the study design.

5.2 Introduction

Following on from chapter 3 and chapter 4, I continue with the topic of *Leishmania* infection in PLHIV, this time focussing on symptomatic VL with the aim of improving clinical care in this population. VL-HIV coinfection presents a challenge to VL elimination efforts on the ISC. Coinfection was shown to be a predictor of an increase in the incidence of VL at a village level in a study in Bihar, India, the site of the study presented in this chapter [159]. In 2019, the prevalence of HIV was estimated to be 0.22% (0.17–0.29%) in India, with the prevalence in Bihar falling just below the national average at 0.18% (0.11-0.24%) [66]. Bihar had the second highest number of new HIV infections behind the state of Maharashtra, with approximately 8,000 new infections in 2019 [66].

As mentioned in chapter 3, much of the data on VL-HIV coinfection comes from studies in East Africa, Brazil, and the Mediterranean. Data on VL-HIV coinfection in India remains limited. In 2014, MSF conducted a consecutive screening of 2,077 people over 13 years of age with presumptive VL infection in Bihar, and found 5.6% of participants to be coinfected with HIV [86]. Currently, coinfection with VL is not classed as a stage four AIDS-defining illness according to World Health Organization (WHO) guidelines, unless a patient presents with disseminated disease. However, it has been recommended by clinicians that VL be included in WHO guidelines due to the severity of disease in coinfected individuals [3,165].

At the MSF treatment centre in Bihar, India, HIV patients presenting with symptoms of VL are screened with an rK39 RDT in combination with a clinical history, as per the WHO definition of a VL case [86,131]. Due to the poor outcomes associated with VL in PLHIV as mentioned in chapter 3 [3,166], the potential for atypical presentations of VL [167], and the increased risk of coexisting or differential diagnoses [14], clinicians are required to be more rigorous before excluding a diagnosis of VL. Where the rK39 RDT is negative, but VL is still strongly suspected or in incidences of VL relapse, patients are further screened by parasitological confirmation of splenic biopsy [131]. Bone marrow aspiration is conducted where splenic biopsy is contraindicated, however, sensitivity is shown to be lower [3,111]. A retrospective analysis of the use of AmBisome and miltefosine combination therapy for coinfected patients, conducted by MSF in Bihar, India, found that 61.8% (n=63) of participants were required to have a confirmatory splenic or bone marrow aspiration [131]. These tissue biopsies are painful, invasive, and require highly trained clinicians in a suitably equipped hospital environment [111]. Biopsies also require microscopy technicians trained in the identification of LD bodies within a Giemsa or Leishman stained sample

[151,168]. In instances where splenic aspiration is carried out in less than suitable conditions, a 1 in 1,000 risk of fatal haemorrhage was previously reported in India [111].

Where VL-HIV coinfection is diagnosed, patients are put on a course of treatment typically lasting between 14 and 38 days on the ISC depending on the drug regimen used [3]. Treatment regimens for VL-HIV where *L. donovani* is the aetiological agent have previously required a 38-day course of LAMB (maximum total dose 40mg/kg) as per WHO recommendation. However, more recently a shorter 14-day course combination therapy of LAMB (AmBisome) and miltefosine (Impavido) was used on a compassionate basis by MSF in VL-HIV patients in India [3,131]. Of the 102 participants recruited to the study, 15.7% (n=16) of participants died during follow-up and the risk of relapse was estimated to be 13.9% at 18 months [131]. Due to the relatively high rates of treatment failure and relapse in VL-HIV, test of cure can be used to determine whether first-line treatment worked and to guide administration of a second-line course of treatment where this cannot be determined clinically [162]. Detection of anti-*Leishmania* antibodies by rK39 serology cannot be used as test of cure. Therefore, test of cure is currently carried out by a second splenic or bone marrow biopsy [15,162]. Alternatives to tissue aspiration are needed to reduce the number of invasive splenic and bone marrow biopsies carried out in PLHIV.

Chapter five summary

Here, we present a protocol to evaluate non- and minimally invasive assays to tissue aspiration for the diagnosis and test of cure for VL in 91 PLHIV attending the RMRIMS in Patna, India. The ethical approval for this study was granted by LSTM, The Institute of Tropical Medicine in Antwerp, the University of Antwerp, and the RMRIMS, Patna. This study was unable to go ahead due to a decision by the Health Ministry's Screening Committee (Indian Council of Medical Research) which approves studies involving collaboration or funding from international organisations. Tests to be evaluated included qPCR in blood and skin microbiopsies, microscopy on peripheral blood buffy coat smear, and the *Leishmania* antigen ELISA. Should the diagnostic accuracy of these assays be high enough they may provide a replacement to invasive bone marrow and splenic aspirates for the diagnosis and test of cure for VL in PLHIV. We are actively engaged to complete this protocol with collaborators at MSF and RMRIMS.

5.3 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-coinfected patients in India: a prospective cohort study

Sophie I Owen[1], Sakib Burza[2], Shiril Kumar[3], Neena Verma[3], Raman Mahajan[2], Amit Harshana[2], Krishna Pandey[3], Kristien Cloots[4], Emily R Adams[1, *], Pradeep Das[3]

[1] Liverpool School of Tropical Medicine, Liverpool, UK

[2] Médecins Sans Frontières, New Delhi, India

[3] Rajendra Memorial Research Institute of Medical Science, Patna, Bihar, India

[4] Institute of Tropical Medicine, Antwerp, Belgium

* Corresponding author

Email: Emily.adams@lstmed.ac.uk (ERA)

Owen S. I. *et al.* BMJ Open (2021) 11: e042519.

Abstract

Introduction: HIV coinfection presents a challenge for diagnosis of visceral leishmaniasis (VL). Invasive splenic or bone marrow aspiration with microscopic visualisation of *Leishmania* parasites remains the gold standard for diagnosis of VL in HIV-coinfected patients. Furthermore, a test of cure by splenic or bone marrow aspiration is required as patients with VL-HIV infection 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.

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.

Trial registration number: REF/2019/01/023677

Strengths and limitations of this study

- This study will evaluate non-invasive and minimally invasive alternatives to splenic or bone marrow aspiration in HIV-infected patients for diagnosis of visceral leishmaniasis (VL) 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 and test of cure of VL in HIV-infected patients could be made.
- The study addresses both issues of initial diagnosis and test of cure.
- This study is limited to HIV-infected patients presenting at hospital who are likely to be presenting with more advanced disease.
- The use of minimally invasive techniques do not have standardised approach methods.

Introduction

In the absence of treatment, visceral leishmaniasis (VL) caused in India by a parasitic infection of *Leishmania donovani* (LD), 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 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, aged ≥14 years, 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 coinfected 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 infections [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). When splenic aspiration is not possible (i.e., unpalpable spleen, low platelet, or haemoglobin (Hb)), bone marrow aspirates (BMAs) have a reasonable sensitivity of between 50 and 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 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-infected patients is currently carried out by parasitological confirmation at day 29 in splenic aspirates where possible, or BMAs when 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 method; however, data on the sensitivity of the rK39 RDT in HIV-coinfected individuals in India do not exist. Kalon Biological (Guilford, UK) has developed an ELISA which detects *Leishmania* antigen excreted in urine, enabling noninvasive 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). *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 noninvasive alternative to tissue aspiration for both diagnosis and ToC [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 also shown that 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-coinfected patients, this may be similar if not higher.

The diagnostic potential of the urine antigen ELISA, peripheral blood and skin qPCR, and peripheral blood buffy coat smear microscopy for diagnosis of VL in HIV-infected 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 ToC for patients coinfected with HIV on the Indian subcontinent (ISC) to potentially eliminate the need for repeated and invasive splenic and bone marrow aspirations.

Study objective(s)

Primary objective(s):

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

Secondary objective(s):

- To evaluate the sensitivity and specificity of the urine antigen ELISA, qPCR (blood and skin microbiopsies) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a ToC at day 15 for symptomatic VL in HIV-positive patients compared with 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 microbiopsies) and peripheral blood buffy coat smear microscopy, singularly or in combination, as a diagnostic and ToC at day 29 for symptomatic VL in HIV-positive patients compared to BMA in a subset of patients contraindicated for splenic aspiration at days 0 and 29, respectively.
- To investigate the kinetics of LD infection during treatment (days 0, 3, 8, 15 and 29) using urine antigen ELISA, qPCR (blood and skin microbiopsies) 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 subset 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).

Furthermore, 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 tuberculosis (TB) (GeneXpert and chest X-ray) will be conducted as standard for these patients.

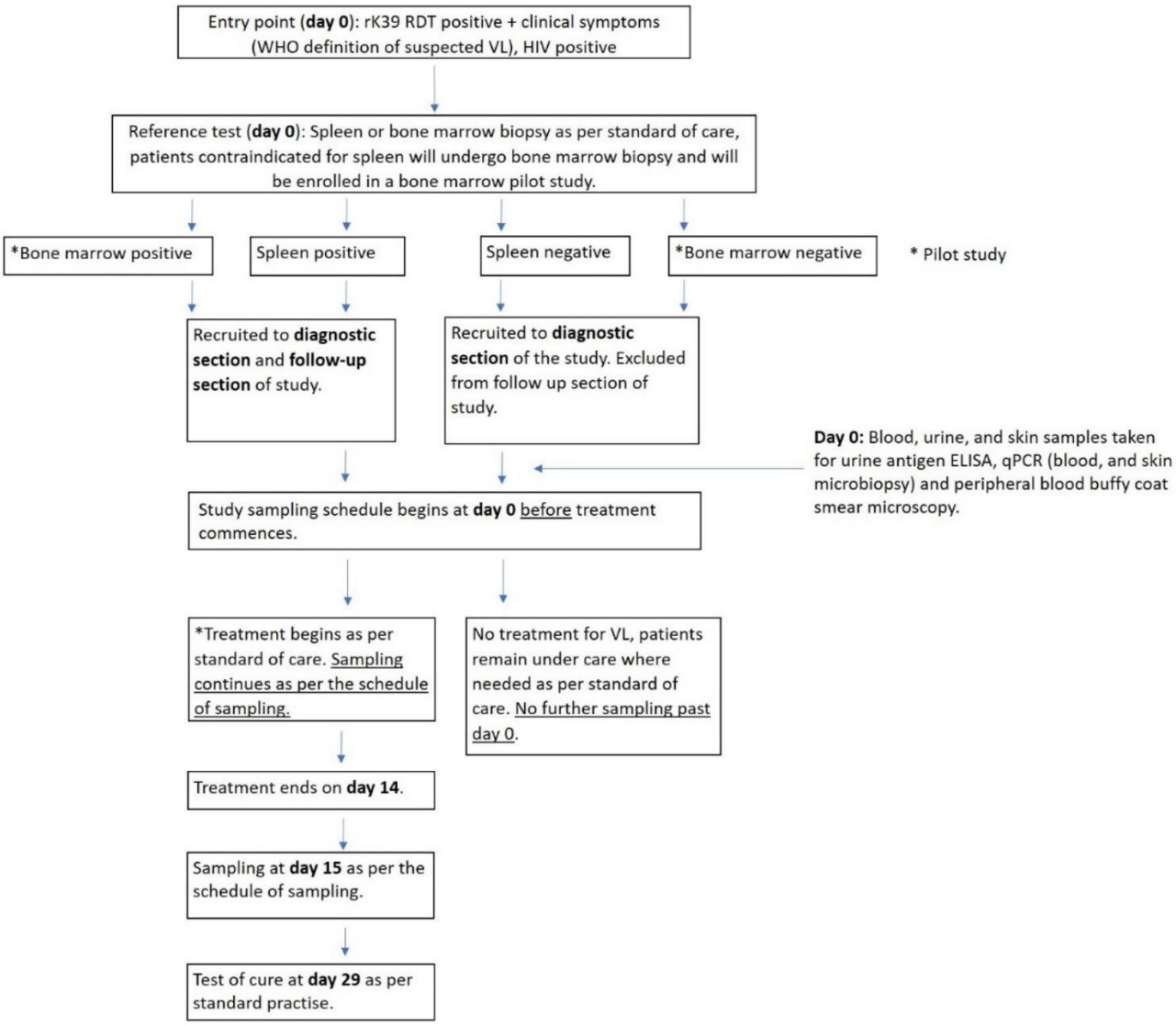


Figure 1. Study workflow.

Main entry criteria

Inclusion criteria:

- WHO definition of a suspected case of VL: rK39 RDT-positive and clinical symptoms.
- Has undergone parasitological confirmation (splenic or bone marrow aspiration) as per the standard of care.
- Diagnosis of HIV as per National AIDS control organization guidelines.
- Patients found positive by parasitological confirmation (splenic aspiration or bone marrow aspiration) in the diagnostic section will be continued to follow-up.
- 18 years of age or above.
- 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 and skin microbiopsy samples) 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 and 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 and skin microbiopsy samples) and peripheral blood buffy coat smear microscopy as a ToC 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)

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

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

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

$$n = 73$$

An additional four 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. 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.

$$n = 91$$

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 RMRIMS, Patna, India or an appropriate quality-assured laboratory. Informed consent will be taken by study staff who have passed the National Institutes of Health (NIH) Protecting Human Research Participants Ethics course (https://phrp.nihtraining.com) or equivalent. A screening and recruitment log will be maintained.

Schedule of events

Suspects will be screened with an rK39 RDT and a clinical examination as per standard practice (WHO definition of a suspected VL case). 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, and where splenic aspiration is not contraindicated. Patients are excluded from splenic aspiration based on an unpalpable spleen, platelet count <40x10^9/L or Hb <50g/L, or with significantly prolonged prothrombin time (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 case record forms (CRFs):

- Sociodemographic information (e.g., sex).
- HIV-related information (e.g., diagnosis, WHO staging and opportunistic infections).
- Antiretroviral therapy (ART)-related information (e.g., CD4 counts, ART regimen, opportunistic infection (OI) treatment and ART adherence).
- VL/post-kala-azar dermal leishmaniasis (PKDL)-related information (e.g., VL/PKDL history and VL/PKDL symptoms).
- Past and current medical conditions (e.g., malaria, chronic comorbidities and 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 and 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.

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 semiquantitative result in relation to a standard curve of known concentration of cultured parasites.

Formal 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 samples 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 microbiopsies	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 LD 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 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 summarised 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 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 days 15 and 29 will be compared to the gold standard ToC (parasitological confirmation) at day 29. Sensitivity and specificity with 95% confidence intervals will be calculated as above.

The continuous variables will be summarised 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 BMA 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 minimised 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 ToC that makes the current invasive splenic or BMAs no longer necessary in the workup of HIV-VL coinfection. This would limit the risks associated to these tests (fatal hemorrhage in case of splenic aspirates of 1/1,000 and painful procedure in the case of BMA) 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 ToC at day 15 is equally accurate as the current (parasitological) ToC at day 29, this would decrease the duration of hospitalisation 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.

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 ToC that are safer, better tolerated and less technically demanding are required. HIV-infected patients diagnosed with VL in India have recently been shown to have better treatment outcomes with a combination therapy of liposomal amphotericin B and miltefosine over 14 days (CTRI/2015/05/005807) [10]. The choice of day 29 for ToC is based on older treatment regimens which were traditionally a month long. As such, evidence for the diagnostic accuracy of a ToC 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 required 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 ISC 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 ToC 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 invasive and noninvasive tools for diagnosis and ToC in VL patients coinfected 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 RMRIMS 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 Guidelines Review Committee (GRC) who are due to provide global updated management guidelines for VL-HIV coinfection in 2021. Reporting of results will follow Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines. Data will be made available on request.

Patient and public involvement

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

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.

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.

3377 3. Le Rutte EA, Coffeng LE, Bontje DM, et al. Feasibility of eliminating visceral leishmaniasis from the
3378 Indian subcontinent: explorations with a set of deterministic age-structured transmission models.
3379 Parasit Vectors. 2016;9:24.

3380 4. National AIDS Control Organisation, Ministry of Health and Family Welfare G of I. National Strategic
3381 Plan for HIV/AIDS and STI 2017 - 2024. 2017. http://naco.gov.in/sites/default/files/Paving the Way for
3382 an AIDS 15122017.pdf

3383 5. Jarvis JN, Lockwood DN. Clinical aspects of visceral leishmaniasis in HIV infection. Curr Opin Infect Dis.
3384 2013;26:1–9.

3385 6. National AIDS Control Organization & ICMR-National Institute of Medical Statistics. India HIV
3386 Estimations 2017: Technical Report. 2018. http://naco.gov.in/sites/default/files/HIV Estimations 2017
3387 Report_1.pdf.

3388 7. Burza S, Mahajan R, Gonzalez Sanz M, et al. HIV and Visceral Leishmaniasis Coinfection in Bihar, India:
3389 An Underrecognized and Underdiagnosed Threat Against Elimination. Clin Infect Dis. 2014;59:552–5.

3390 8. Akuffo H, Costa C, van Griensven J, et al. New insights into leishmaniasis in the immunosuppressed.
3391 PLoS Negl Trop Dis. 2018;12:e0006375.

3392 9. Vallur AC, Tutterrow YL, Mohamath R, et al. Development and comparative evaluation of two antigen
3393 detection tests for Visceral Leishmaniasis. BMC Infect Dis. 2015;15:1–10.

3394 10. Burza S, Croft SL, Boelaert M. Leishmaniasis. The Lancet: 2018;10.1016/S0140-6736(18)31204-2.

3395 11. Srivastava P, Dayama A, Mehrotra S, et al. Diagnosis of visceral leishmaniasis. Trans R Soc Trop Med
3396 Hyg. 2011;105:1–6.

3397 12. Adams ER, Schoone G, Versteeg I, et al. Development and Evaluation of a Novel Loop-Mediated
3398 Isothermal Amplification Assay for Diagnosis of Cutaneous and Visceral Leishmaniasis. J Clin Microbiol.
3399 2018;56:e00386-18.

3400 13. Courtenay O, Carson C*, Calvo-Bado L, et al. Heterogeneities in *Leishmania infantum* Infection: Using
3401 Skin Parasite Burdens to Identify Highly Infectious Dogs. PLoS Negl Trop Dis. 2014;8:e2583.

3402 14. Aslan H, Oliveira F, Meneses C, et al. New Insights Into the Transmissibility of *Leishmania infantum*
3403 From Dogs to Sand Flies: Experimental Vector-Transmission Reveals Persistent Parasite Depots at Bite
3404 Sites. J Infect Dis 2016;213:1752–61.

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. 2014.

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.

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.

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.

Author Statement

SIO, SB, ERA: conceptualised the study; SIO, SB, SK, NV, RM, AH, KP, KC: methodology of the study; SIO, SB: writing of the original draft; SIO, SB, SK, NV, RM, AH, KP, KC, ERA, PD: writing, review and editing of the manuscript; ERA: funding acquisition.

Acknowledgements

We would like to thank the field teams and the patients.

Funding

The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Conflicts of Interests

None declared.

Chapter 6. Alternative antigen detection tests for visceral leishmaniasis

6.1 Acknowledgement of work done

Sophie I. Owen developed the workplan for evaluation of the mAbs. Sophie I. Owen conducted all laboratory work, data analysis, and writing. Emmanuel Moreau and Chris Williams provided guidance in the validation of the mAbs and the kala-azar antigen detection test. Antonio Campos-Netos provided the protocol for the kala-azar antigen detection test (DetectoGen Inc., USA). The mAbs validated in this chapter belong to FIND. With thanks to Francisco Javier Moreno Nuncio from the Instituto de Salud Carlos III, Madrid, Spain for providing the *L. donovani* strains used for culture.

6.2 Abstract

Background: An antigen test in rapid lateral flow assay (LFA) format is yet to be successfully developed for *Leishmania*, yet there have been several successful developments of antigen assays in enzyme-linked immunosorbent assay (ELISA) format. This may be due to a lack of sensitive antibody pairings and a low antigen load in VL patients, where ELISA tends to have a higher sensitivity to a comparative LFA. Here I evaluate thirteen monoclonal antibodies (mAb) targeting the *Leishmania donovani* complex from the Foundation for Innovative New Diagnostics' (FIND) archives for their suitability in an LFA. I then go on to evaluate the kala-azar antigen detection test (DetectoGen Inc., USA) on which there is no published performance data on the Indian subcontinent.

Methods: Thirteen mAb candidates were screened by dot blot, western blot, and ELISA against a commercial direct agglutination test (DAT) antigen (50,000,000 promastigotes/ml) (AMC Medical Research BV, Amsterdam, Netherlands), pooled *Leishmania* antigen positive urine from individuals with VL, *Leishmania* antigen negative urine from an individual with no history of VL, and matched sera from the FIND biobank. The kala-azar antigen detection test (DetectoGen Inc., USA) was run as per manufacturer's instructions on urine samples from the FIND biobank and manufacturer provided standards.

Results: Weak binding was seen in all thirteen mAbs with the strongest signal seen in mAbs one, five, and twelve. High cross-reactivity against negative controls was seen with mAb one. MAb five showed the strongest signal with low background against positive sera. MAb twelve showed signal against positive urine, positive serum, and DAT, but with high background. Proteins ranging from approximately 30- 250 kDa were identified by all thirteen mAbs in pooled *Leishmania* antigen positive urine. Optimal running conditions for the kala-azar antigen detection test (DetectoGen Inc., USA) were identified but could not be replicated in positive clinical urine samples.

Conclusion(s): No mAbs were identified as suitable candidates for a LFA using the discussed techniques. The kala-azar antigen detection test (DetectoGen Inc., USA) is a promising ELISA for the detection of *Leishmania* antigen, however further work is needed to improve assay robustness and sensitivity in non-manufacturer laboratories.

6.3 Introduction

Following on from previous chapters, in this chapter I continue with the subject of antigen tests for *Leishmania*, suitable for use in asymptomatic populations and PLHIV in an elimination setting. In chapter 2 I presented data on the potential use of antigen tests in the screening of contacts of VL and PKDL cases for ALI in Bangladesh. In chapter 3 and chapter 4 I presented data on the use of antigen tests in the screening of PLHIV for ALI in India. In chapter 5, I presented a protocol to evaluate antigen tests in VL-HIV coinfection. In this chapter (6), I focus on the early stages of development of an antigen test in collaboration with FIND, including the evaluation of antigen ELISA tests for *Leishmania* infection not evaluated elsewhere.

Existing antigen detection assays, generally detect *Leishmania* carbohydrates or protein excreted in urine [112,120]. The kinetics of renal damage following *L. donovani* infection were studied in hamster models [169]. Golden hamsters infected with *L. donovani* amastigotes (n=42) showed changes in the glomerular of the kidneys followed by a progressive deposition of the protein amyloid, when compared to uninfected hamsters (n=28) [169]. *L. donovani* antigen and Ig deposited in the renal mesangium was correlated with glomerulonephritis [169]. *L. donovani* infection causes an increase in Ig concentration in the urine, from day 14 post infection, which declines between days 42-49 post infection, and in serum peaking between days 21-28 post infection [169].

A further study of Ig eluted from the kidneys of hamsters detected the presence of *L. donovani* IgG1, IgG2, IgA, and IgM [170]. A 134kDa protein, recognised by rabbit anti-*L. donovani* promastigote and rabbit anti-*L. donovani* amastigote antisera, was found to be eluted from the kidneys of hamsters infected with *L. donovani* compared to kidney eluate from normal hamsters [170]. It was postulated that this 134KDa protein was *L. donovani* acid phosphatase [170]. These studies of renal damage as a result of *L. donovani* infection in hamster models and the studies presented in section 1.10.4 led to the development of the kala-azar antigen detection test (DetectoGen Inc., USA) (section 1.10.4). There are no published data on the performance of the kala-azar antigen detection test (DetectoGen Inc., USA) on the ISC.

The KAtex (Clin-Tech, UK), *Leishmania* antigen ELISA (Clin-Tech, UK), *Leishmania* antigen detect™ (InBios International Inc., USA), and Kala-azar antigen immunochromatographic test (ICT) (Xinjier Biotechnology Co., Ltd, China) introduced in sections 1.10.1, 1.10.2, 1.10.3, and 1.10.5 respectively, represent all the other antigen tests developed for VL at the time of writing. Both the *Leishmania* antigen ELISA (Clin-

Tech, UK) and the *Leishmania* antigen detect™ (InBios International Inc., USA) assays have shown promising diagnostic accuracy, but are yet to be adapted into LFAs.

Chapter six summary

Here, I evaluate a panel of thirteen mAbs provided by FIND, raised against *L. donovani* complex. In this chapter I focus on screening of candidate mAbs in ELISA format with the aim of identifying candidates suitable for LFA. I then go onto evaluate the kala-azar antigen detection test (DetectoGen Inc., USA), facilitated by FIND's sample biobank.

6.4 Materials and Methods

L. donovani culture

L. donovani strains S-719 from Kenya (IMRT/KE/62/LRC-L57) and DD8 from India (MHOM/IN/80/DD8) were received from the Instituto de Salud Carlos III, Madrid, Spain. Cultures were shipped to Liverpool from Madrid in Novy-MacNeal-Nicolle medium and incubated immediately upon arrival. Parasites were cultured as described by Meredith *et al.* (1995) [100].

Preparation of Leishmania antigen as per the DAT

Leishmania antigen was prepared up to the point at which promastigotes were stained [100,171]. Briefly, promastigote culture was harvested and washed in Locke solution containing 0.9% NaCl, 0.25% glucose, 0.04% KCl, 0.02% $CaCl_2$, and 0.02% $NaHCO_3$. Parasites were subsequently incubated in Locke solution with 0.4% trypsin for 45 minutes at 37°C. Parasites were then fixed in Locke solution containing 1% (wt/vol) formaldehyde for 20 hours at 4°C. Parasites were then washed in saline-citrate solution containing 0.9% NaCl and 1% (wt/vol) sodium citrate.

Protein Assay

The Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) as used as per manufacturer's instructions.

Concentration of Leishmania antigen preparation

Leishmania antigen preparation was concentrated by centrifuging parasite preparation through a 3kDa filter at 4,000 RPM for 20 minutes.

FIND mAbs

Thirteen mouse mAbs against the *L. donovani* complex were received from FIND, as described in Table 1.

Table 1. FIND's mAbs against *Leishmania donovani* complex.

mAb number	Ig Subclass	Immunising *Leishmania* Strain	Further information
1	IgG1, Kappa	*L. chagasi.* Strain: MHOM/BR/00/Edmael	ATCC deposit. Promastigotes and amastigotes.
2	IgG1, Kappa	*L. infantum.* Strain: MHOM/ET/67/HV3	
3	IgG1	*L. chagasi.* Strain: MHOM/BR/00/Edmael	
4	IgG1	*L. donovani* promastigotes. Strains: WR352, LV9, WR168c	
5	IgG2b, Kappa	*L. donovani* promastigotes. Strains: WR352, LV9, WR168c	
6	IgG1	*L. infantum.* Strain: MHOM/ET/67/HV3	ECACC deposit. Recognises GP63.
7	IgG1	*L. donovani* promastigotes. Strains: WR352, LV9, WR168c	
8	IgG1	*L. donovani* promastigotes. Strains: WR352, LV9, WR168c	
9	IgG1	*L. donovani* promastigotes. Strains: WR352, LV9, WR168c	
10	IgG1	*L. chagasi.* Strain: MHOM/BR/00/Edmael	
11	IgG1	*L. donovani* promastigotes. Strains: WR352, LV9, WR168c	
12	IgG1	*L. chagasi.* Strain: MHOM/BR/00/Edmael	
13	IgG1	*L. donovani* promastigotes. Strains: WR352, LV9, WR168c	

Ethics statement

Ethical approval for the use of biobank samples was given by the Ethical Review Committee (ERC) of the icddr,b (PR-14093). Written informed consent was given by all adult participants. Informed consent was provided by the participant's parent or guardian for participants under 18 years of age. All participants consented for biobanking anonymized samples for the development and evaluation of diagnostic tests for *Leishmania*. Samples were transferred to LSTM under MTAs [172,173].

Biobanking of samples

Sample collection for the biobank is described in chapter 2 and appendix 1. Briefly, serum, peripheral blood, dry blood spots, and urine samples were collected from September 2016 until March 2018 from people with clinically confirmed VL (n=80) and clinically healthy household and neighbouring contacts of people with VL or PKDL (n=720). All participants resided in endemic districts in Bangladesh. Healthy participants had no symptoms or history of VL and PKDL.

Dot blots

Antigen preparation or sample (5, 10, or 15µl) was applied to Amersham™ Protran™ 0.45µm nitrocellulose blotting membrane (GE Healthcare Life Sciences, Amersham, UK) and allowed to dry for 10 minutes at room temperature. Membranes were blocked for 1 hour at room temperature in blocking buffer made up of 5% skimmed milk PBS-Tween (0.1%). MAbs diluted 1 to 2,000 or 1 to 1,000 in blocking buffer were applied to the membranes and incubated at room temperature for 1 hour. Following addition of mAbs, membranes were washed in PBS-Tween (0.1%), twice for 20 seconds and 3 times for 5 minutes. Secondary antibodies, rat anti-mouse IgG2b conjugated to alkaline phosphatase (Southern Biotech, Birmingham, AL, USA) and rat anti-mouse IgG1 conjugated to alkaline phosphatase (Southern Biotech, USA), or rat anti-mouse kappa light chain conjugated to alkaline phosphatase (Southern Biotech, USA) were diluted in 2.5% skimmed milk PBS-Tween (0.1%) and incubated on the membranes for 1 hour at room temperature. Following addition of secondary antibodies, membranes were washed in PBS-Tween (0.1%) as before. Sigma*FAST*™ BCIP®/NBT alkaline phosphatase substrate (Merck, Kenilworth, NJ, USA) was added to the membranes and allowed to develop for 24 hours or until dots were clearly visible. Membranes were rinsed in distilled water to stop the reaction.

Indirect ELISA

The antigen preparation or sample were diluted in bicarbonate coating buffer. Invitrogen™ Nunc Maxisorp™ flat-bottom 96-well ELISA plates (Thermo Fisher Scientific, USA) were coated with 100µl per well of diluted antigen and incubated overnight at 4°C. Plates were washed three times in PBS-Tween (0.05%), blocked in 5% skimmed milk PBS-Tween (0.1%) for two hours at room temperature, and then washed three times in PBS-Tween (0.05%). MAbs were diluted in 1% skimmed milk PBS-Tween (0.1%) and incubated for two hours at 37°C. Plates were washed four times in PBS-Tween (0.05%), and then incubated with rat anti-mouse kappa conjugated to alkaline phosphatase (Southern Biotech, USA) for two hours at 37°C. Plates were washed four times in PBS-Tween (0.05%), and then incubated with Sigma*FAST*™ p-Nitrophenyl phosphate (Merck, USA) in the dark at room temperature. OD was read within 30 minutes at 405nm.

Western blots

The sample or antigen preparation (15µl) were mixed with 3µl Laemmli SDS sample buffer (6x) (Alfa Aesar, MA, USA), heated at 95°C for ten minutes, and loaded onto a 12% Mini-PROTEAN® TGX™ precast gel (Bio-Rad laboratories, Hercules, CA, USA). This preparation was then fractionated using SDS-PAGE for 40 minutes at 200v and transferred onto a Trans-Blot Turbo Mini 0.2µm nitrocellulose membrane (Bio-Rad laboratories, USA) using the Trans-Blot Turbo system (Bio-Rad laboratories, USA). The membranes were then blocked in 5% skimmed milk PBS-Tween (0.1%) for one hour at room temperature. Blots were rinsed once in PBS-Tween (0.1%). MAbs diluted 1:50 in 1% skimmed milk PBS-Tween (0.1%) were applied to the blots and incubated for one hour at room temperature. Blots were then washed in PBS-Tween (0.1%) twice for 20 seconds followed by three times for five minutes. Rat anti-mouse kappa conjugated to alkaline phosphatase (Southern Biotech, USA) diluted 1:1,000 in 1% skimmed milk PBS-Tween (0.1%) was incubated on the blots for one hour at room temperature and washed as before. Sigma*FAST*™ BCIP®/NBT alkaline phosphatase substrate (Merck, USA) was added overnight. Blots were rinsed in distilled water to stop the reaction and allowed to dry.

Conjugation of mAbs

MAbs were conjugated to a LINKBRIGHT™ using the Conjugated Polymer Nanoparticles (CPN) - Amine IgG Antibody Conjugation Kit (Stream Bio Ltd., Nether Alderley, UK) according to manufacturer's instructions. Briefly, mAbs were purified to remove ammonium ions, primary amines, or sodium azide preservatives from the buffer. For 10µl of each mAb (1mg/ml), 8µl of IgG Antibody Purification Reagent

was added and incubated for five minutes at room temperature. Antibodies were then centrifuged at 13,000xg for five minutes and supernatant was discarded. Antibodies were resuspended in 10µl of Solution R. Ten microlitres of antibody was added 150µl of LINKBRIGHT CPN™-Amine and incubated at room temperature for 30 minutes. Solution SG (6µl) was added and incubated for five minutes at room temperature. A further 6µl of Solution Z (protein stabiliser) was added. CPN™ 610 (Orange) was excited at 480nm and emitted at 610nm.

Sandwich ELISA

MAbs (1mg/ml) (capture and detection) were diluted in PBS at 0.5, 1, 2, and 5µg/ml and 100µl of capture antibody at each concentration was added to an Invitrogen™ Nunc Maxisorp™ flat-bottom 96-well ELISA plate (Thermo Fisher Scientific, USA). Plates were incubated overnight at 4°C, aspirated, and then 200µl of blocking buffer (1% skimmed milk PBS-Tween (0.1%)) was added to each well and incubated for one hour at room temperature. Sera were diluted 1:50 and 1:100 in 1% skimmed milk PBS-Tween (0.1%) and 100µl of diluted sera and 1% skimmed milk PBS-Tween (0.1%) negative control was added to each well and incubated at room temperature for two and a half hours. Plates were washed three times with wash buffer (PBS-Tween (0.1%)). LINKBRIGHT CPN™ conjugated detection antibodies were serially diluted 1:200, 1:1,000, 1:5,000, and 1:25,000 in 1% skimmed milk PBS-Tween (0.1%) and 100µl per well were added to each plate and incubated for one and a half hours at room temperature. Plates were washed three times. CPN™ 610 (Orange) was excited at 480nm and emitted at 610nm.

Kala-azar antigen detection test (DetectoGen Inc., USA)

The kala-azar antigen detection test (DetectoGen Inc., USA) was performed as per manufacturer's instructions. Briefly, a pool of six recombinant *Leishmania* antigens (1µg/ml) provided as a positive control was diluted 1 in 50 in 1% skimmed milk PBS. Plates pre-coated with a pool of capture mAbs were coated in 100µl per well of urine, diluted positive control, and 1% skimmed milk PBS used as a negative control. Plates were covered and incubated overnight at 4°C. Wells were washed six times with PBS-Tween (0.1%). A pool of biotinylated mAbs were provided. Biotinylated mAbs were diluted in 1% skimmed milk-PBS and 100µl per well was applied to the plate. Plates were incubated for one hour at room temperature. Plates were washed as before. Streptavidin-Peroxidase Polymer (catalogue number Sigma S2438, Merck, USA) was diluted in 1% skimmed milk PBS and 100µl per well was applied to the plate. Again, plates were incubated for one hour at room temperature. Wells were washed as before.

Plates were incubated with 100 µl per well ofTMB substrate solution (Merck, USA) in the dark at room temperature for 10-30 minutes to allow a blue colour to develop. The reaction was stopped with the addition of 100µl per well of 0.25M hydrochloric acid (HCl) solution and a yellow colour was observed. The OD of the plates was read within 30 minutes at 450nm.

6.5 Results

Dot blots

All thirteen mAbs were found to not bind two in-house *Leishmania* antigen preparations (prepared from *L. donovani* strains IMRT/KE/62/LRC-L57 and MHOM/IN/80/DD8) when secondary antibodies rat anti-mouse IgG_1 and rat anti-mouse IgG_{2b} were diluted 1:8,000 (as recommended by the manufacturer), and mAbs were diluted 1:5000. Protein was not detected in the in-house DAT antigen. The DAT preparation was concentrated through a filter, and the protein assay repeated, however no protein was detected.

Four urine samples from the FIND biobank belonging to individuals with VL, which were highly positive for urinary antigen by *Leishmania* antigen ELISA (Clin-Tech, UK, formerly Kalon Biological, UK), were pooled for further testing. Signals could be seen for all thirteen anti-*Leishmania* mAbs against this pool (Figure 1). No signal was seen for a negative urine sample from an individual from an endemic region with no symptoms of VL and negative for urinary antigen by *Leishmania* antigen ELISA (Clin-Tech, UK) (Figure 1). The strongest visual signals were seen with mAbs one, five, six, and eight (Figure 1). However, signals were slow to develop, and membranes were left overnight before a signal could be visualised.

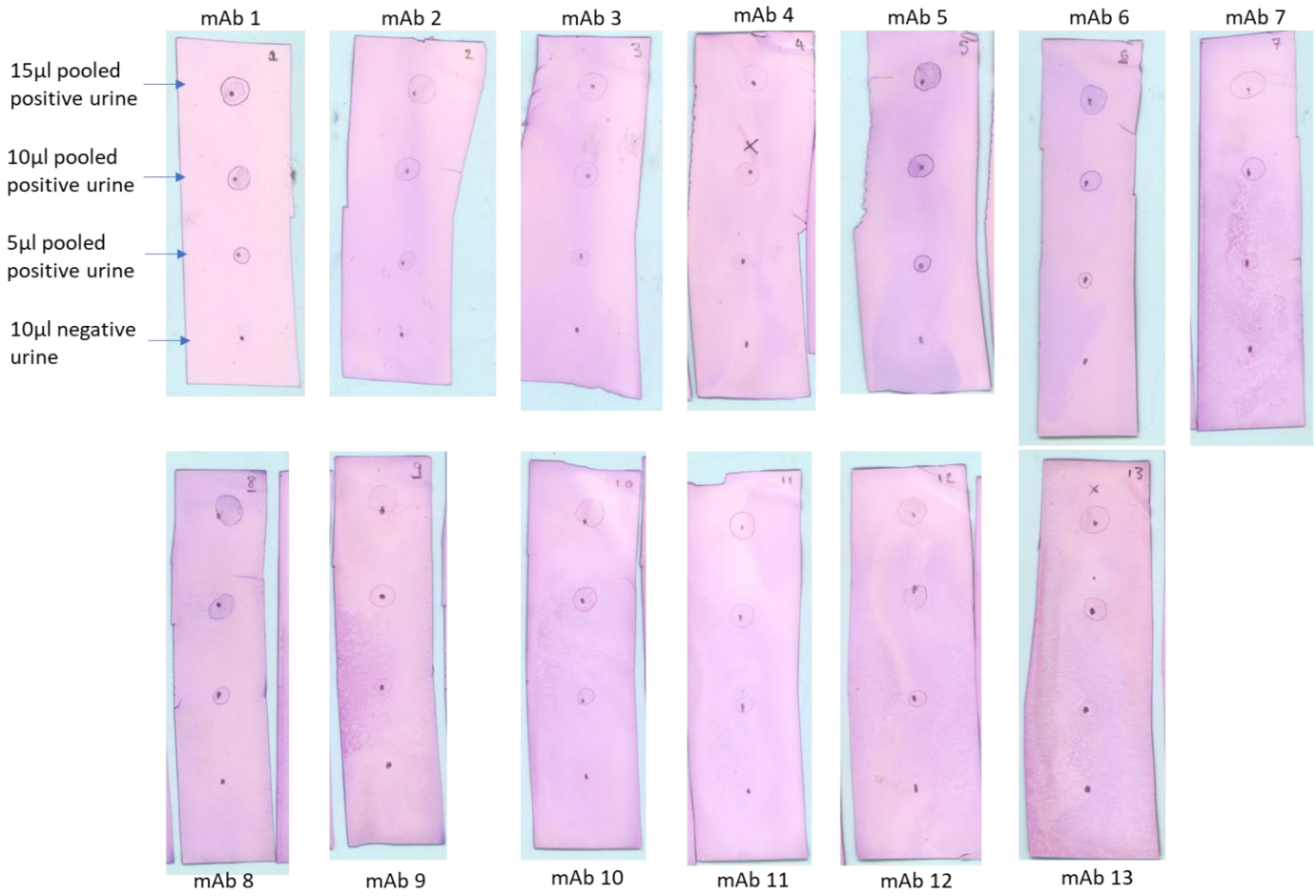
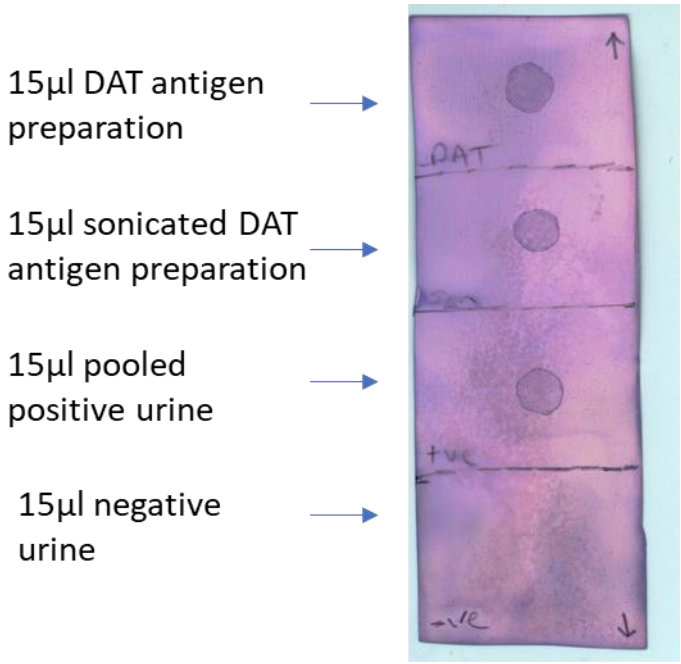


Figure 1. MAbs one to thirteen on dot blots Thirteen mAbs against fifteen, ten, and five microlitres of pooled *Leishmania* antigen positive urine from four individuals with VL, and ten microlitres of *Leishmania* antigen negative urine from an individual with no history of VL, applied to each membrane from top to bottom. MAbs were diluted 1:2000 and secondary antibodies rat anti-mouse IgG_1-alkaline phosphatase and rat anti-mouse IgG_{2b}-alkaline phosphatase were diluted 1:4000. The substrate was left overnight to develop a visible signal.

A commercial preparation of DAT antigen (50,000,000 promastigotes/ml) (AMC Medical Research BV, Amsterdam, Netherlands) was procured, and the dot blot of mAb number eight was repeated to confirm a signal with the change in antigen (Figure 2). Additionally, a new secondary antibody, rat anti-mouse kappa-alkaline phosphatase (Southern Biotech, USA) was tested. Signal could be seen for DAT antigen, sonicated DAT antigen, and pooled positive urine. As before, no signal was seen for negative urine (Figure 2). The signal was slow to develop, and the membrane was left overnight before a signal could be visualised.

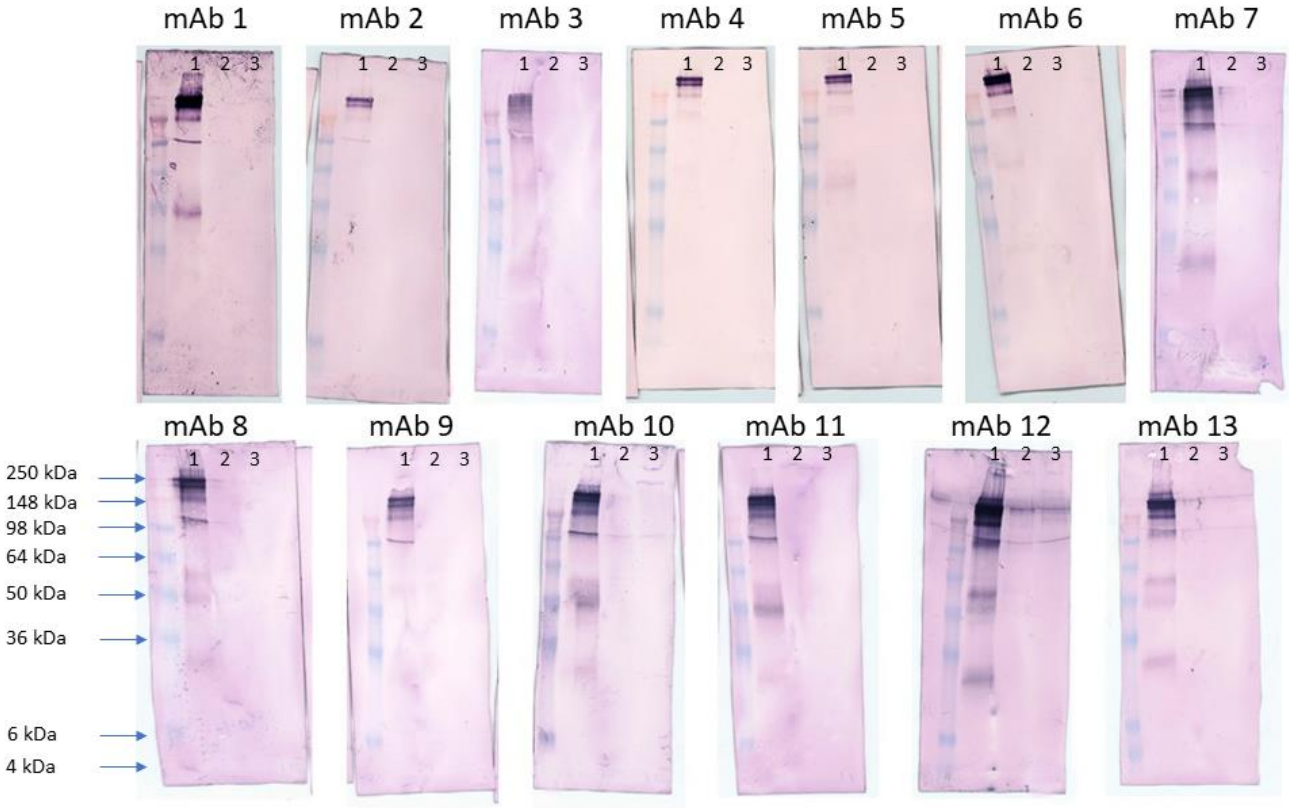


mAb 8

Figure 2. MAb number eight against 15 microlitres of commercial DAT antigen preparation, sonicated commercial DAT antigen preparation, pooled *Leishmania* antigen positive urine from four individuals with VL, and *Leishmania* antigen negative urine from an individual with no history of VL, applied to the membrane from top to bottom. MAbs were diluted 1:1000 and secondary antibody rat anti-mouse kappa light chain-alkaline phosphatase was diluted 1:2000. The substrate was left overnight to develop a visible signal.

Western blot

Proteins were detected in pooled positive urine by all thirteen mAbs by western blot (Figure 3). Antibodies ten and twelve detected proteins in the commercial DAT antigen preparation (Figure 3). Antibodies, ten, seven, and twelve had faint signals against proteins in pooled negative urine (Figure 3). Pooled positive serum at a range of dilutions was trialed as a sample type, with a one in ten dilution of serum giving the cleanest blot but was deemed not necessary to be taken forward with the remaining mAbs (Figure 4).



3741

3742 **Figure 3. MAbs one to thirteen on western blot.** Lane 1: pooled *Leishmania* antigen positive urine from
3743 four individuals with VL; Lane 2: pooled *Leishmania* antigen negative urine from four individuals with no
3744 history of VL; Lane 3: commercial DAT antigen preparation.

3745

3746

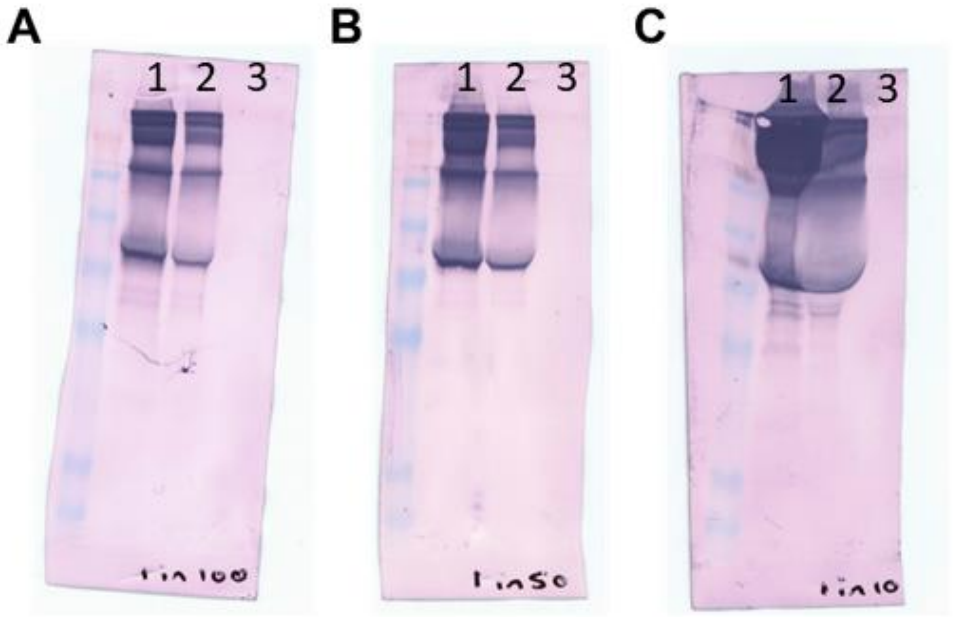


Figure 4. Trial of serum on western blot with mAb number five. Lane 1: pooled sera from four individuals with VL; Lane 2: pooled sera from four individuals with no history of VL; Lane 3: sample buffer. Sera were diluted **(A)** 1 in 100, **(B)** 1 in 50, or **(C)** 1 in 10.

Indirect ELISA

A screening of mAbs using an indirect ELISA method was established to identify optimal concentrations of antigen and antibody dilutions. Initial dilutions of 1:400, 1:800, and 1:1600 produced negative results, so dilutions of 1:20, 1:50, 1:100, and 1:200 were tested overnight at 4°C. A visible colour change was seen only in the 1:50 dilution of mAb eight in wells coated with DAT and pooled positive urine when Sigma*FAST*™ p-Nitrophenyl phosphate was left overnight to develop (data not shown). No colour change was seen in wells containing antigen coating and secondary antibody only (data not shown).

Steps were taken to reduce incubation times and trial serum as an antigen. In addition to the pooled urine, a further four matched serum samples from individuals with VL were used to make a pooled serum from the biobank. Plates were coated overnight with DAT, pooled positive urine, and serum. Two sets of conditions were run in parallel, the first being overnight at 4°C and full day incubations as before, and the second being incubation for two hours at 37°C. MAb eight was diluted 1:50, 1:100, 1:200, 1:400, and the secondary antibody rat anti-mouse kappa light chain-alkaline phosphatase was diluted 1:1000. Sigma*FAST*™ p-Nitrophenyl phosphate solution was added, and plates incubated in the dark. The OD405nm was read at five hours and 18 hours. Signal was seen at 1:50 mAb dilution with the shorter 37°C incubations of primary and secondary antibodies when read at five and 18 hours (Figure 6). A stronger signal was observed after 18 hours. Serum showed the highest signal with longer incubations of primary and secondary antibodies, but a longer incubation also resulted in the highest background, followed by urine, and then DAT. Under all conditions, the signal was slow to develop.

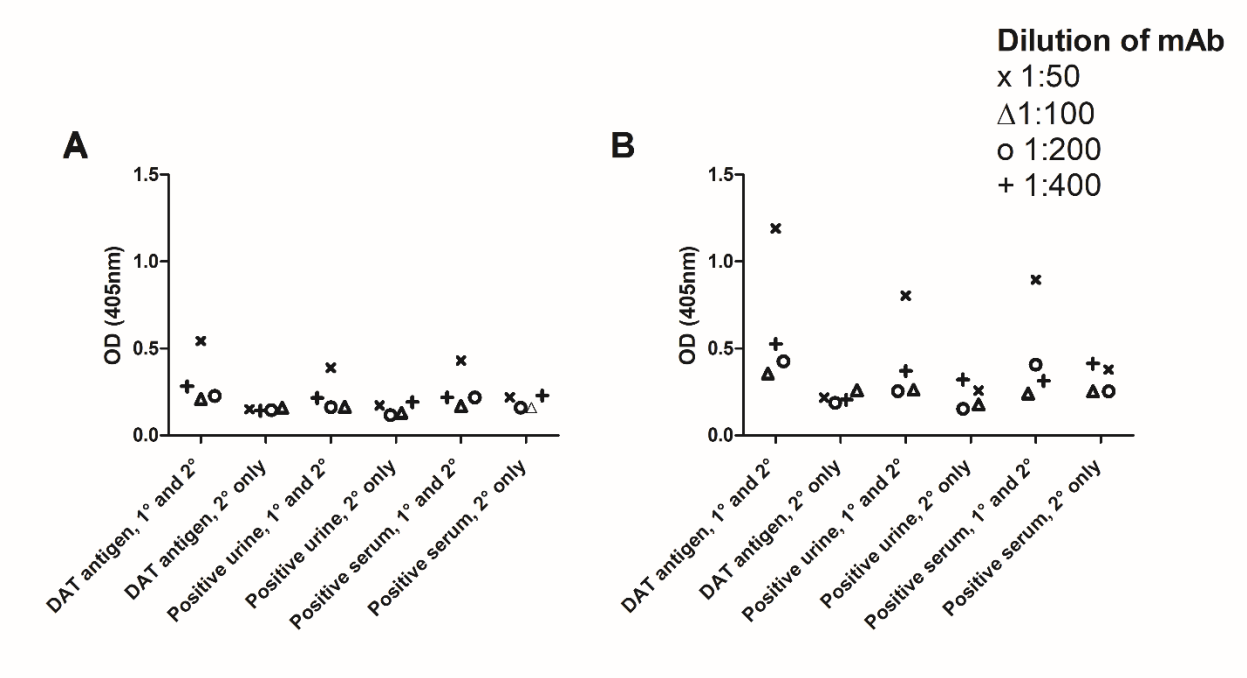


Figure 6. Optimisation of assay to reduce incubation times. Plates were coated with commercial DAT antigen preparation, pooled *Leishmania* antigen positive urine from four individuals with VL, and pooled sera from four individuals with VL. MAb (1º antibody) number eight was diluted 1:50, 1:100, 1:200 and 1:400 and secondary antibody (2º antibody) rat anti-mouse kappa light chain-alkaline phosphatase was diluted 1:1000. Shorter two-hour 37°C antibody incubation steps were trialed. Sigma*FAST*™ p-Nitrophenyl phosphate was left to develop for **(A)** 5 hours or **(B)** 18 hours to develop.

All antibodies were then run under the shorter 37°C incubation conditions of the primary and secondary antibodies with the same antibody dilutions as before. Sigma*FAST*™ p-Nitrophenyl phosphate was allowed to develop for five or 18 hours. The best signal was seen at 1:50 dilutions of mAbs and when the substrate was allowed to develop for 18 hours (Figure 7). Overall, sera showed higher signal than and commercial DAT antigen (Figure 7).

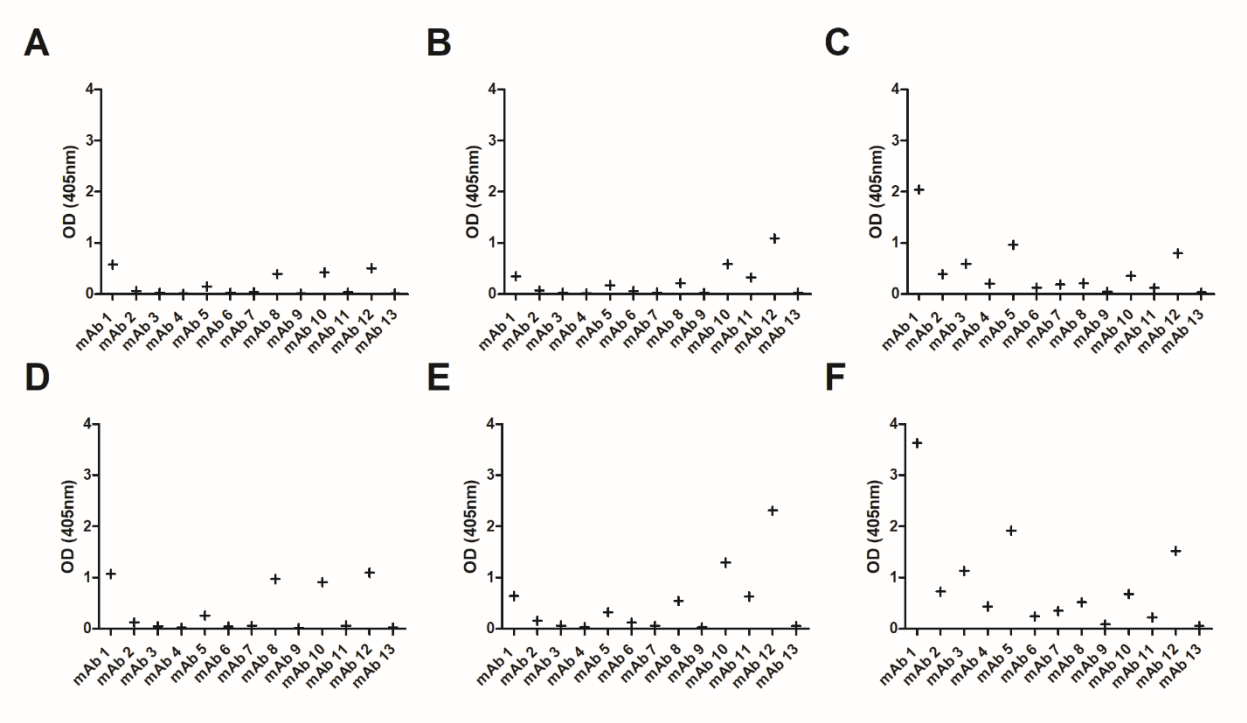


Figure 7. Plates were coated with (A) commercial DAT antigen, (B) pooled *Leishmania* antigen positive urine from four individuals with VL, and (C) pooled sera from four individuals with VL and Sigma*FAST*™ p-Nitrophenyl phosphate was allowed to develop for five hours, or plates were coated with (D) commercial DAT antigen, (E) pooled *Leishmania* antigen positive urine from four individuals with VL, and (F) pooled sera from four individuals with VL and Sigma*FAST*™ p-Nitrophenyl phosphate was allowed to develop for 18 hours. All mAbs were diluted 1:50 and secondary antibody rat anti-mouse kappa light chain-alkaline phosphatase was diluted 1:1000. Data were corrected on the secondary-only reading.

All mAbs were tested with pooled VL negative urine and serum under the same conditions. Non-specific binding was observed with mAbs one, ten, and twelve against urine, and one, ten, and twelve against serum, with higher OD values with the longer incubation times (Figure 8).

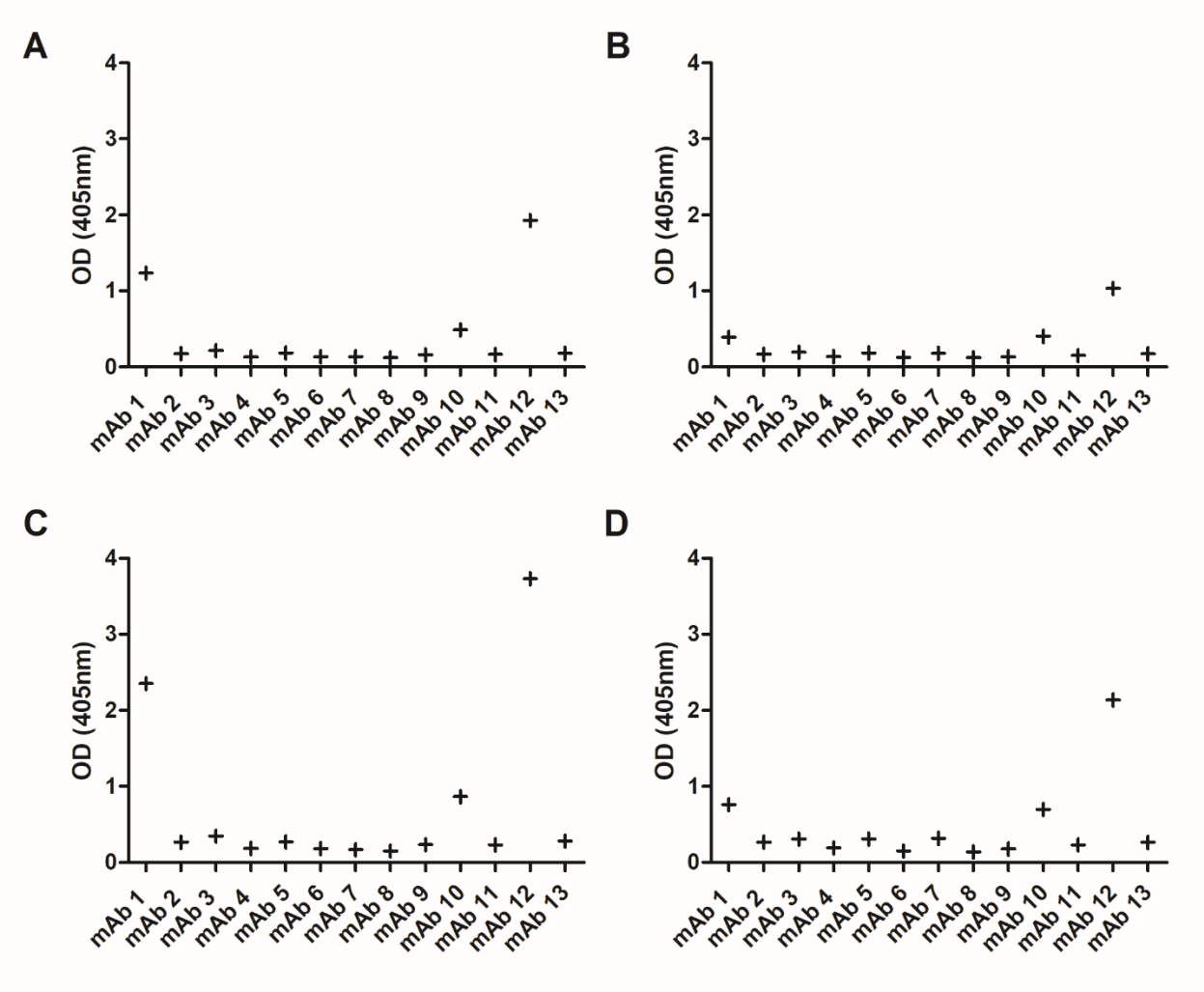
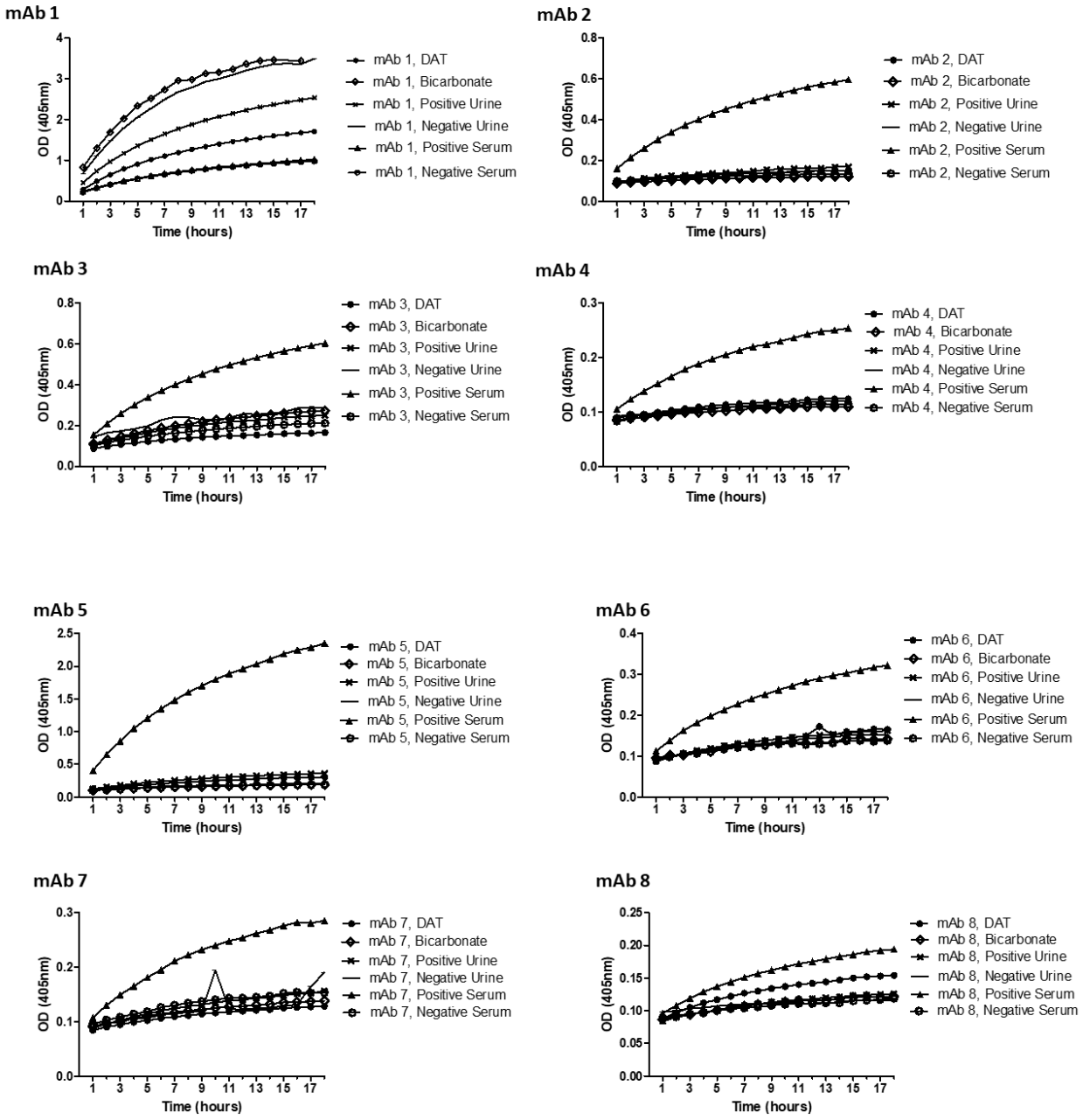


Figure 8. Plates were coated with (A) pooled negative urine and (B) pooled negative sera and Sigma*FAST*™ p-Nitrophenyl phosphate was allowed to develop for five hours, or plates were coated with (C) pooled negative urine and (D) pooled negative sera and Sigma*FAST*™ p-Nitrophenyl phosphate was allowed to develop for 18 hours. All mAbs were diluted 1:50 and secondary antibody rat anti-mouse kappa light chain-alkaline phosphatase was diluted 1:1000.

Next, all mAbs were run on the same plate with DAT, pooled positive urine and serum, bicarbonate and, pooled negative urine and serum. The OD405nm was read every 30 minutes from 1 hour post addition of Sigma*FAST*™ p-Nitrophenyl phosphate for 18 hours. MAbs two to eight and ten to thirteen showed low signal against positive serum, with mAb five showing the strongest signal with little background (Figure 9). MAb twelve was the only antibody to show signal against DAT and positive urine and serum (Figure 9). MAbs one, five, and twelve showed higher signal read outs than other mAbs, however mAb one showed high non-specific binding (Figure 9). The signal was slow to develop.



3842

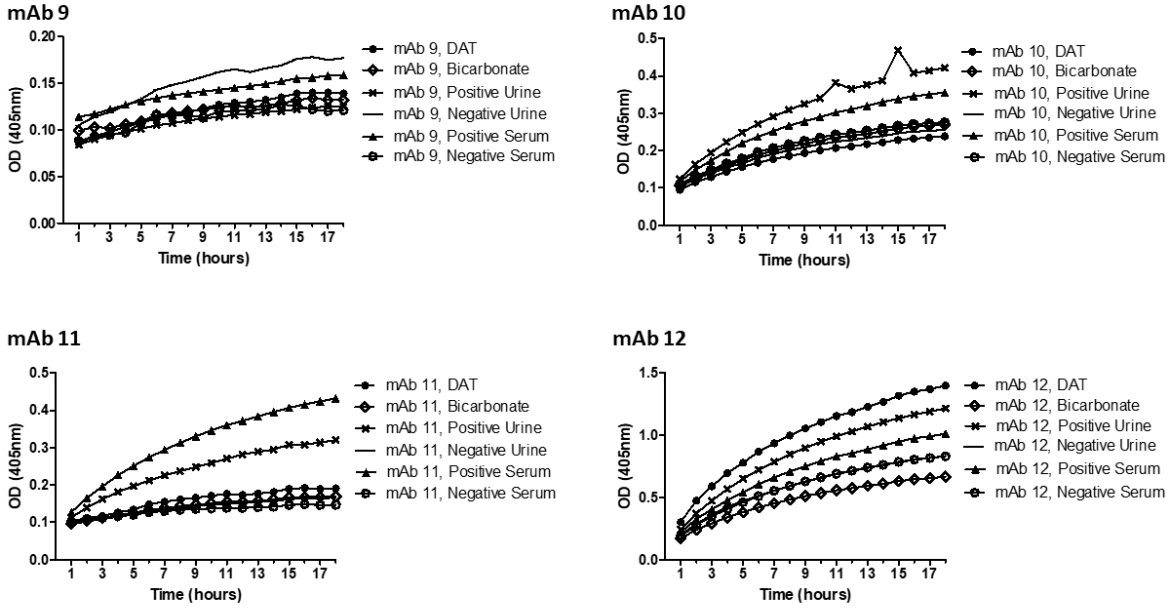
3843

3844 **Figure 9. MAbs one to thirteen diluted 1:50 and secondary antibody diluted 1:1000.** The OD405nm was

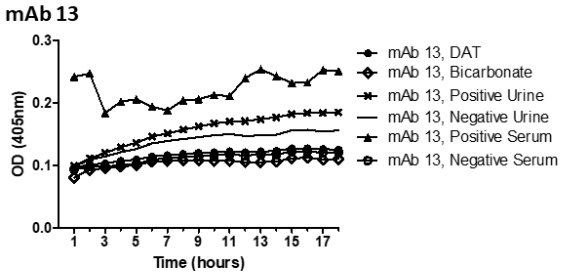
3845 read every 30 minutes from one hour post addition of substrate until 18 hours.

3846

3847



3848



3849

3850 **Figure 9. Continued.**

3851

3852

3853

3854

3855

3856

3857

Sandwich ELISA

A secondary antibody cannot be used in sandwich ELISA format because the mAbs are all the same species, therefore selected antibodies were taken forward for conjugation to fluorescent nanoparticles. Fluorescent nanoparticles were chosen to boost signal given the time taken for signals to develop in previous experiments. MAbs five and twelve were taken forward for conjugation based on the data presented in this report. A standard protocol for the method development for a sandwich ELISA was followed. Both mAbs five and twelve were trialed as the coating and detection antibodies. No signal was detected.

Kala-azar antigen detection test (DetectoGen Inc., USA)

MAb and streptavidin concentrations were optimised for signal to background ratio against serial dilutions of the assay positive control (Figure 10). MAb diluted at 1 to 500 and streptavidin diluted 1 to 4000 gave the highest signal to background ratio, but discrepancies between replicates were seen across the plate particularly at higher antigen concentrations (Figure 10).

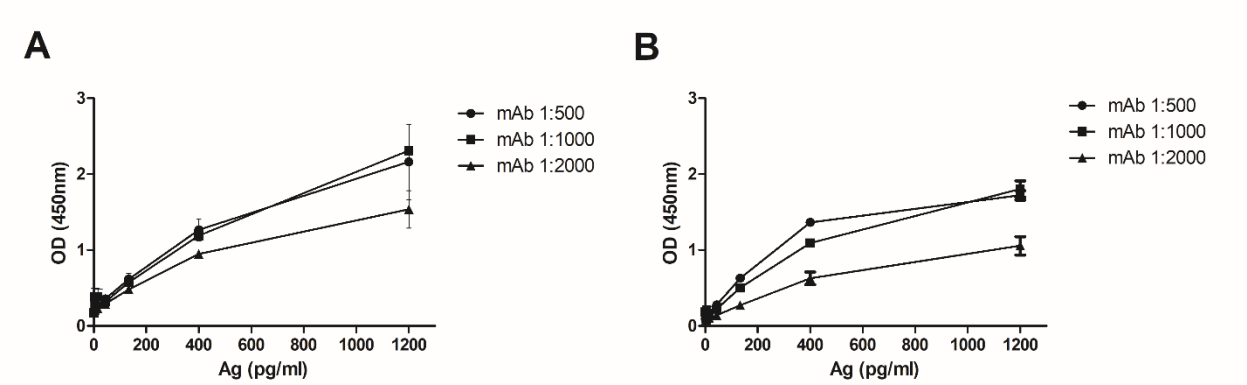


Figure 10. Optimisation of conditions for the kala-azar antigen detection test (DetectoGen Inc., USA). Detectogen Inc. (USA) mAbs were diluted 1:500, 1:1000 and 1:2000, and **(A)** Streptavidin was 1 to 200 or **(B)** Streptavidin 1 to 4000.

The best conditions were then replicated with negative urine and fifteen positive urine samples, both centrifuged and uncentrifuged. Only two of the centrifuged samples and one uncentrifuged sample were positive when the cut-off was calculated as the mean plus three standard deviations. An overall drop in signal was seen and discrepancies between replicates.

Optimisation plates were then re-run. Overall signal was lower and discrepancies between replicates were observed. The highest signal was seen with mAb diluted 1 to 500 and streptavidin diluted 1 to 200, albeit not with the highest signal to background ratio (Figure 11).

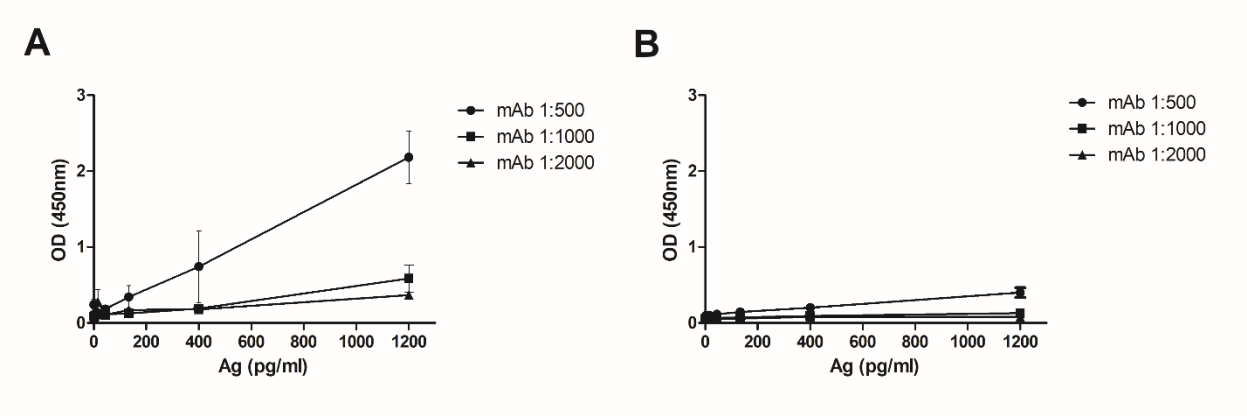


Figure 11. Optimisation of conditions for the DetectoGen ELISA. (A) Streptavidin 1 to 200. (B) Streptavidin 1 to 4000.

These conditions were then replicated with negative urine samples and eleven positive urine samples both diluted one in two and neat. No samples were positive when the cut-off was calculated as the mean plus three standard deviations and an overall drop in signal from the optimisation run was seen.

The optimisation plate was then repeated with new streptavidin and TMB substrate. The signal improved but the signal to background ratio was poor (Figure 12).

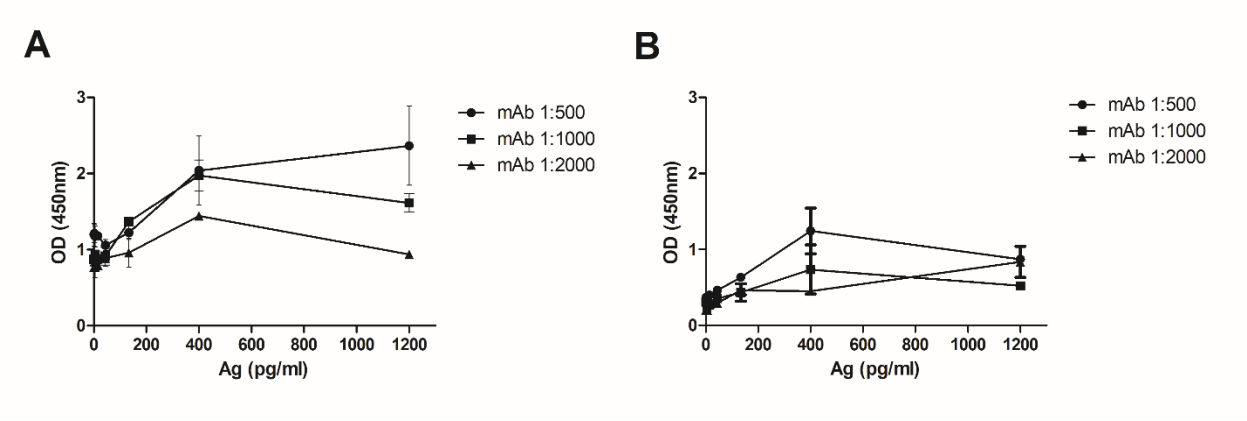


Figure 12. Optimisation of conditions for the DetectoGen ELISA. (A) Streptavidin 1 to 200. (B) Streptavidin 1 to 4000.

Plates were then run as before with negative urine and eleven positive urine samples both diluted one in two and neat, again with mAb diluted 1 to 500 and streptavidin diluted 1 to 200, but with the new streptavidin and TMB substrate. Three of eleven undiluted samples were positive with a cut-off calculated as the mean plus three standard deviations. No diluted samples were above the cut-off.

In the final run 30 positive urine samples were run undiluted, with mAb diluted 1 to 500 and streptavidin diluted 1 to 200. Overall signal was higher, but only two samples were above the cut-off when the cut-off was calculated as the mean plus three standard deviations.

6.6 Discussion

Antigen tests have been shown to be of benefit in detecting acute *Leishmania* infection for the detection of *Leishmania* infection in PLHIV, monitoring response to treatment, and detection of ALI [112,113,172]. There are several antigen tests in ELISA format [112,117], however, there are no assays with a LFA format. Development and evaluation of assays or candidate mAbs able to detect *Leishmania* antigen, either protein or carbohydrate, in a sample such as urine will aid the development of an LFA and enable the management and follow-up of patients in primary healthcare settings and the community.

The screening of a panel of thirteen mAbs against the *L. donovani* complex using a combination of dot blot, western blot, and ELISAs indicated that none of the mAbs were suitable. Overall, mAbs had weak bindings with signal slow to develop, and non-specific binding in some instances, with sera showing particularly high non-specific binding. The techniques employed are commonly used to screen

antibodies and have been used in the development of RDTs, such as the use of ELISA in the screening of mAbs for the development of an RDT for Ebola virus infections [174].

Proteins of approximately 30 to 250 kDa were identified in pooled *Leishmania* antigen positive urine by all thirteen mAbs compared to negative urine and DAT. Renal injury including glomerulonephritis and amyloidosis was seen in hamster models of *L. donovani* infection, with IgG1, IgG2, IgA, and IgM detected in the urine from 21 days post infection [169,170]. Proteins of 134, 82, 52, 31, and 26 kDa were detected by western blot in the urine of hamster models of *L. donovani* infection [170]. The 134 kDa protein was thought to be *L. donovani* acid phosphatase [170], suggesting relatively large molecular weight antigens are able to pass through the nephrons and into urine. *L. infantum* antigens in urine of VL patients were demonstrated by mass spectrometry to be iron superoxide dismutase (21.5 kDa), tryparedoxin (16.7 kDa), and nuclear transport factor 2 (13.9 kDa) [118].

L. donovani infected rats have circulating immune complexes in sera, and proteins of 45, 47, and 58 kDa can be detected in urine bound by anti-amastigote Ig [175]. An antigen of low molecular weight (5-20 kDa), the antigen used in the KAtex, was found in the urine of VL patients with *Leishmania* in Nepal, Brazil, Sudan, and Spain, but not in patients diagnosed with other parasitic disease such as malaria and schistosomiasis or non-parasitic diseases, giving it high specificity [176]. Rats infected with *L. donovani* were positive by KAtex one week following infection with rapid decline post-treatment [177]. MAbs were screened with DAT antigen preparation, which uses *L. donovani* promastigotes [100,171], and patients' samples from the ISC, where *L.* donovani is the aetiological agent. MAbs were raised against members of the *L. donovani* complex and did not show specificity for the immunising strain, with *Leishmania* spp. immunogens showing better binding than some *L. donovani* immunogens against *L. donovani* samples, highlighting the potential pan-species capabilities of these antibodies.

Currently, there are no antigen tests recommended for specific geographic regions. However, variability in diagnostic accuracy is well documented across endemic regions for the rK39 RDTs [129]. The performance of antibodies in ELISA formats do not necessarily correlate with their performance in lateral flow formats. There are however other methods to screen antibodies, but these methods require specialist equipment such as the Instrument for Biomolecular Interaction Sensing MultipleX 96 (IBIS-MX96) Surface Plasmon Resonance (SPR) Imager, which were not available to us.

The kala-azar antigen detection test (DetectoGen Inc., USA) remains in development stages and is yet to be provided in a commercial kit. Discrepancies were seen between data collected in laboratories at

DetectoGen Inc. and replicates were highly variable. The assay was sent by cold chain however, there may have been a loss in sensitivity during transport. There were no other antigen tests available for evaluation. I was unable to procure the *Leishmania* antigen detect™ (InBios International Inc., USA), as the test is not currently being manufactured. Similarly, I was unable to procure the Kala-azar antigen immunochromatographic test (ICT) (Xinjier Biotechnology Co., Ltd, China) as the company was not available for contact.

6.7 Conclusion

With no suitable mAb candidates identified and no ELISA yet to be developed into LFA format, further epitope discovery and screening of candidate antibodies is needed. A major challenge to this evaluation was the identification of a mAb of high enough sensitivity.

Chapter 7. General discussion

7.1 Introduction

People with ALI and PLHIV are considered key populations to the VL elimination campaign on the ISC. Diagnostics that are suitable for the detection of ALI and acute VL, to monitor treatment responses and relapse, and surveillance are needed to support the efforts of the elimination campaign. Widely used diagnostic tests, such as rK39 serology and the DAT, do not distinguish between current and previous infection, presenting challenges in populations without symptoms or where relapse is frequent. There are several assays that detect current infection and may complement serology, however, data on performance and utility in these key populations are lacking.

This thesis contributes data on the performance and utility of diagnostic assays in asymptomatic populations on the ISC and further explores their use in PLHIV. The main objectives were:

- To determine the prevalence and determinants of ALI in an elimination setting and to determine the clinical utility and diagnostic accuracy of antigen, molecular, and serological tests for ALI in an elimination setting.
- To determine the prevalence and determinants of ALI in PLHIV in an elimination setting and to determine the clinical utility of the antigen, molecular, and serological tests in this population.
- To determine the rate and risk factors for progression to VL and the utility of the antigen, molecular, and serological tests as markers for progression to VL in a cohort of PLHIV with ALI in an elimination setting.
- To establish a protocol to determine the clinical utility of the *Leishmania* antigen ELISA, blood smear microscopy and qPCR in blood and skin microbiopsies for diagnosis and test of cure for VL in PLHIV.
- To evaluate a panel of thirteen monoclonal antibodies for use in an alternative antigen detection test for VL and to evaluate other currently available antigen tests for VL.

7.2 Discussions of key findings

7.2.1 Prevalence of ALI in an elimination setting including PLHIV

ALI was detected by a combination of antigen, molecular, and serological methods in both an immunocompromised and immunocompetent population in India and Bangladesh, respectively. The data suggests there were a small proportion of individuals, residing in VL endemic areas, presenting asymptomatically with markers of an effective immune response to *Leishmania* infection and/or markers of an active infection. This could indicate recent *Leishmania* transmission at the time of the studies resulting in asymptomatic infections, although it is not possible to ascertain the exact time of infection in these individuals.

At a population level, individuals with ALI may represent a source of *Leishmania* transmission, although this was found not to be the case in a recent xenodiagnosis study in which asymptomatic individuals were not infectious to sandflies [23]. Given the poor outcomes associated with VL-HIV coinfection, the presence of ALI in PLHIV in India is of concern. At an individual level, detection of ALI in PLHIV could be used to improve clinical management should these individuals develop VL at a higher rate or have a poorer prognosis than PLHIV with no detectable ALI. It remains to be determined whether PLHIV with ALI are infectious to sandflies and hence their importance in an epidemiological context is still to be elucidated.

There remains a lack of consensus on the definition of ALI, making study to study comparisons of prevalence difficult. Generally, serology, PCR, and/or the LST are used to establish the prevalence of ALI, however the assays used, and the exact combination vary by study [161,178]. The assays used to detect ALI are often primarily used in the diagnosis of VL and hence are not developed for use in asymptomatic individuals. A guideline for defining and detecting ALI would enable researchers to make better comparisons of ALI between populations and timepoints and enable the evaluation of assays not previously evaluated in an asymptomatic population.

7.2.2 Determinants of ALI in an elimination setting

In Bangladesh, we found a moderately strong association between having a household or neighbouring contact with PKDL and being positive by at least one of the four tests used to determine ALI. These data may suggest that individuals in proximity to an index VL or PKDL case are also exposed to similar factors that put the individuals with clinical disease at risk for *Leishmania* infection, however, clinical disease did not develop in these individuals for reasons not explored in this study. These data could also suggest

that contacts of individuals with PKDL and VL are at risk of indirect transmission from a household or neighbouring index case. In an immunocompromised population, low CD4 counts, and a larger household size were found to be risk factors for ALI. The results might suggest that a lower CD4 count puts an individual at risk of ALI or conversely having ALI could lower the CD4 count. There could be several factors linked to household size. These could include an increased likelihood of an individual with *Leishmania* infection residing within the same house, which is in keeping with the clustering nature of *Leishmania* infections [34]. Alternatively, the number of people per room or household size could be an indicator of socioeconomic status which has previously been linked to an increased risk of transmission [34,35].

Studies to measure determinants of ALI give a better understanding of those individuals most at risk of infection and can inform public health intervention strategies. Furthermore, these data can be used to update guidelines for the management of PLHIV in VL endemic areas, should ALI be associated with poor prognosis in PLHIV. The data presented in chapter 2 are in keeping with previous studies which found that close proximity to a person with VL increases the risk of *Leishmania* infection, including asymptomatic infection [34]. Modelling has shown that people with PKDL contribute to transmission and xenodiagnoses studies have shown that individuals with PKDL are infectious to sand flies [153,179]. Together, these data highlight the need for rapid identification and treatment of individuals with VL and PKDL to prevent onward transmission. Further, the follow-up of contacts of individuals with PKDL and VL may help with the early detection of VL cases in India, where time to diagnosis can be delayed by between 35 and 59 days [180].

Study to study comparisons of risk factors for *Leishmania* infection can be challenging as risk factors are not standardised, with the same risk factor described in different ways i.e., household size or people per room, or stratified differently between studies. The data from our study in PLHIV in which CD4 counts were a risk for ALI are contradictory to those seen in other studies of PLHIV. A study of PLHIV with *L. infantum* infections in Brazil found that the prevalence of asymptomatic infection was distributed similarly across CD4 count groups [160]. Similarly, a longitudinal study of PLHIV in Ethiopia did not find CD4 count to be a risk factor for baseline ALI [161]. Both CD4 count and household size could be confounded by other factors, but both may serve as easy to measure markers for identifying those at risk of ALI in PLHIV in India.

7.2.3 Clinical utility and diagnostic accuracy of tests for ALI

The studies presented in chapters 2 and 3 show that two tests in combination, the *Leishmania* antigen ELISA and DAT, and the *Leishmania* antigen ELISA and rK39 ELISA, respectively capture all participants positive by more than one test where four tests were used. As mentioned previously, the optimal diagnostic algorithm for detecting ALI in immunocompetent and immunocompromised populations has not yet been established. The majority of studies investigating the prevalence of ALI in a population use approximately four tests [160,161], but this is time consuming and expensive in a real-world application. A two-test approach such as a combination of serology with an antigen test e.g., the *Leishmania* antigen ELISA could maximise the number of individuals with ALI detected, and reduce costs associated with running several assays. The *Leishmania* antigen ELISA and serological assays such as the rK39 ELISA, rK39 RDT, and DAT require minimal laboratory equipment and/or expertise compared to PCR. A further advantage of the *Leishmania* antigen ELISA is the use of a non-invasive sample type, making sample collection straightforward in comparison to invasive techniques.

Studies of asymptomatically infected individuals, including the studies presented in chapters 2 and 3, have shown moderate to poor agreement between tests [55,123,160,172]. Given that the tests used have imperfect specificity, it would concur that there are several false positives within the dataset which could in part account for the poor agreement. The *Leishmania* antigen ELISA and serology along with a clinical history may be useful for the follow up of contacts of individuals with VL and PKDL, who are known to be at an increased risk of *Leishmania* infection [153,179]. However, there is yet to be a treatment for *Leishmania* infection that would be suitable for use in an otherwise healthy individual, particularly given the lack of concordance between tests. As the prevalence of *Leishmania* infection declines in an elimination setting, the positive predictive value of a given assay declines, and as such the diagnosis of ALI at an individual level and any further intervention would be challenging.

Furthermore, multiple tests are likely needed to capture individuals in different biological stages of infection [160], and hence antigen or molecular tests may complement serology in detecting current infection. Repeated surveys of individuals to detect indicators of *Leishmania* infection can be used in monitoring and surveillance to give a picture of changes in transmission and inform intervention strategies. However, there were no repeat surveys in the studies presented in chapters 2 and 3 and hence the utility of these tests for monitoring and surveillance cannot be fully elucidated. Finally, the rK39 RDT remains the only field compatible test for *Leishmania* infection. For all other assays, samples

need to be transported to the laboratory for testing and hence the development of an antigen test in RDT format would be of benefit to the elimination campaign.

7.2.4 Progression of ALI to VL in PLHIV

In chapter 4, four (3.7%) of 108 PLHIV progressed from ALI to VL within 18-months of follow-up, two of whom were positive by three of four tests, and the remaining two of whom were positive by all four tests. In all four individuals who developed VL, anti-*Leishmania* antibody titers and *Leishmania* antigenuria were higher than in asymptomatic non-progressors at baseline. These data suggest serology and antigenuria to be quantitative markers of progression to VL. This is in keeping with a study in India and Nepal which found individuals with high titer DAT and/or rK39 ELISA results were at an increased risk of progression to VL [61]. In the 94 participants presented in chapter 4 with markers of ALI at enrolment, *Leishmania* markers persisted in 32 (34.0%) participants after 18 months. These data suggest a persistence of *Leishmania* markers in a substantial proportion of individuals and help us to understand the dynamics of *Leishmania* infection over time. Similarly, in Ethiopia,16 (34.8%) of 46 PLHIV with asymptomatic infections at baseline reverted to negative over the course of follow-up [161].

Furthermore, we saw a three of four (75.0%) of participants remain positive for the *Leishmania* antigen ELISA at 18-months where matched samples were available, albeit in a small sample size. Antigenuria remained high at 18-months follow-up in these individuals with little change from baseline. Two of three (66.6%) asymptomatic participants remained positive by qPCR at 18-months follow-up. These data suggest a persistent *Leishmania* infection. It may be that in an immunocompromised population, without treatment for *Leishmania*, infections are not cleared by the immune system and antigenuria and parasitaemia remain detectable for long periods. Univariate analysis found that all-cause mortality was higher in PLHIV with ALI among other variables. On further multivariate analysis, ALI was no longer associated with mortality, and low CD4 counts, having a WHO clinical stage three HIV infection, and not being on ART at baseline were significantly associated with mortality, suggesting confounding factors for the severity of HIV infection.

This study represents the first to monitor for progression of ALI to VL in PLHIV on the ISC and could be used to inform clinical management in a population at risk of poor outcomes associated with VL. As mentioned previously, xenodiagnoses studies of the infectiousness of asymptomatic individuals to sand flies do not corroborate modelling studies, which suggest asymptomatic individuals play a role in transmission [36,154,181]. However, this is yet to be determined in PLHIV. Regardless of whether

asymptomatic individuals are infectious to sand flies, given the anthroponotic nature of disease reservoirs on the ISC and that a number of individuals progress to clinical disease, albeit in low numbers [124,161], they may represent an important population for disease elimination. A study of PLHIV in Italy found HAART reduced the incidence of VL, associated with a restoration of immune function [182]. The adherence to HAART was high in the cohort of PLHIV and could account for the low number of individuals who developed VL in chapter 4.

A positive *Leishmania* antigen ELISA result at baseline was not included in the criteria for in person follow-up in the study presented in chapter 4 owing to the lack of data on the assay at the time of the study design. All asymptomatic participants followed up in person over 18-months had anti-*Leishmania* antibodies detected at baseline by rK39 ELISA. As such, we are unable to calculate time to seroconversion following detection of antigenuria by *Leishmania* antigen ELISA or parasitaemia by qPCR. All asymptomatic progressors in chapter 4 were positive by at least three of the four tests used. However, given the low number of progressors the study lacks the statistical power. The incidence of ALI could not be calculated as the non-asymptomatic cohort was not followed up for further samples over the 18-month follow-up. Given the resources required for in person follow-up, only individuals testing positive for ALI at baseline were followed up in person, and as such may represent a bias in the data. Measurement of anti-*Leishmania* antibody titers, *Leishmania* antigenuria, and *Leishmania* kDNA could be a useful way of determining those individuals most at risk of developing VL. However, the limited number of individuals progressing to VL in this study restricted the analysis of cut-offs for quantitative markers of infection.

7.2.5 A protocol to determine the clinical utility of the Leishmania antigen ELISA, blood smear microscopy, and qPCR in blood and skin microbiopsies for diagnosis and test of cure for VL in PLHIV

The study presented in chapter 5 was unable to go ahead and as such the protocol has been published to allow other groups to conduct the study should they have access to the right patient population and resources. Here we aimed to establish the clinical utility of the *Leishmania* antigen ELISA, blood smear microscopy, and qPCR in blood and skin microbiopsies for diagnosis and test of cure for VL in PLHIV in India. Given the effectiveness of antigen tests for monitoring response to treatment in an immunocompetent population [112,173], it would concur that the *Leishmania* antigen ELISA would perform well in an immunocompromised population. Furthermore, the KAtex was found to predict treatment failure and relapse in HIV patients in Ethiopia [113,162]. To the best of our knowledge this study would be the first to evaluate the *Leishmania* antigen ELISA in PLHIV. This protocol is limited by

the lack of follow-up visits to monitor for relapse of VL, and this should be considered should this protocol be taken up.

7.2.6 Potential of mAbs as an alternative antigen detection test for VL and other antigen tests for VL

Further development of *Leishmania* antigen tests is required, particularly a field compatible RDT. In the final chapter, we were unable to identify a suitable monoclonal antibody candidate in a panel belonging to FIND. However, we were limited to assays such as ELISA, western blot, and dot blot in the screening process. Further antigen discovery studies are needed to identify suitable antigen candidates for generation of monoclonal antibodies, with strong industry links for the production and screening of monoclonal antibodies. The kala-azar antigen detection test (DetectoGen Inc., USA) did not perform well under evaluation and requires further optimisation before validation by external groups. There were no other antigen tests available at the time of evaluation.

The introduction of the In Vitro Diagnostic Regulation (IVDR) creates a challenge for manufacturers of diagnostics for VL to viably place or keep tests on the market [183]. The manufacturers of the only effective rK39 RDT for the diagnosis of VL in East Africa are to discontinue production [184]. Given the rK39 RDT is a widely established test with a large evidence base, the challenges to keep or place a *Leishmania* antigen test on the market are potentially even greater. Viable alternative paths to market need to be identified for existing and novel VL diagnostics.

7.3 Future work

There are two biobanks available which could support future diagnostic development and evaluation efforts, including, antigen discovery, and test validation. Further studies are needed to evaluate prognostic markers for development of VL in PLHIV, including those with asymptomatic *Leishmania* infection and those at risk of relapse, such as work to evaluate CD40 ligand and neopterin which have shown promise [185]. Lastly, we invite teams capable of conducting the protocol presented in chapter 5 to take on the study and provide much needed data on diagnostics and test of cure for VL in PLHIV in India.

7.4 Final words

Here, we presented several studies on the development and evaluation of diagnostics for *Leishmania* infection with a primary focus on asymptomatic populations and PLHIV in an elimination setting. Antigen tests may still provide a useful tool for detection of *Leishmania* infection in these populations. We presented evidence for the follow-up of contacts of individuals with VL and PKDL using a combination of

serology and antigen testing to monitor for ALI and highlighted the importance of rapid VL and PKDL case detection (chapter 2). We then show a combination of serology, antigen, and molecular testing to be useful for detection of ALI and stratification of risk for progression to VL in PLHIV (chapters 3 and 4). We then presented future work on the diagnosis and test of cure for VL in PLHIV in India (chapter 5). Lastly, we presented the challenges associated with development and evaluation of novel tests for VL (chapter 6).

References

1. World Health Organization. WHO Technical Report Series CONTROL OF THE LEISHMANIASES. 2010;978: 22–26. Available: http://apps.who.int/iris/bitstream/handle/10665/44412/WHO_TRS_949_eng.pdf?sequence=1&isAllowed=y

2. Cameron MM, Acosta-Serrano A, Bern C, Boelaert M, den Boer M, Burza S, 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

3. Burza S, Croft SL, Boelaert M. Leishmaniasis. 2018 [cited 27 Nov 2018]. doi:10.1016/S0140-6736(18)31204-2

4. Ready PD. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 2014;6: 147–54. doi:10.2147/CLEP.S44267

5. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. Lancet. 2005;366: 1561–1577. doi:10.1016/S0140-6736(05)67629-5

6. Reithinger R, Dujardin J-C, Louzir H, Pirmez C, Alexander B, Brooker S. Review Cutaneous leishmaniasis. 2007. doi:10.1016/S1473-3099(07)70209-8

7. CDC - Centers for Disease Control and. CDC - Leishmaniasis - Biology. 2019 [cited 21 Feb 2020]. Available: https://www.cdc.gov/parasites/leishmaniasis/biology.html

8. World Health Organization. Status of endemicity of visceral leishmaniasis: 2020. 2020 [cited 1

Sep 2021]. Available:
https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html

9. World Health Organization. Leishmaniasis. 2020 [cited 1 Sep 2021]. Available:
https://www.who.int/news-room/fact-sheets/detail/leishmaniasis

10. DNDi. TOWARDS A NEW GENERATION OF TREATMENTS FOR LEISHMANIASIS. Available:
https://unitingtocombatntds.org/wp-content/uploads/2017/11/london_

11. Diro E, Lynen L, Ritmeijer K, Boelaert M, Hailu A, Van Griensven J. Visceral Leishmaniasis and HIV Coinfection in East Africa. PLoS Negl Trop Dis. 2014;8. doi:10.1371/journal.pntd.0002869

12. Al-Salem W, Herricks JR, Hotez PJ. A review of visceral leishmaniasis during the conflict in South Sudan and the consequences for East African countries. Parasit Vectors. 2016;9: 460. doi:10.1186/s13071-016-1743-7

13. Muniaraj M. The lost hope of elimination of Kala-azar (visceral leishmaniasis) by 2010 and cyclic occurrence of its outbreak in India, blame falls on vector control practices or co-infection with human immunodeficiency virus or therapeutic modalities? Trop Parasitol. 2014;4: 10–9. doi:10.4103/2229-5070.129143

14. Alvar J, Cañavate C, Gutiérrez-Solar B, Jiménez M, Laguna F, López-Vélez R, et al. Leishmania and human immunodeficiency virus coinfection: the first 10 years. Clin Microbiol Rev. 1997;10: 298–319. doi:10.1128/CMR.10.2.298

15. Alvar J, Aparicio P, Aseffa A, Boer M Den, Cañavate C, Dedet J-P, et al. The Relationship between Leishmaniasis and AIDS: the Second 10 Years. Clin Microbiol Rev. 2008;21: 334–359. doi:10.1128/CMR.00061-07

16. WHO. Guidelines for diagnosis, treatment and prevention of visceral leishmaniasis in South Sudan. 2012. Available:
https://www.who.int/leishmaniasis/burden/Guidelines_for_diagnosis_treatment_and_preventio
n_of_VL_in_South_Sudan.pdf

17. van Griensven J, Carrillo E, López-Vélez R, Lynen L, Moreno J. Leishmaniasis in immunosuppressed individuals. Clin Microbiol Infect. 2014;20: 286–299. doi:10.1111/1469-0691.12556

18. Lins Guedes D, Medeiros Z, Dionísio da Silva E, Violeta Martins de Vasconcelos A, Santana da Silva M, Almerice Lopes da Silva M, et al. Visceral Leishmaniasis in Hospitalized HIV-Infected Patients in Pernambuco, Brazil. Am J Trop Med Hyg. 2018; 1–6. doi:10.4269/ajtmh.17-0787

19. 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

20. Ceccarelli M, Rullo EV, Condorelli F, Vitale F, Marco V Di, Nunnari G, et al. Unusual Signs and Symptoms in HIV-Positive Patients Coinfected with Leishmania spp: The Importance of Neglected Tropical Disease in Differential Diagnosis. Open access Maced J Med Sci. 2018;6: 843–847. doi:10.3889/oamjms.2018.186

21. de Araújo VEM, Morais MHF, Reis IA, Rabello A, Carneiro M. Early Clinical Manifestations Associated with Death from Visceral Leishmaniasis. Reithinger R, editor. PLoS Negl Trop Dis. 2012;6: e1511. doi:10.1371/journal.pntd.0001511

22. Abongomera C, Ritmeijer K, Vogt F, Buyze J, Mekonnen Z, Admassu H, et al. Development and external validation of a clinical prognostic score for death in visceral leishmaniasis patients in a high HIV co-infection burden area in Ethiopia. Satoskar AR, editor. PLoS One. 2017;12: e0178996. doi:10.1371/journal.pone.0178996

23. Petersen CA, Heather M, Greenlee W. Neurologic Manifestations of Leishmania spp. Infection. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3110707/pdf/nihms298622.pdf

24. Diro E, van Griensven J, Mohammed R, Colebunders R, Asefa M, Hailu A, et al. Atypical manifestations of visceral leishmaniasis in patients with HIV in north Ethiopia: a gap in guidelines for the management of opportunistic infections in resource poor settings. Lancet Infect Dis. 2015;15: 122–129. doi:10.1016/S1473-3099(14)70833-3

25. Célia MF, Gontijo / +, Pacheco RS, Oréfice F, Lasmar E, Silva ES, et al. Concurrent Cutaneous, Visceral and Ocular Leishmaniasis Caused by Leishmania (Viannia) braziliensis in a Kidney Transplant Patient. Mem Inst Oswaldo Cruz, Rio Janeiro. 2002. Available: http://www.scielo.br/pdf/mioc/v97n5/4474.pdf

26. Reiter-Owona I, Rehkaemper-Schaefer C, Arriens S, Rosenstock P, Pfarr K, Hoerauf A. Specific K39 antibody response and its persistence after treatment in patients with imported leishmaniasis. Parasitol Res. 2016;115: 761–9. doi:10.1007/s00436-015-4801-8

27. Frequently Asked Questions on Visceral Leishmaniasis (Kala-azar). 2013. Available: http://www.searo.who.int/entity/world_health_day/2014/SEA-CD-274.pdf

28. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis Worldwide and Global Estimates of Its Incidence. Kirk M, editor. PLoS One. 2012;7: e35671. doi:10.1371/journal.pone.0035671

29. WHO. Process of validation of elimination of kala-azar as a public health problem in South-East Asia. 2016. Available: https://img1.wsimg.com/blobby/go/c5156b45-48df-4ba4-ab15-be2bb6261d20/downloads/1bu1begqv_714167.pdf

30. Government of Nepal Ministry of Health and Population. National Guideline on Kala-azar Elimination Program (Updated) 2019. 2019.

31. Fitzpatrick A, Al-Kobaisi NSMS, Beitman Maya J, Ren Chung Y, Duhan S, Elbegdorj E, et al. Sustaining visceral leishmaniasis elimination in Bangladesh – Could a policy brief help? Picado A, editor. PLoS Negl Trop Dis. 2017;11: e0006081. doi:10.1371/journal.pntd.0006081

32. Directorate National Vector Borne Disease Control Programme. Accelerated Plan for Kala-azar Elimination 2017. 2017. Available: https://www.who.int/leishmaniasis/resources/Accelerated-Plan-Kala-azar1-Feb2017_light.pdf?ua=1

33. WHO. Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030. 2020.

34. Bern C, Courtenay O, Alvar J. Of Cattle, Sand Flies and Men: A Systematic Review of Risk Factor Analyses for South Asian Visceral Leishmaniasis and Implications for Elimination. Boelaert M, editor. PLoS Negl Trop Dis. 2010;4: e599. doi:10.1371/journal.pntd.0000599

35. Boelaert M, Meheus F, Sanchez A, Singh SP, Vanlerberghe V, Picado A, et al. The poorest of the poor: a poverty appraisal of households affected by visceral leishmaniasis in Bihar, India. Trop Med Int Heal. 2009;14: 639–644. doi:10.1111/j.1365-3156.2009.02279.x

36. Le Rutte EA, Coffeng LE, Bontje DM, Hasker EC, Ruiz Postigo JA, Argaw D, 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

37. Selvapandiyanid A, Croft SL, Rijal S, Nakhasiid HL, Ganguly NK. Innovations for the elimination and control of visceral leishmaniasis. PLoS Negl Trop Dis. 2019;9. doi:10.1371/journal.pntd.0007616

38. Cloots K, Uranw S, Ostyn B, Bhattarai NR, Le Rutte E, Khanal B, et al. Impact of the visceral leishmaniasis elimination initiative on Leishmania donovani transmission in Nepal: a 10-year repeat survey. Lancet Glob Heal. 2020;8: e237–e243. doi:10.1016/S2214-109X(19)30536-4

39. Ostyn B, Uranw S, Bhattarai NR, Das ML, Rai K, Tersago K, et al. Transmission of Leishmania donovani in the Hills of Eastern Nepal, an Outbreak Investigation in Okhaldhunga and Bhojpur Districts. Warburg A, editor. PLoS Negl Trop Dis. 2015;9: e0003966. doi:10.1371/journal.pntd.0003966

40. Banjara MR, Joshi AB. Evidence for visceral leishmaniasis elimination in Nepal. The Lancet Global Health. Elsevier Ltd; 2020. pp. e161–e162. doi:10.1016/S2214-109X(19)30538-8

41. Muniaraj M. The lost hope of elimination of Kala-azar (visceral leishmaniasis) by 2010 and cyclic occurrence of its outbreak in India, blame falls on vector control practices or co-infection with human immunodeficiency virus or therapeutic modalities? Trop Parasitol. 2014;4: 10–9. doi:10.4103/2229-5070.129143

42. Chowdhury R, Mondal D, Chowdhury V, Faria S, Alvar J, Nabi SG, et al. How Far Are We from Visceral Leishmaniasis Elimination in Bangladesh? An Assessment of Epidemiological Surveillance Data. Ghedin E, editor. PLoS Negl Trop Dis. 2014;8: e3020. doi:10.1371/journal.pntd.0003020

43. Chowdhury R, Mondal D, Chowdhury V, Faria S, Alvar J, Nabi SG, et al. How Far Are We from Visceral Leishmaniasis Elimination in Bangladesh? An Assessment of Epidemiological Surveillance Data. PLoS Negl Trop Dis. 2014;8. doi:10.1371/journal.pntd.0003020

44. Rahman KM, Islam N. Resurgence of visceral leishmaniasis in Bangladesh. Bull World Health Organ. 1983;61: 113–6. Available: http://www.ncbi.nlm.nih.gov/pubmed/6601534

45. Zijlstra EE. The immunology of post-kala-azar dermal leishmaniasis (PKDL). Parasites and Vectors. BioMed Central Ltd.; 2016. p. 464. doi:10.1186/s13071-016-1721-0

46. NATIONAL ROAD MAP FOR KALA-AZAR ELIMINATION. 2014. Available: https://nvbdcp.gov.in/Doc/Road-map-KA_2014.pdf

47. Addy1 M, Nandy2 A. Ten years of kala-azar in West Bengal, Part 1. Did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-Parganas? Bull World Health Organ. 1992;70: 341–346.

48. World Health Organization. Leprosy elimination campaigns: impact on case detection. Wkly Epidemiol Rec / Heal Sect Secr Leag Nations. 2003.

49. Rinaldi A. The Global Campaign to Eliminate Leprosy. PLoS Med. 2005;2: e341. doi:10.1371/journal.pmed.0020341

50. Lockwood DNJ, Shetty V, Oliveira G. Hazards of setting targets to eliminate disease: Lessons from the leprosy elimination campaign. BMJ. 2014;348. doi:10.1136/bmj.g1136

51. Feenstra P. Strengths and weaknesses of leprosy-elimination campaigns. Lancet. Elsevier Limited; 2000 Jun. doi:10.1016/S0140-6736(00)02370-9

52. Fichoux Y le, Quaranta J-F, Aufeuvre J-P, Lelievre A, Marty P, Suffia I, et al. Occurrence of Leishmania infantum Parasitemia in Asymptomatic Blood Donors Living in an Area of Endemicity in Southern France. J Clin Microbiol. 1999;37: 1953–1957. Available: https://jcm.asm.org/content/37/6/1953.long

53. Badaro R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, et al. New Perspectives on a Subclinical Form of Visceral Leishmaniasis. J Infect Dis. 1986;154: 1003–1011. doi:10.1093/infdis/154.6.1003

54. Riera C, Fisa R, Udina M, Gállego M, Portus M. Detection of Leishmania infantum cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans R Soc Trop Med Hyg. 2004;98: 102–110. doi:10.1016/S0035-9203(03)00015-4

55. Hasker E, Kansal S, Malaviya P, Gidwani K, Picado A, Singh RP, et al. Latent Infection with Leishmania donovani in Highly Endemic Villages in Bihar, India. Warburg A, editor. PLoS Negl Trop Dis. 2013;7: e2053. doi:10.1371/journal.pntd.0002053

56. Bern C, Haque R, Chowdhury R, Ali M, Kurkjian KM, Vaz L, et al. The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. Am J Trop Med Hyg. 2007;76: pp 909-914. doi:10.4269/ajtmh.2007.76.909

57. Ostyn B, Gidwani K, Khanal B, Picado A, Chappuis F, Singh SP, et al. Incidence of Symptomatic and

Asymptomatic Leishmania donovani Infections in High-Endemic Foci in India and Nepal: A Prospective Study. Milon G, editor. PLoS Negl Trop Dis. 2011;5: e1284. doi:10.1371/journal.pntd.0001284

58. Badaro R, Jones TC, Lorenco R, Cerf BJ, Sampaio D, Carvalho EM, et al. A Prospective Study of Visceral Leishmaniasis in an Endemic Area of Brazil. J Infect Dis. 1986;154: 639–649. doi:10.1093/infdis/154.4.639

59. Zulstra EE, El-Hassan AM, Ismael A, Ghalib AW. ENDEMIC KALA-AZAR IN EASTERN SUDAN: A LONGITUDINAL STUDY ON THE INCIDENCE OF CLINICAL AND SUBCLINICAL INFECTION AND POST-KALA-AZAR DERMAL LEISHMANIASIS. Am J Trop Med Hyg. 1994. Available: http://www.ajtmh.org/docserver/fulltext/14761645/51/6/TM0510060826.pdf?expires=1559319590&id=id&accname=11582&checksum=670928C746842F24C2968F2DDBA792F0

60. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S. Asymptomatic Leishmania Infection: A New Challenge for Leishmania Control. Clin Infect Dis. 2014;58: 1424–1429. doi:10.1093/cid/ciu102

61. Hasker E, Malaviya P, Gidwani K, Picado A, Ostyn B, Kansal S, et al. Strong Association between Serological Status and Probability of Progression to Clinical Visceral Leishmaniasis in Prospective Cohort Studies in India and Nepal. Satoskar AR, editor. PLoS Negl Trop Dis. 2014;8: e2657. doi:10.1371/journal.pntd.0002657

62. Cohen MS, Shaw GM, Mcmichael AJ, Ch B, Haynes BF. Acute HIV-1 Infection. N Engl J Med. 2011. Available: https://www.nejm.org/doi/pdf/10.1056/NEJMra1011874?articleTools=true

63. Clark SJ, Saag MS, Decker WD, Campbell-Hill S, Roberson JL, Veldkamp PJ, et al. High Titers of Cytopathic Virus in Plasma of Patients with Symptomatic Primary HIV-1 Infection. N Engl J Med. 1991;324: 954–960. doi:10.1056/NEJM199104043241404

64. Daar ES, Moudgil T, Meyer RD, Ho DD. Transient High Levels of Viremia in Patients with Primary Human Immunodeficiency Virus Type 1 Infection. N Engl J Med. 1991;324: 961–964. doi:10.1056/NEJM199104043241405

65. Epstein FH, Pantaleo G, Graziosi C, Fauci AS. The Immunopathogenesis of Human Immunodeficiency Virus Infection. N Engl J Med. 1993;328: 327–335. doi:10.1056/NEJM199302043280508

66. National AIDS Control Organization & ICMR National Institute of Medical Statistics. India HIV Estimates 2019: Report. 2020.

67. National AIDS Control Organization & ICMR-National Institute of Medical Statistics. India HIV Estimations 2017: Technical Report. 2018. Available: http://naco.gov.in/sites/default/files/HIV Estimations 2017 Report_1.pdf

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

69. WHO. WHO CASE DEFINITIONS OF HIV FOR SURVEILLANCE AND REVISED CLINICAL STAGING AND IMMUNOLOGICAL CLASSIFICATION OF HIV-RELATED DISEASE IN ADULTS AND CHILDREN HIV/AIDS Programme. 2007. Available: https://www.who.int/hiv/pub/guidelines/HIVstaging150307.pdf?ua=1

70. 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

71. Andreani G, Lodge R, Richard D, Tremblay MJ. Mechanisms of interaction between protozoan parasites and HIV. Curr Opin HIV AIDS. 2012;7: 276–282. doi:10.1097/COH.0b013e32835211e9

72. Ali N, John A, Luty F, Kariithi HM, Adriaensen W, Dorlo TPC, et al. Immunomodulatory Therapy of Visceral Leishmaniasis in Human Immunodeficiency Virus-Coinfected Patients. Front Immunol. 2018;8: 1. doi:10.3389/fimmu.2017.01943

73. Stein RA. Super-spreaders in infectious diseases. Int J Infect Dis. 2011;15: e510–e513. doi:10.1016/J.IJID.2010.06.020

74. WHO | Leishmaniasis and HIV coinfection. WHO. 2014 [cited 16 Jun 2019]. Available: https://www.who.int/leishmaniasis/burden/hiv_coinfection/burden_hiv_coinfection/en/

75. WHO. Report of the Fifth Consultative Meeting on Leishmania/HIV Coinfection. 2007. Available: https://www.who.int/leishmaniasis/resources/Leishmaniasis_hiv_coinfection5.pdf

76. Cruz I, Morales M, Noguer I, Rodriguez A, Alvar J. Leishmania in discarded syringes from intravenous drug users. Lancet. 2002;359: 1124–1125. doi:10.1016/S0140-6736(02)08160-6

77. Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, et al. Re-emergence of

leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. 2013;18. Available: www.eurosurveillance.org:pii=20546.Availableonline:http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20546

78. UNAIDS. Brazil | UNAIDS. [cited 16 Jun 2019]. Available: https://www.unaids.org/en/regionscountries/countries/brazil

79. de Oliveira Santos G, Priscila Sales de Jesus N, Vasconcelos Cerqueira-Braz J, Santana Santos V, Mara Dolce de Lemos L. Prevalence of HIV and associated factors among visceral leishmaniasis cases in an endemic area of Northeast Brazil. J Brazilian Soc Trop Med. 2018;52. doi:10.1590/0037-8682-0257-2018

80. Van Griensven J, Simegn T, Endris M, Diro E. Visceral Leishmaniasis and HIV Co-Infection in Northwest Ethiopia: Antiretroviral Treatment and Burden of Disease among Patients Enrolled in HIV Care. Am J Trop Med Hyg. 2018;98: 486–491. doi:10.4269/ajtmh.17-0142

81. Yimer M, Abera B, Mulu W, Zenebe Y, Bezabih B. Proportion of Visceral leishmaniasis and human immune deficiency virus co-infection among clinically confirmed visceral leishmaniasis patients at the endemic foci of the Amhara National Regional State, north-west Ethiopia. Am J Biomed Life Sci. 2014;2: 1–7. doi:10.11648/j.ajbls.20140201.11

82. Hurissa Z, Gebre-Silassie S, Hailu W, Tefera T, Lalloo DG, Cuevas LE, et al. Clinical characteristics and treatment outcome of patients with visceral leishmaniasis and HIV co-infection in northwest Ethiopia. Trop Med Int Heal. 2010;15: 848–855. doi:10.1111/j.1365-3156.2010.02550.x

83. Tarekegn B, Tamene A. Clinical and laboratory profiles of visceral leishmaniasis among adult patients admitted to Felege Hiwot Hospital, Bahir Dar, Ethiopia. SAGE Open Med. 2021;9: 1–6. doi:10.1177/20503121211036787

84. Lyons S, Veeken H, Long J. Visceral leishmaniasis and HIV in Tigray, Ethiopia. Trop Med Int Health. 2003;8: 733–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/12869095

85. ter Horst R, Collin SM, Ritmeijer K, Bogale A, Davidson RN. Concordant HIV Infection and Visceral Leishmaniasis in Ethiopia: The Influence of Antiretroviral Treatment and Other Factors on Outcome. Clin Infect Dis. 2008;46: 1702–1709. doi:10.1086/587899

86. Burza S, Mahajan R, Gonzalez Sanz M, Sunyoto T, Kumar R, Mitra G, et al. HIV and Visceral Leishmaniasis Coinfection in Bihar, India: An Underrecognized and Underdiagnosed Threat Against Elimination. Clin Infect Dis. 2014;59: 552–555. doi:10.1093/cid/ciu333

87. WHO TDR. The Use of Visceral Leishmaniasis Rapid Diagnostic Tests. 2008. Available: http://apps.who.int/iris/bitstream/handle/10665/44012/9789241597357_eng.pdf?sequence=1

88. Horst R ter, Tefera T, Assefa G, Ebrahim AZ, Davidson RN, Ritmeijer K, 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. doi:80/6/929 [pii]

89. Chulay JD, Bryceson ADM. Quantitation of Amastigotes of Leishmania Donovani in Smears of Splenic Aspirates from Patients with Visceral Leishmaniasis *. Am J Trop Med Hyg. 1983;32: 475–479. doi:10.4269/ajtmh.1983.32.475

90. Salam MA, Khan MGM, Bhaskar KRH, Afrad MH, Huda MM, Mondal D. Peripheral blood buffy coat smear: a promising tool for diagnosis of visceral leishmaniasis. J Clin Microbiol. 2012;50: 837–40. doi:10.1128/JCM.05067-11

91. Kassa M, Abdellati S, Cnops L, Bremer Hinckel BC, Yeshanew A, Hailemichael W, et al. Diagnostic accuracy of direct agglutination test, rK39 ELISA and six rapid diagnostic tests among visceral leishmaniasis patients with and without HIV coinfection in Ethiopia. PLoS Negl Trop Dis. 2020;14: 1–13. doi:10.1371/journal.pntd.0008963

92. Burns JM, Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG. Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. Proc Natl Acad Sci U S A. 1993;90: 775–779. doi:10.1073/pnas.90.2.775

93. Boelaert M, Verdonck K, Menten J, Sunyoto T, van Griensven J, Chappuis F, et al. Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. Cochrane database Syst Rev. 2014; CD009135. doi:10.1002/14651858.CD009135.pub2

94. Schroeder HW, Jr, Cavacini L. Structure and Function of Immunoglobulins. J Allergy Clin Immunol. 2010;125: S41. doi:10.1016/J.JACI.2009.09.046

95. Bhattacharyya T, Ayandeh A, Falconar AK, Sundar S, El-Safi S, Gripenberg MA, et al. IgG1 as a Potential Biomarker of Post-chemotherapeutic Relapse in Visceral Leishmaniasis, and Adaptation to a Rapid Diagnostic Test. Picado A, editor. PLoS Negl Trop Dis. 2014;8: e3273. doi:10.1371/journal.pntd.0003273

96. Marlais T, Bhattacharyya T, Singh OP, Mertens P, Gilleman Q, Thunissen C, et al. Visceral Leishmaniasis IgG1 Rapid Monitoring of Cure vs. Relapse, and Potential for Diagnosis of Post Kala-Azar Dermal Leishmaniasis. Front Cell Infect Microbiol. 2018;8: 427. doi:10.3389/fcimb.2018.00427

97. Boelaert M, Rijal S, Regmi S, Singh R, Karki B, Jacquet D, et al. A COMPARATIVE STUDY OF THE EFFECTIVENESS OF DIAGNOSTIC TESTS FOR VISCERAL LEISHMANIASIS. 2004.

98. Medrano FJ, Avate CC, Leal M, Rey CN, Lissen E, Alvar J. THE ROLE OF SEROLOGY IN THE DIAGNOSIS AND PROGNOSIS OF VISCERAL LEISHMANIASIS IN PATIENTS COINFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE-1. Am J Trop Med Hyg. 1998;59: 155–162. Available: http://www.ajtmh.org/docserver/fulltext/14761645/59/1/9684645.pdf?expires=1526635663&id=id&accname=guest&checksum=C23E796915362AFE92F60CC52217E366

99. Cota GF, de Sousa MR, Demarqui FN, Rabello A. The Diagnostic Accuracy of Serologic and Molecular Methods for Detecting Visceral Leishmaniasis in HIV Infected Patients: Meta-Analysis. Boelaert M, editor. PLoS Negl Trop Dis. 2012;6: e1665. doi:10.1371/journal.pntd.0001665

100. Meredith SEO, Kroon NCM, Sondorp E, Seaman J, Goris MGA, Van Ingen CW, et al. Leish-KIT, a Stable Direct Agglutination Test Based on Freeze-Dried Antigen for Serodiagnosis of Visceral Leishmaniasis. J Clin Microbiol. 1995. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC228261/pdf/331742.pdf

101. Adams ER, Jacquet D, Schoone G, Gidwani K, Boelaert M, Cunningham J. Leishmaniasis Direct Agglutination Test: Using Pictorials as Training Materials to Reduce Inter-Reader Variability and Improve Accuracy. PLoS Negl Trop Dis. 2012;6: e1946. doi:10.1371/journal.pntd.0001946

102. Boelaert M, El Safi S, Mousa H, Githure J, Mbati P, Gurubacharya VL, et al. Multi-centre evaluation of repeatability and reproducibility of the direct agglutination test for visceral leishmaniasis. Trop Med Int Heal. 1999;4: 31–37. doi:10.1046/j.1365-3156.1999.00348.x

103. Chappuis F, Rijal S, Soto A, Menten J, Boelaert M. A meta-analysis of the diagnostic performance

of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. BMJ. 2006;333: 723. doi:10.1136/bmj.38917.503056.7C

104. Zijlstra EE, Daifalla NS, Kager † P A, Khalil EAG, El-Hassan AM, Reed SG, et al. rK39 Enzyme-Linked Immunosorbent Assay for Diagnosis of Leishmania donovani Infection. Clin Diagn Lab Immunol. 1998. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC95645/pdf/cd000717.pdf

105. Tadese D, Hailu A, Bekele F, Belay S. An epidemiological study of visceral leishmaniasis in North East Ethiopia using serological and leishmanin skin tests. Schriefer A, editor. PLoS One. 2019;14: e0225083. doi:10.1371/journal.pone.0225083

106. Moral L, Rubio EM, Moya M. A leishmanin skin test survey in the human population of I'Alacanti Region (Spain): implications for the epidemiology of Leishmania infanturn infection in southern Europe. Trans R Soc Trop Med Hyg. 2002. Available: https://academic.oup.com/trstmh/article-abstract/96/2/129/1910531

107. Pattabhi S, Whittle J, Mohamath R, El-Safi S, Moulton GG, Guderian JA, et al. Design, Development and Evaluation of rK28-Based Point-of-Care Tests for Improving Rapid Diagnosis of Visceral Leishmaniasis. Louzir H, editor. PLoS Negl Trop Dis. 2010;4: e822. doi:10.1371/journal.pntd.0000822

108. Sundar S, Singh RK, Bimal SK, Gidwani K, Mishra A, Maurya R, et al. Comparative evaluation of parasitology and serological tests in the diagnosis of visceral leishmaniasis in India: a phase III diagnostic accuracy study. Trop Med Int Heal. 2006;0: 061127095204008-??? doi:10.1111/j.1365-3156.2006.01775.x

109. Bremer Hinckel BC, Marlais T, Airs S, Bhattacharyya T, Imamura H, Dujardin J-C, et al. Refining wet lab experiments with in silico searches: A rational quest for diagnostic peptides in visceral leishmaniasis. Ajjampur SS, editor. PLoS Negl Trop Dis. 2019;13: e0007353. doi:10.1371/journal.pntd.0007353

110. Riera C, Fisa R, Lopez P, Ribera E, Carrio J, Falco V, et al. Evaluation of a latex agglutination test (KAtex) for detection of Leishmania antigen in urine of patients with HIV-Leishmania coinfection: value in diagnosis and post-treatment follow-up. Eur J Clin Microbiol Infect Dis. 2004;23: 899–904. doi:10.1007/s10096-004-1249-7

111. Boelaert M, El-Safi S, Hailu A, Mukhtar M, Rijal S, Sundar S, et al. Diagnostic tests for kala-azar: a

multi-centre study of the freeze-dried DAT, rK39 strip test and KAtex in East Africa and the Indian subcontinent. Trans R Soc Trop Med Hyg. 2008;102: 32–40. doi:10.1016/j.trstmh.2007.09.003

112. Vallur AC, Tutterrow YL, Mohamath R, Pattabhi S, Hailu A, Abdoun AO, 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

113. van Griensven J, Mengesha B, Mekonnen T, Fikre H, Takele Y, Adem E, et al. Leishmania Antigenuria to Predict Initial Treatment Failure and Relapse in Visceral Leishmaniasis/HIV Coinfected Patients: An Exploratory Study Nested Within a Clinical Trial in Ethiopia. Front Cell Infect Microbiol. 2018;8: 94. doi:10.3389/fcimb.2018.00094

114. Sundar S, Agrawal S, Pai K, Chance M, Hommel M. DETECTION OF LEISHMANIAL ANTIGEN IN THE URINE OF PATIENTS WITH VISCERAL LEISHMANIASIS BY A LATEX AGGLUTINATION TEST. Am J Trop Med Hyg. 2005;73: 269–271. Available: http://www.ajtmh.org/docserver/fulltext/14761645/73/2/0730269.pdf?expires=1557484595&id=id&accname=guest&checksum=3BF04621F19118F30A5FE3A08CD103CA

115. Salam MAA, Khan MGMGM, Mondal D. Urine antigen detection by latex agglutination test for diagnosis and assessment of initial cure of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2011;105: 269–272. doi:10.1016/j.trstmh.2010.12.007

116. Abeijon C, Campos-Neto A. Potential Non-invasive Urine-Based Antigen (Protein) Detection Assay to Diagnose Active Visceral Leishmaniasis. Louzir H, editor. PLoS Negl Trop Dis. 2013;7: e2161. doi:10.1371/journal.pntd.0002161

117. Abeijon C, Alves F, Monnerat S, Wasunna M, Mbui J, Viana AG, et al. Development of a multiplexed assay for detection of leishmania donovani and leishmania infantum protein biomarkers in urine samples of patients with visceral leishmaniasis. J Clin Microbiol. 2019;57. doi:10.1128/JCM.02076-18

118. Abeijon C, Kashino SS, Silva FO, Costa DL, Fujiwara RT, Costa CHN, et al. Identification and diagnostic utility of Leishmania infantum proteins found in urine samples from patients with visceral leishmaniasis. Clin Vaccine Immunol. 2012;19: 935–943. doi:10.1128/CVI.00125-12

119. Abeijon C, Singh OP, Chakravarty J, Sundar S, Campos-Neto A. Novel Antigen Detection Assay to Monitor Therapeutic Efficacy of Visceral Leishmaniasis. Am J Trop Med Hyg. 2016;95: 800–802.

doi:10.4269/ajtmh.16-0291

120. Abeijon C, Alves F, Verine Monnerat S, Mbui J, Viana AG, Almeida RM, et al. Urine-based antigen detection assay for diagnosis of visceral leishmaniasis using monoclonal antibodies specific for six protein biomarkers of Leishmania infantum / Leishmania donovani. 2020 [cited 27 Apr 2020]. doi:10.1371/journal.pntd.0008246

121. Hossain F, Ghosh P, Khan MAA, Duthie MS, Vallur AC, Picone A, 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

122. Sudarshan M, Singh T, Chakravarty J, Sundar S. A Correlative Study of Splenic Parasite Score and Peripheral Blood Parasite Load Estimation by Quantitative PCR in Visceral Leishmaniasis. J Clin Microbiol. 2015;53: 3905–7. doi:10.1128/JCM.01465-15

123. Kaushal H, Bhattacharya SK, Verma S, Salotra P. Serological and Molecular Analysis of Leishmania Infection in Healthy Individuals from Two Districts of West Bengal, India, Endemic for Visceral Leishmaniasis. Am J Trop Med Hyg. 2017;96: 1448–1455. doi:10.4269/ajtmh.16-0592

124. Chakravarty J, Hasker E, Kansal S, Singh OP, Malaviya P, Singh AK, et al. Determinants for progression from asymptomatic infection to symptomatic visceral leishmaniasis: A cohort study. PLoS Negl Trop Dis. 2018;13. doi:10.1371/journal.pntd.0007216

125. Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: Current status and future applications. J Clin Microbiol. 2007;45: 21–25. doi:10.1128/JCM.02029-06

126. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000;28: 63e – 63. doi:10.1093/nar/28.12.e63

127. Adams ER, Schoone G, Versteeg I, Gomez MA, Diro E, Mori Y, 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

128. Mukhtar M, Ali SS, Boshara SA, Albertini A, Monnerat S, Bessell P, et al. Sensitive and less invasive confirmatory diagnosis of visceral leishmaniasis in Sudan using loop-mediated

isothermal amplification (LAMP). Bañuls A-L, editor. PLoS Negl Trop Dis. 2018;12: e0006264. doi:10.1371/journal.pntd.0006264

129. Cunningham J, Hasker E, Das P, El Safi S, Goto H, Mondal D, et al. A global comparative evaluation of commercial immunochromatographic rapid diagnostic tests for visceral leishmaniasis. Clin Infect Dis. 2012;55: 1312–9. doi:10.1093/cid/cis716

130. World Health Organisation. Control of the leishmaniases: Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22–26 March 2010. 2010. Available: https://apps.who.int/iris/bitstream/handle/10665/44412/WHO_TRS_949_eng.pdf;jsessionid=EE3C4A24A9AB60389E8DB6BF610DEDFF?sequence=1

131. Mahajan R, Das P, Isaakidis P, Sunyoto T, Sagili KD, Lima MA, et al. Combination Treatment for Visceral Leishmaniasis Patients Coinfected with Human Immunodeficiency Virus in India. Clin Infect Dis. 2015;61: 1255–1262. doi:10.1093/cid/civ530

132. Nair M, Kumar P, Pandey S, Kazmi S, Moreto-Planas L, Ranjan A, et al. Quality of life perceptions amongst patients co-infected with Visceral Leishmaniasis and HIV: A qualitative study from Bihar, India. Finlayson K, editor. PLoS One. 2020;15: e0227911. doi:10.1371/journal.pone.0227911

133. Olliaro PL, Guerin PJ, Gerstl S, Haaskjold AA, Rottingen JA, Sundar S. Treatment options for visceral leishmaniasis: A systematic review of clinical studies done in India, 1980-2004. Lancet Infectious Diseases. Elsevier; 2005. pp. 763–774. doi:10.1016/S1473-3099(05)70296-6

134. Sundar S. Drug resistance in Indian visceral leishmaniasis. Trop Med Int Heal. 2001;6: 849–854. doi:10.1046/j.1365-3156.2001.00778.x

135. Thakur CP, Narayan S. A comparative evaluation of amphotericin B and sodium antimony gluconate, as first-line drugs in the treatment of Indian visceral leishmaniasis. Ann Trop Med Parasitol. 2004;98: 129–138. doi:10.1179/000349804225003154

136. van Griensven J, Balasegaram M, Meheus F, Alvar J, Lynen L, Boelaert M. Combination therapy for visceral leishmaniasis. The Lancet Infectious Diseases. Elsevier; 2010. pp. 184–194. doi:10.1016/S1473-3099(10)70011-6

137. Jha TK, Olliaro P, Thakur CPN, Kanyok TP, Singhania BL, Singh IJ, et al. Randomised controlled trial of aminosidine (paromomycin) v sodium stibogluconate for treating visceral leishmaniasis in

North Bihar, India. BMJ. 1998;316: 1200–1207. doi:10.1136/bmj.316.7139.1200

138. Sundar S, Chakravarty J. Paromomycin in the treatment of leishmaniasis. Expert Opin Investig Drugs. 2008;17: 787–794. doi:10.1517/13543784.17.5.787

139. Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fischer C, et al. Miltefosine, an Oral Agent, for the Treatment of Indian Visceral Leishmaniasis. N Engl J Med. 1999;341: 1795–1800. doi:10.1056/nejm199912093412403

140. Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, Ostyn B, et al. Efficacy of Miltefosine in the Treatment of Visceral Leishmaniasis in India After a Decade of Use. Clin Infect Dis. 2012;55: 543–550. doi:10.1093/cid/cis474

141. Bhandari V, Kulshrestha A, Deep DK, Stark O, Prajapati VK, Ramesh V, et al. Drug Susceptibility in Leishmania Isolates Following Miltefosine Treatment in Cases of Visceral Leishmaniasis and Post Kala-Azar Dermal Leishmaniasis. Ghedin E, editor. PLoS Negl Trop Dis. 2012;6: e1657. doi:10.1371/journal.pntd.0001657

142. Sundar S, Mondal D, Rijal S, Bhattacharya S, Ghalib H, Kroeger A, et al. Implementation research to support the initiative on the elimination of kala azar from Bangladesh, India and Nepal - the challenges for diagnosis and treatment. Trop Med Int Heal. 2008;13: 2–5. doi:10.1111/j.1365-3156.2007.01974.x

143. van Griensven J, Diro E. Visceral Leishmaniasis: Recent Advances in Diagnostics and Treatment Regimens. Infectious Disease Clinics of North America. W.B. Saunders; 2019. pp. 79–99. doi:10.1016/j.idc.2018.10.005

144. Sundar S, Chakravarty J, Agarwal D, Rai M, Murray HW. Single-Dose Liposomal Amphotericin B for Visceral Leishmaniasis in India. N Engl J Med. 2010;362: 504–512. doi:10.1056/nejmoa0903627

145. Sinha PK, van Griensven J, Pandey K, Kumar N, Verma N, Mahajan R, et al. Liposomal Amphotericin B for Visceral Leishmaniasis in Human Immunodeficiency Virus-Coinfected Patients: 2-Year Treatment Outcomes in Bihar, India. Clin Infect Dis. 2011;53: e91–e98. doi:10.1093/cid/cir521

146. Burza S, Mahajan R, Sinha PK, van Griensven J, Pandey K, Lima MA, et al. Visceral Leishmaniasis and HIV Co-infection in Bihar, India: Long-term Effectiveness and Treatment Outcomes with

Liposomal Amphotericin B (AmBisome). PLoS Negl Trop Dis. 2014;8: e3053. doi:10.1371/JOURNAL.PNTD.0003053

147. van Griensven J, Zijlstra EE, Hailu A. Visceral Leishmaniasis and HIV Coinfection: Time for Concerted Action. PLoS Negl Trop Dis. 2014;8: e3023. doi:10.1371/journal.pntd.0003023

148. Molina I, Falco V, Crespo M, Riera C, Ribera E, Curran A, et al. Efficacy of liposomal amphotericin B for secondary prophylaxis of visceral leishmaniasis in HIV-infected patients. J Antimicrob Chemother. 2007;60: 837–842. doi:10.1093/jac/dkm294

149. Diro E, Ritmeijer K, Boelaert M, Alves F, Mohammed R, Abongomera C, et al. Use of Pentamidine As Secondary Prophylaxis to Prevent Visceral Leishmaniasis Relapse in HIV Infected Patients, the First Twelve Months of a Prospective Cohort Study. Louzir H, editor. PLoS Negl Trop Dis. 2015;9: e0004087. doi:10.1371/journal.pntd.0004087

150. Diro E, Ritmeijer K, Boelaert M, Alves F, Mohammed R, Abongomera C, 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: 444–451. doi:10.1093/cid/cix807

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

152. Srivastava P, Gidwani K, Picado A, Van der Auwera G, Tiwary P, Ostyn B, et al. Molecular and serological markers of *Leishmania donovani* infection in healthy individuals from endemic areas of Bihar, India. Trop Med Int Heal. 2013;18: 548–554. doi:10.1111/tmi.12085

153. Hirve S, Boelaert M, Matlashewski G, Mondal D, Arana B, Kroeger A, et al. Transmission Dynamics of Visceral Leishmaniasis in the Indian Subcontinent – A Systematic Literature Review. Chatterjee M, editor. PLoS Negl Trop Dis. 2016;10: e0004896. doi:10.1371/journal.pntd.0004896

154. Singh OP, Tiwary P, Kushwaha AK, Singh SK, Singh DK, Lawyer P, et al. Xenodiagnosis to evaluate the infectiousness of humans to sandflies in an area endemic for visceral leishmaniasis in Bihar, India: a transmission-dynamics study. The Lancet Microbe. 2021;2: e23–e31. doi:10.1016/s2666-5247(20)30166-x

155. Dinesh DS, Kar SK, Kishore K, Palit A, Verma N, Gupta K, et al. Screening sandflies for natural

infection with Leishmania donovani, using a non-radioactive probe based on the total DNA of the parasite. Ann Trop Med Parasitol. 2000;94: 447–451. doi:10.1080/00034983.2000.11813563

156. Addy M, Nandy A. Ten years of kala-azar in West Bengal, Part I. Did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-Parganas? Bull World Health Organ. 1992;70: 341–346. Available: /pmc/articles/PMC2393278/?report=abstract

157. Picado A, Das ML, Kumar V, Kesari S, Dinesh DS, Roy L, et al. Effect of Village-wide Use of Long-Lasting Insecticidal Nets on Visceral Leishmaniasis Vectors in India and Nepal: A Cluster Randomized Trial. Dinglasan RR, editor. PLoS Negl Trop Dis. 2010;4: e587. doi:10.1371/journal.pntd.0000587

158. Salam MA, Huda MM, Khan MGM, Shomik MS, Mondal D. Evidence-based diagnostic algorithm for visceral leishmaniasis in Bangladesh. Parasitol Int. 2021;80: 102230. doi:10.1016/j.parint.2020.102230

159. Cloots K, Marino P, Burza S, Gill N, Boelaert M, Hasker E. Visceral Leishmaniasis-HIV Coinfection as a Predictor of Increased Leishmania Transmission at the Village Level in Bihar, India. Front Cell Infect Microbiol. 2021;11. doi:10.3389/fcimb.2021.604117

160. Orsini M, Canela JR, Disch J, Maciel F, Greco D, Toledo A, et al. High frequency of asymptomatic Leishmania spp. infection among HIV-infected patients living in endemic areas for visceral leishmaniasis in Brazil. Trans R Soc Trop Med Hyg. 2012;106: 283–288. doi:10.1016/j.trstmh.2012.01.008

161. van Griensven J, van Henten S, Mengesha B, Kassa M, Adem E, Endris Seid M, et al. Longitudinal evaluation of asymptomatic Leishmania infection in HIV-infected individuals in North-West Ethiopia: A pilot study. Schallig HDFH, editor. PLoS Negl Trop Dis. 2019;13: e0007765. doi:10.1371/journal.pntd.0007765

162. Vogt F, Mengesha B, Asmamaw H, Mekonnen T, Fikre H, Takele Y, et al. Antigen Detection in Urine for Noninvasive Diagnosis and Treatment Monitoring of Visceral Leishmaniasis in Human Immunodeficiency Virus Coinfected Patients: An Exploratory Analysis from Ethiopia. Am J Trop Med Hyg. 2018;99: 957–966. doi:10.4269/ajtmh.18-0042

163. García-García JA, Martín-Sánchez J, Gállego M, Rivero-Román A, Camacho A, Riera C, et al. Use of noninvasive markers to detect Leishmania infection in asymptomatic human immunodeficiency

virus-infected patients. J Clin Microbiol. 2006;44: 4455–4458. doi:10.1128/JCM.00921-06

164. Wolday D, Akuffo H, Fessahaye G, Valantine A, Britton S. Live and Killed Human Immunodeficiency Virus Type-1 Increases the Intracellular Growth of Leishmania donovani in Monocyte-derived Cells. Scand J Infect Dis. 1998;30: 171. doi:10.1080/003655498750002268

165. Pasquau F, Ena J, Sanchez R, Cuadrado JM, Amador C, Flores J, et al. Leishmaniasis as an opportunistic infection in HIV-infected patients: determinants of relapse and mortality in a collaborative study of 228 episodes in a Mediterreanean region. Eur J Clin Microbiol Infect Dis. 2005;24: 411–418. doi:10.1007/s10096-005-1342-6

166. Burza S, Mahajan R, Sinha PK, Griensven J van, Pandey K, Lima MA, et al. Visceral Leishmaniasis and HIV Co-infection in Bihar, India: Long-term Effectiveness and Treatment Outcomes with Liposomal Amphotericin B (AmBisome). PLoS Negl Trop Dis. 2014;8: e3053. doi:10.1371/JOURNAL.PNTD.0003053

167. Angelo J, Lindoso L, Cunha MA, Thiago Queiroz I, Henrique C, Moreira V. Leishmaniasis-HIV coinfection: current challenges. 2016 [cited 2 May 2019]. doi:10.2147/HIV.S93789

168. Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol. 2002;9: 951–958. doi:10.1128/CDLI.9.5.951-958.2002

169. Oliveira A V., Roque-Barreira MC, Sartori A, Campos-Neto A, Rossi MA. Mesangial proliferative glomerulonephritis associated with progressive amyloid deposition in hamsters experimentally infected with Leishmania donovani. Am J Pathol. 1985;120: 256. Available: /pmc/articles/PMC1887832/?report=abstract

170. Sartori A, Roque-Barreira MC, Coe J, Campos-Neto A. Immune complex glomerulonephritis in experimental kala-azar II: Detection and characterization of parasite antigens and antibodies eluted from kidneys of Leishmania donovani-infected hamsters. Clin exp Immunol. 1991;87: 386–392.

171. El Harith A, Hero A, Kolk,' J, Leeuwenburg J, Muigai R, Huigen,' E, et al. Improvement of a Direct Agglutination Test for Field Studies of Visceral Leishmaniasis. J Clin Microbiol. 1988. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC266601/pdf/jcm00079-0087.pdf

172. Owen SI, Hossain F, Ghosh P, Chowdhury R, Sakhawat Hossain M, Jewell C, et al. Detection of

asymptomatic Leishmania infection in Bangladesh by antibody and antigen diagnostic tools shows an association with post-kala-azar dermal leishmaniasis (PKDL) patients. Parasites Vectors 2021 141. 2021;14: 1–7. doi:10.1186/s13071-021-04622-8

173. Hossain F, Picado A, Owen SI, Ghosh P, Chowdhury R, Maruf S, et al. Evaluation of Loopamp™ Leishmania Detection Kit and Leishmania Antigen ELISA for Post-Elimination Detection and Management of Visceral Leishmaniasis in Bangladesh. Artic 670759 1 Front Cell Infect Microbiol. 2021;11: 670759. doi:10.3389/fcimb.2021.670759

174. Yoshida R, Muramatsu S, Akita H, Saito Y, Kuwahara M, Kato D, et al. Development of an Immunochromatography Assay (QuickNavi-Ebola) to Detect Multiple Species of Ebolaviruses. J Infect Dis. 2016;214: S185–S191. doi:10.1093/INFDIS/JIW252

175. Azazy AA, Chance ML, Devaney E. A time-course study of circulating antigen and parasite-specific antibody in cotton rats infected with Leishmania donovani. Ann Trop Med Parasitol. 1997;91: 153–162. doi:10.1080/00034983.1997.11813125

176. Sarkari B, Chance M, Hommel M. Antigenuria in visceral leishmaniasis: Detection and partial characterisation of a carbohydrate antigen. Acta Trop. 2002;82: 339–348. doi:10.1016/S0001-706X(02)00043-8

177. Attar ZJ, Chance ML, El-Safi S, Carney J, Azazy A, El-Hadi M, et al. Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. Acta Trop. 2000;78: 11–16. doi:10.1016/S0001-706X(00)00155-8

178. Guedes DL, Justo AM, Júnior WLB, da Silva ED, de Aquino SR, Junior MS da CL, et al. Asymptomatic Leishmania infection in HIV-positive outpatients on antiretroviral therapy in Pernambuco, Brazil. PLoS Negl Trop Dis. 2021;15: e0009067. doi:10.1371/JOURNAL.PNTD.0009067

179. Molina R, Ghosh D, Carrillo E, Monnerat S, Bern C, Mondal D, et al. Infectivity of Post-Kala-azar Dermal Leishmaniasis Patients to Sand Flies: Revisiting a Proof of Concept in the Context of the Kala-azar Elimination Program in the Indian Subcontinent. Clin Infect Dis. 2017;65: 150–153. doi:10.1093/cid/cix245

180. Kuehn BM. India Takes Aim at Eliminating Visceral Leishmaniasis. JAMA. 2021;326: 999–999. doi:10.1001/JAMA.2021.15467

181. Stauch A, Sarkar RR, Picado A, Ostyn B, Sundar S, Rijal S, et al. Visceral leishmaniasis in the indian subcontinent: Modelling epidemiology and control. PLoS Neglected Tropical Diseases. PLoS Negl Trop Dis; 2011. doi:10.1371/journal.pntd.0001405

182. Tumbarello M, Tacconelli E, Bertagnolio S, Cauda R. Highly active antiretroviral therapy decreases the incidence of visceral leishmaniasis in HIV-infected individuals. AIDS. AIDS; 2000. pp. 2948–2949. doi:10.1097/00002030-200012220-00021

183. European Commission. Regulation (EU) 2017/746 of the European parliament and of the council on in vitro diagnostic medical devices. Off J Eur Union. 2017;5: 117–176.

184. Dahl EH, Hamdan HM, Mabrouk L, Matendechero SH, Mengistie TB, Elhag MS, et al. Control of visceral leishmaniasis in East Africa: fragile progress, new threats. BMJ Glob Heal. 2021;6: e006835. doi:10.1136/BMJGH-2021-006835

185. Alcami J, Vallejo A, Angelo Lauletta Lindoso J, Adriaensen W, Abdellati S, van Henten S, et al. Serum Levels of Soluble CD40 Ligand and Neopterin in HIV Coinfected Asymptomatic and Symptomatic Visceral Leishmaniasis Patients. Front Cell Infect Microbiol | www.frontiersin.org. 2018;8: 428. doi:10.3389/fcimb.2018.00428

186. World Health Organisation. Coronavirus Disease 2019 (COVID-19) Situation Report - 51. 2020.

187. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. Nat Med. 2020;26: 672–675. doi:10.1038/s41591-020-0869-5

188. Liu Y, Yan L-M, Wan L, Xiang T-X, Le A, Liu J-M, et al. Viral dynamics in mild and severe cases of COVID-19. Lancet Infect Dis. 2020;20: 656–657. doi:10.1016/S1473-3099(20)30232-2

189. FIND. GENERAL INFORMATION ON Ag-AND Ab-DETECTION RDTs FOR COVID-19. 2020. Available: www.finddx.org/covid-19

190. Long Q-X, Liu B-Z, Deng H-J, Wu G-C, Deng K, Chen Y-K, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. Nat Med. 2020; 1–4. doi:10.1038/s41591-020-0897-1

191. Staines HM, Kirwan DE, Clark DJ, Adams ER, Augustin Y, Byrne RL, et al. Dynamics of IgG seroconversion and pathophysiology of COVID-19 infections. medRxiv. 2020; 2020.06.07.20124636. doi:10.1101/2020.06.07.20124636

192. Ma H, Zeng W, He H, Zhao D, Jiang D, Zhou P, et al. Serum IgA, IgM, and IgG responses in COVID-

19. Cell Mol Immunol. 2020;17: 773–775. doi:10.1038/s41423-020-0474-z

193. Padoan A, Sciacovelli L, Basso D, Negrini D, Zuin S, Cosma C, et al. IgA-Ab response to spike glycoprotein of SARS-CoV-2 in patients with COVID-19: A longitudinal study. Clin Chim Acta. 2020;507: 164–166. doi:10.1016/j.cca.2020.04.026

194. Landis JR, Koch GG. The Measurement of Observer Agreement for Categorical Data. Biometrics. 1977;33: 159. doi:10.2307/2529310

195. Adams ER, Augustin Y, Byrne RL, Clark DJ, Cocozza M, Cubas-Atienzar AI, et al. Rapid development of COVID-19 rapid diagnostics for low resource settings: accelerating delivery through transparency, responsiveness, and open collaboration. medRxiv. 2020; 2020.04.29.20082099. doi:10.1101/2020.04.29.20082099

196. Frasier SL. False Positive Alarm. Sci Am. 2020;323: 12–13. Available: https://www.scientificamerican.com/article/coronavirus-antibody-tests-have-a-mathematical-pitfall/?utm_source=Nature+Briefing&utm_campaign=2426c56bed-briefing-dy-20200617&utm_medium=email&utm_term=0_c9dfd39373-2426c56bed-44191917

197. Pouwels KB, House T, Robotham J V, Birrell PJ, Gelman A, Bowers N, et al. Community prevalence of SARS-CoV-2 in England: Results from the ONS Coronavirus Infection Survey Pilot. medRxiv. 2020; 1–13.

Appendix 1: *Leishmania* diagnostic evaluation – further work and peer-reviewed publications

Publications

Publications arising during this PhD, not presented in this thesis:

1. Hossain F, Picado A, **Owen SI**, Ghosh P, Chowdhury R, Maruf S, Ashfaq Khan A, Nath R, Baker J, Rashid U, Ghosh D, Hossain S, Duthie MS, Adams ER, Cruz I, Mondal D. 2021. Evaluation of Loopamp™ *Leishmania* Detection Kit and *Leishmania* Antigen ELISA for Post-Elimination Detection and Management of Visceral Leishmaniasis in Bangladesh. Front Cell Infect Microbiol 11:670759.

Consultancy for WHO-TDR

Co-sponsored by United Nations Children's Fund (UNICEF), the United Nations Development Programme (UNDP), The World Bank, and the WHO, the Special Programme for Research and Training in Tropical Diseases (TDR) aims to support research and training in infectious diseases associated with poverty. I conducted consultancy for WHO-TDR on VL research projects in India and Bangladesh. My role was to ensure the studies were being properly conducted and documented on behalf of WHO-TDR. During a site visit in Bangladesh, major tasks included: discussing protocol amendments and protocol compliance; following up on study enrolment; verifying the procedure for informed consent; checking a subset of study consent forms; checking a subset of case record forms against source documents and for completion; trouble-shooting laboratory issues. Pre-visit preparation included writing and sharing agendas. A post-visit monitoring report was submitted, and interim technical reports submitted for the duration of the project.

Oral Presentations:

1. Nov 2018: International Conference on Innovations for the Elimination and Control of Visceral Leishmaniasis (IEC-VL), Delhi. Oral presentation on behalf of Dr Emily Adams titled 'Diagnostics for Leishmaniasis in elimination settings'.

Poster Presentations:

1. Sept 2019: European Congress on Tropical Medicine and International Health (ECTMIH), Liverpool. Two poster presentations titled 'Non-invasive alternatives for diagnosis and test of cure for visceral leishmaniasis in HIV co-infected patients' and 'Urinary antigen for detection of asymptomatic visceral leishmaniasis in HIV positive patients'.
2. Nov 2018: International Conference on Innovations for the Elimination and Control of Visceral Leishmaniasis (IEC-VL), Delhi. Poster presentation titled 'Development and evaluation of diagnostics for visceral leishmaniasis (VL) in HIV patients in elimination settings'.

Appendix 2: Secondment to diagnostic research for SARS-CoV-2 infection

Summary of experiences and achievements during secondment

During the COVID-19 pandemic, I seconded to the diagnostic evaluation response being carried out in the Centre for Drugs and Diagnostics at LSTM led by Dr Emily Adams. Over the course of 6 months, I assisted in multiple studies recruiting within community and healthcare settings including the diagnostics studies Facilitating A SARS CoV-2 TEst for Rapid triage (FASTER), FALCON, and COVID-LIV, and drug study AGILE-ACCORD, from which evidence was submitted for three separate diagnostic products to the World Health Organisations Emergency Use Listing. I led an evaluation of 21 point of care lateral flow immunoassays as part of an internal evaluation and an international multi-centre evaluation for the Foundation for Innovative New Diagnostics (FIND). I assisted in multiple matrix equivalency studies to assess the use of capillary blood on lateral flow immunoassays in comparison to venous whole blood, plasma or serum. I assisted the set-up of plaque reduction neutralisation assays using live SARS CoV-2 to better understand the immune response to SARS CoV-2 infection. Over the time period I was trained and became independent working to biosafety level (BSL) 3 requirements.

Publications

Publications arising during secondment, not presented in this thesis:

1. Cubas-Atienzar AI, Bell F, Byrne RL, Buist K, Clark DJ, Cocozza M, Collins AM, Cuevas LE, Duvoix A, Easom N, Edwards T, Ferreira DM, Fletcher T, Groppelli E, Hyder-Wright A, Kadamus E, Kirwan DE, Kontogianni K, Krishna S, Kluczna D, Mark J, Mensah-Kane J, Miller E, Mitsi E, Norton D, O'Connor E, **Owen SI**, Planche T, Shelley S, Staines HM, Tate D, Thompson CR, Walker G, Williams CT, Wooding D, Fitchett JRA, Adams ER. 2021. Accuracy of the Mologic COVID-19 rapid antigen test: a prospective multi-centre analytical and clinical evaluation. Wellcome Open Res 6:132.
2. Setiabudi W, Hungerford D, Subramaniam K, Vaselli NM, Shaw VE, Wilton M, Vivancos R, Aston S, Platt G, Moitt T, Jones AP, Gabbay M, Buchan I, Carrol ED, Iturriza-Gomara M, Solomon T, Greenhalf W, Naisbitt DJ, Adams ER, Cunliffe NA, Turtle L, French N, on behalf of the **COVID-LIV Study Group**. 2021. Prospective observational study of SARS-CoV-2 infection, transmission and

immunity in a cohort of households in Liverpool City Region, UK (COVID-LIV): A study protocol. BMJ Open 11:48317.

3. Kay GA, **Owen SI**, Giorgi E, Clark DJ, Williams CT, Menzies S, Cuevas LE, Davies BMO, Eckersley NM, Hughes GL, Kirwan DE, Krishna S, Patterson EI, Planche T, Staines HM, Adams ER. 2022. SARS-CoV-2 enzyme-linked immunosorbent assays as proxies for plaque reduction neutralisation tests. Sci Rep 12:1–9.
4. Edwards T, Kay GA, Aljayyoussi G, **Owen SI**, Harland AR, Pierce NS, Calder JDF, Fletcher TE, Adams ER. 2022. SARS-CoV-2 viability on sports equipment is limited, and dependent on material composition. Sci Rep 12:1–8.
5. Brown L, Byrne RL, Fraser A, **Owen SI**, Cubas-Atienzar AI, Williams CT, Kay GA, Cuevas LE, Fitchett JRA, Fletcher T, Garrod G, Kontogianni K, Krishna S, Menzies S, Planche T, Sainter C, Staines HM, Turtle L, Adams ER. 2021. Self-sampling of capillary blood for SARS-CoV-2 serology. Sci Rep 11:7754.
6. Byrne RL, Kay GA, Kontogianni K, Aljayyoussi G, Brown L, Collins AM, Cuevas LE, Ferreira DM, Fraser AJ, Garrod G, Hill H, Hughes GL, Menzies S, Mitsi E, **Owen SI**, Patterson EI, Williams CT, Hyder-Wright A, Adams ER, Cubas-Atienzar AI. 2020. Saliva Alternative to Upper Respiratory Swabs for SARS-CoV-2 Diagnosis. Emerg Infect Dis 26:2770–2771.
7. Staines HM, Kirwan DE, Clark DJ, Adams ER, Augustin Y, Byrne RL, Cocozza M, Cubas-Atienzar AI, Cuevas LE, Cusinato M, Davies BMO, Davis M, Davis P, Duvoix A, Eckersley NM, Forton D, Fraser AJ, Garrod G, Hadcocks L, Hu Q, Johnson M, Kay GA, Klekotko K, Lewis Z, Macallan DC, Mensah-Kane J, Menzies S, Monahan I, Moore CM, Nebe-von-Caron G, **Owen SI**, Sainter C, Sall AA, Schouten J, Williams CT, Wilkins J, Woolston K, Fitchett JRA, Krishna S, Planche T. 2021. IgG Seroconversion and Pathophysiology in Severe Acute Respiratory Syndrome Coronavirus 2 Infection. Emerg Infect Dis 27.
8. Adams ER, Augustin Y, Byrne RL, Clark DJ, Cocozza M, Cubas-Atienzar AI, Cuevas LE, Cusinato M, Davies BMO, Davies M, Davies P, Duvoix A, Eckersley NM, Edwards T, Fletcher T, Fraser A j, Garrod G, Hadcocks L, Hu Qi, johnson M, Kay GA, Keymer K, Kirwan D, Klekotko K, Lewis Z, Mason J, Mensah-Kane J, Menzies S, Monahan I, Moore CM, Nebe-von-Caron G, **Owen SI**, Planche T, Sainter C, Schouten J, Staines HM, Turtle L, Williams C, Wilkins J, Woolston K, Sall AA, Fitchett JRA, Krishna S. 2020. Rapid development of COVID-19 rapid diagnostics for low resource settings: accelerating delivery through transparency, responsiveness, and open collaboration. medRxiv 2020.04.29.20082099.

The following publications which arose during secondment are presented in this thesis due to the significant overlap between PhD work and work related to COVID-19:

Comparative evaluation of ten lateral flow immunoassays to detect SARS-CoV-2 antibodies

Sophie I. Owen[a,*], Gala Garrod[a,*], J. Kenneth Baillie[b], Lisa Baldwin[a], Lottie Brown[a], Rachel L. Byrne[a], Ana I. Cubas-Atienzar[a], Luis E. Cuevas[a], Alice J. Fraser[a], Thomas Fletcher[c,d], Lynsey Goodwin[e], Grant A. Kay[a], ISARIC4C Investigators[¥], Konstantina Kontogianni[a], Jenifer Mason[f], Peter J.M. Openshaw[g], Stefanie Menzies[a], Shona C. Moore[e], Malcolm G. Semple[e], Joseph Taylor[f], Lance C.W. Turtle[e], Christopher T. Williams[a], Emily R. Adams[a,#]

[a] Centre for Drugs and Diagnostics Research, Liverpool School of Tropical Medicine (LSTM), Liverpool, UK

[b] Genetics and Genomics, Roslin Institute, University of Edinburgh, Edinburgh, UK

[c] Tropical and Infectious Diseases Unit, Royal Liverpool University Hospital, Prescot Street, Liverpool L7 8XP, United Kingdom

[d] Clinical Sciences, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, United Kingdom

[e] National Institute for Health Research (NIHR) Health Protection Research Unit (HPRU) in Emerging and Zoonotic Infections, University of Liverpool, Liverpool, UK

[f] Liverpool Clinical Laboratories, Liverpool University Hospital NHS Foundation Trust, Liverpool, UK

[g] National Heart and Lung Institute, Imperial College London, London, UK

[¥] ISARIC4C Investigators listed in end material

Running Head: Lateral flow assays to detect SARS-CoV-2 antibodies

#Address correspondence to Emily R. Adams, Emily.Adams@lstmed.ac.uk

***Gala Garrod and Sophie I. Owen contributed equally to this work. Author order was determined alphabetically.**

Garrod G. & Owen S.I. *et al.* Wellcome Open Res (2021) 6:18

Abstract

Background: Rapid mobilisation from industry and academia following the outbreak of the novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), led to the development and availability of SARS-CoV-2 lateral flow immunoassays (LFAs). High quality LFAs are urgently needed at the point of care to add to currently available diagnostic tools. In this study, we provide evaluation data for ten LFAs suitable for use at the point of care.

Methodology/Principal Findings: COVID-19 positive patients (N=45), confirmed by reverse transcription – quantitative polymerase chain reaction (RT-qPCR), were recruited through the International Severe Acute Respiratory and Emerging Infection Consortium - Coronavirus Clinical Characterisation Consortium (ISARIC4C) study. Sera collected from patients with influenza A (N=20), tuberculosis (N=5), individuals with previous flavivirus exposure (N=21), and healthy sera (N=4), collected pre-pandemic, were used as negative controls. Ten LFAs manufactured or distributed by ASBT Holdings Ltd, Cellex, Fortress Diagnostics, Nantong Egens Biotechnology, Mologic, NG Biotech, Nal von Minden and Suzhou Herui BioMed Co. were evaluated. Compared to RT-qPCR, sensitivity of LFAs ranged from 87.0-95.7%. Specificity against pre-pandemic controls ranged between 92.0-100%. Compared to IgG ELISA, sensitivity and specificity ranged between 90.5-100% and 93.2-100%, respectively. Percentage agreement between LFAs and IgG ELISA ranged from 89.6-92.7%. Inter-test agreement between LFAs and IgG ELISA ranged between kappa=0.792-0.854.

Conclusions/Significance: LFAs may serve as a useful tool for rapid confirmation of ongoing or previous infection in conjunction with clinical suspicion of COVID-19 in patients attending hospital. Impartial validation prior to commercial sale provides users with data that can inform best use settings.

Introduction

In December 2019, an outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China before spreading globally, with the World Health Organization (WHO) declaring its pandemic status in March 2020 (WHO, 2020). The reference standard for diagnosis of SARS-CoV-2 are reverse transcription – quantitative polymerase chain reaction (RT-qPCR) assays. However, although a successful RT-qPCR amplification confirms an infection, the peak viral load is short lived and often occurs prior to or in the initial days after symptom onset and therefore the timing of the test is crucial [187,188].

Patients tested after several days of illness may already have decreasing viral loads and negative RT-qPCR results and other markers of infection, such as immunoglobulins (Ig) could play an adjunct role in diagnosis, particularly in cases presenting ≥10 days from onset of symptoms [189]. SARS-CoV-2 IgG and IgM can be detected in over 90% and 95% of hospitalised patients with confirmed infections respectively, with Ig presenting with simultaneous or sequential conversion [190,191]. Immunoglobulins are usually well established after 21 days of infection, but levels can be detectable at earlier timepoints [192,193].

Global mobilisation in response to the pandemic resulted in the rapid development of lateral flow immunoassays (LFAs) for SARS-CoV-2. These assays can detect IgG, IgM, and occasionally IgA antibodies, are relatively simple to use and generate results in 10-15 minutes, making them appropriate for the point of care. LFAs identify individuals who have formerly experienced infections (with or without symptoms), to document the prevalence of infection in the population. Moreover, LFAs could also complement the information generated by RT-qPCR for the diagnosis of patients with presumptive COVID-19, detecting an early rise of antibodies at the time that viral loads have become undetectable.

Most LFAs are evaluated in-house by the manufacturer. However, the WHO recommends these evaluations should be complemented by independent evaluations of diagnostic accuracy. Here, we report an evaluation of ten SARS-COV-2 LFAs in a cohort of patients with RT-qPCR confirmed SARS-CoV-2 infections; in hospitalised patients with other conditions, and in healthy individuals.

Materials and Methods

Ethics statement

The study was based on samples collected for the International Severe Acute Respiratory and Emerging Infection Consortium - Coronavirus Clinical Characterisation Consortium (ISARIC4C) study. ISARIC4C is a prospective study based in 309 hospitals in England, Scotland, and Wales. The protocols, case report forms and details of its Independent Data and Material Access Committee are available online. Ethical approval for ISARIC4C was given by the South Central - Oxford C Research Ethics Committee in England (Ref 13/SC/0149), the Scotland A Research Ethics Committee (Ref 20/SS/0028), and the WHO Ethics Review Committee (RPC571 and RPC572, 25th April 2013). The study protocol is available at http://isaric4c.net/protocols; study registry https://www.isrctn.com/ISRCTN66726260 and at the ISARIC4C website (https://isaric4c.net/).

Participants

Forty-five participants with RT-qPCR-confirmed SARS-CoV-2 infections admitted to National Health Service (NHS) hospitals in the UK, were recruited through the ISARIC4C study. RT-qPCRs were run by UK National Health Service (NHS) accredited laboratories. Patient sera were collected ≥4 days post-symptom onset and one patient provided samples at two time points. Serum samples from a further 50 patients with influenza A (N=20), tuberculosis (N=5), documented previous flavivirus exposure through vaccination or infection (N=21) or healthy sera (N=4) that had been collected pre-pandemic were included as controls.

IgG enzyme-linked immunosorbent assay (ELISA)

All samples were screened by ELISA for the detection of anti-SARS-CoV-2 IgG (Omega Diagnostics, Alva, UK), as per the manufacturer's instructions.

LFAs evaluated

All LFAs were evaluated using patient sera and compared to ELISA results. The LFAs included IgG/IgM RDT 1 (ASBT Holdings Ltd, Norfolk, UK), IgG/IgM RDT 2 (ASBT Holdings Ltd, Norfolk, UK), COVID-19 Total Ab Device (Fortress Diagnostics, Antrim, UK), Rapid COVID-19 IgG/IgM Antibody Test (Suzhou Herui BioMed Co., Ltd, China), NADAL COVID-19 IgG/IgM rapid test (Nal von Minden, Moers, Germany), qSARS-CoV-2 IgG/IgM Rapid Test (Cellex, Durham, NC, USA), COVID-19 IgG/IgM Rapid Test (Nantong Egens Biotechnology, Nantong, China), NG-Test IgG-IgM COVID-19 (NG Biotech, Guipry, France), Generation one RDT prototype (Mologic, Bedfordshire, UK), and Triple Antibody RDT (Mologic, Bedfordshire, UK). All tests detect anti-SARS-CoV-2 IgG and IgM, except the Mologic LFAs which additionally detect anti-SARS-CoV-2 IgA. Serum samples for the evaluation had been stored at -80°C and

were thawed to room temperature before use. LFAs were run at room temperature according to the manufacturer's instructions, as summarised in Table 1. In brief, 5-10µl serum were added to the LFA well, followed by 70-100µl of the proprietary test diluent in the same or a separate buffer well. Test lines were scored as positive or negative by two independent readers at the specified reading time. The readers were blinded to each other's results and discrepant scores were resolved by a third reader.

Statistical analysis

Sensitivity was calculated as the proportion of positive tests in convalescent sera of patients with RT-qPCR confirmed infections. Specificity was calculated as the proportion of negative tests among SARS-CoV-2 negative controls collected pre-pandemic. Further calculations of sensitivity and specificity were made when using the IgG ELISA as the reference standard. The levels of agreement were calculated using Cohen's Kappa statistic [194].

Results

The 45 participants with confirmed SARS-CoV-2 infections had a median age of 58 years (IQR: 19) and 25 (56%) were male. Sensitivity of the LFAs ranged from 87% to 96%, with IgG ranging from 83% to 94% and IgM between 24% and 96%. Specificity ranged from 92% to 100% for both IgG and IgM (Table 2). Differences in sensitivity and specificity across the tests were not statistically different. Sensitivity of the LFAs up to 3 weeks post-symptom onset ranged from 85% to 91% and from 83% to 100% after 3 weeks post-symptom onset. All tests except Generation one RDT prototype (Mologic, Bedfordshire, UK) recorded higher sensitivity over 3 weeks post-symptom onset (Table S1).

Forty-two (91%) of the 45 participants with qPCR confirmed SARS-CoV-2 infections were IgG ELISA positive and 44 (88%) of the 50 controls were IgG ELISA negative. Using ELISA as the reference, LFA IgG sensitivity ranged from 91% [95% CI: 77-97%] to 100% [95% CI: 92-100%] and specificity from 93% [95% CI: 81-99%] to 100% [95% CI: 92-100%] (Table S2). Differences across the tests were not statistically different. The percentage agreement between LFAs and ELISA ranged from 90% to 93%, as shown in Table 3. The greatest agreement between ELISA and a LFA was seen with the IgG/IgM RDT 1 (ASBT Holdings Ltd), COVID-19 Total Ab Device (Fortress Diagnostics) and Rapid COVID-19 IgG/IgM Antibody Test (Suzhou Herui BioMed Co., Ltd) (kappa=0.854 for all three, corresponding to very good agreement).

Discussion

LFAs are potential tools for disease surveillance and the assessment of presence of antibodies to infection, which are rapid and easily conducted. In this study, we evaluated ten LFAs using sera from RT-qPCR confirmed SARS-CoV-2 infections and sera collected pre-pandemic. Although differences between tests were not statistically significant, the Fortress Total Ab Device had the highest overall sensitivity when compared to RT-qPCR. Across all tests, IgM had the widest sensitivity range (from 26% to 96%) while IgG LFAs had similar ranges between 83% and 94%. Specificity was high across all assays and four out of the ten LFAs had specificity ≥ 98%. The IgG ELISA's sensitivity and specificity (Omega, UK) are 95% (≥7 days) and 97% (≥10 days) post-diagnosis, respectively [195] and thus, as expected, the sensitivity and specificity of the LFAs was higher when ELISA was considered the reference standard. All LFAs had very high agreement with IgG ELISA. These data suggest LFAs can provide valuable data that is highly correlated to ELISAs, with an acknowledged small loss in sensitivity. LFAs have the advantage of being rapid and simple to run with no requirement for a laboratory or trained operators. This makes them highly suitable for low resource settings, self-testing, surveillance of the prevalence of infection or when rapid screening is required.

LFAs should have high specificity, especially in settings where infection rates are low, to avoid high numbers of false positives [196]. COVID-19 clinical presentation may be indistinguishable from other respiratory illnesses and LFAs could complement the information generated by RT-qPCR assays, with the tests combined identifying a larger number of individuals with current and previous SARS-CoV-2 infections. Moreover, with seasonal influenza likely coinciding with high COVID-19 incidence, these assays could play a significant role to differentiate SARS-CoV-2 from other viral infections and facilitate more targeted strategies for the management and quarantining of symptomatic patients.

We acknowledge this validation has several limitations, as it included a small sample size, without enough power to find small differences in the performance of the assays, and therefore our findings can only be shown to be indicative of the likely findings of appropriately powered evaluations. All SARS-CoV-2 positive samples were obtained from hospitalised patients as a marker of severe COVID-19 who may be expected to have high levels of antibodies, and our findings need to be replicated in asymptomatic individuals and in patients presenting with mild symptoms and in community settings. Furthermore, all testing was run under laboratory conditions by laboratory staff and the results may vary when conducted at the point of need and self-testing by untrained individuals. Finally, we were constrained to use serum, and further evaluations are needed to evaluate their performance on whole venous or

capillary blood. Test performance on capillary blood, including a comparison of self-testing and laboratory testing, would inform the potential for use of less invasive sample collection methods.

Validation of test performance in people with presumptive SARS-COV-2 infection who are asymptomatic or are experiencing mild infection is of particular interest as reports indicate a large proportion of individuals testing positive by RT-PCR have no symptoms on the day of sampling, a week prior and a week after a positive result [197]. LFAs ease of use and their fast time to results lends them to self-testing outside a clinical or laboratory environment. However, validation of test performance under these more challenging and less controlled environments is needed. Further evaluations of LFAs should include other coronaviruses and respiratory illnesses with overlapping signs and symptoms of COVID-19, including other causes of pneumonia, upper and lower respiratory infection, rhinoviruses, respiratory syncytial virus and influenza.

The impartial evaluation of LFAs, as recommended by the WHO, can confirm the evaluations of the developer's validation data, and inform best-use settings. LFAs are a valuable tool which could be applied at the point of need in conjunction with other assays to provide a more holistic diagnosis and to monitor the prevalence of infection.

Acknowledgements

ISARIC4C Investigators

Consortium Lead Investigator: J Kenneth Baillie, Chief Investigator: Malcolm G Semple, Co-Lead Investigator: Peter JM Openshaw. ISARIC Clinical Coordinator: Gail Carson. Co-Investigators: Beatrice Alex, Benjamin Bach, Wendy S Barclay, Debby Bogaert, Meera Chand, Graham S Cooke, Annemarie B Docherty, Jake Dunning, Ana da Silva Filipe, Tom Fletcher, Christopher A Green, Ewen M Harrison, Julian A Hiscox, Antonia Ying Wai Ho, Peter W Horby, Samreen Ijaz, Saye Khoo, Paul Klenerman, Andrew Law, Wei Shen Lim, Alexander J Mentzer, Laura Merson, Alison M Meynert, Mahdad Noursadeghi, Shona C Moore, Massimo Palmarini, William A Paxton, Georgios Pollakis, Nicholas Price, Andrew Rambaut, David L Robertson, Clark D Russell, Vanessa Sancho-Shimizu, Janet T Scott, Thushan de Silva, Louise Sigfrid, Tom Solomon, Shiranee Sriskandan, David Stuart, Charlotte Summers, Richard S Tedder, Emma C Thomson, AA Roger Thompson, Ryan S Thwaites, Lance CW Turtle, Maria Zambon. Project Managers: Hayley Hardwick, Chloe Donohue, Ruth Lyons, Fiona Griffiths, Wilna Oosthuyzen. Data Analysts: Lisa Norman, Riinu Pius, Tom M Drake, Cameron J Fairfield, Stephen Knight, Kenneth A Mclean, Derek Murphy, Catherine A Shaw. Data and Information System Managers: Jo Dalton, James Lee, Daniel

Plotkin, Michelle Girvan, Egle Saviciute, Stephanie Roberts, Janet Harrison, Laura Marsh, Marie Connor, Sophie Halpin, Clare Jackson, Carrol Gamble. Data integration and presentation: Gary Leeming, Andrew Law, Murray Wham, Sara Clohisey, Ross Hendry, James Scott-Brown. Material Management: William Greenhalf, Victoria Shaw, Sarah McDonald. Patient engagement: Seán Keating Outbreak Laboratory Staff and Volunteers: Katie A. Ahmed, Jane A Armstrong, Milton Ashworth, Innocent G Asiimwe, Siddharth Bakshi, Samantha L Barlow, Laura Booth, Benjamin Brennan, Katie Bullock, Benjamin WA Catterall, Jordan J Clark, Emily A Clarke, Sarah Cole, Louise Cooper, Helen Cox, Christopher Davis, Oslem Dincarslan, Chris Dunn, Philip Dyer, Angela Elliott, Anthony Evans, Lorna Finch, Lewis WS Fisher, Terry Foster, Isabel Garcia-Dorival, Willliam Greenhalf, Philip Gunning, Catherine Hartley, Antonia Ho, Rebecca L Jensen, Christopher B Jones, Trevor R Jones, Shadia Khandaker, Katharine King, Robyn T. Kiy, Chrysa Koukorava, Annette Lake, Suzannah Lant, Diane Latawiec, L Lavelle-Langham, Daniella Lefteri, Lauren Lett, Lucia A Livoti, Maria Mancini, Sarah McDonald, Laurence McEvoy, John McLauchlan, Soeren Metelmann, Nahida S Miah, Joanna Middleton, Joyce Mitchell, Shona C Moore, Ellen G Murphy, Rebekah Penrice-Randal, Jack Pilgrim, Tessa Prince, Will Reynolds, P. Matthew Ridley, Debby Sales, Victoria E Shaw, Rebecca K Shears, Benjamin Small, Krishanthi S Subramaniam, Agnieska Szemiel, Aislynn Taggart, Jolanta Tanianis-Hughes, Jordan Thomas, Erwan Trochu, Libby van Tonder, Eve Wilcock, J. Eunice Zhang. Local Principal Investigators: Kayode Adeniji, Daniel Agranoff, Ken Agwuh, Dhiraj Ail, Ana Alegria, Brian Angus, Abdul Ashish, Dougal Atkinson, Shahedal Bari, Gavin Barlow, Stella Barnass, Nicholas Barrett, Christopher Bassford, David Baxter, Michael Beadsworth, Jolanta Bernatoniene, John Berridge, Nicola Best, Pieter Bothma, David Brealey, Robin Brittain-Long, Naomi Bulteel, Tom Burden, Andrew Burtenshaw, Vikki Caruth, David Chadwick, Duncan Chambler, Nigel Chee, Jenny Child, Srikanth Chukkambotla, Tom Clark, Paul Collini, Catherine Cosgrove, Jason Cupitt, Maria-Teresa Cutino-Moguel, Paul Dark, Chris Dawson, Samir Dervisevic, Phil Donnison, Sam Douthwaite, Ingrid DuRand, Ahilanadan Dushianthan, Tristan Dyer, Cariad Evans, Chi Eziefula, Chrisopher Fegan, Adam Finn, Duncan Fullerton, Sanjeev Garg, Sanjeev Garg, Atul Garg, Effrossyni Gkrania-Klotsas, Jo Godden, Arthur Goldsmith, Clive Graham, Elaine Hardy, Stuart Hartshorn, Daniel Harvey, Peter Havalda, Daniel B Hawcutt, Maria Hobrok, Luke Hodgson, Anil Hormis, Michael Jacobs, Susan Jain, Paul Jennings, Agilan Kaliappan, Vidya Kasipandian, Stephen Kegg, Michael Kelsey, Jason Kendall, Caroline Kerrison, Ian Kerslake, Oliver Koch, Gouri Koduri, George Koshy, Shondipon Laha, Steven Laird, Susan Larkin, Tamas Leiner, Patrick Lillie, James Limb, Vanessa Linnett, Jeff Little, Michael MacMahon, Emily MacNaughton, Ravish Mankregod, Huw Masson, Elijah Matovu, Katherine McCullough, Ruth McEwen, Manjula Meda, Gary Mills, Jane Minton, Mariyam Mirfenderesky, Kavya Mohandas, Quen Mok, James Moon, Elinoor Moore, Patrick

Morgan, Craig Morris, Katherine Mortimore, Samuel Moses, Mbiye Mpenge, Rohinton Mulla, Michael Murphy, Megan Nagel, Thapas Nagarajan, Mark Nelson, Igor Otahal, Mark Pais, Selva Panchatsharam, Hassan Paraiso, Brij Patel, Natalie Pattison, Justin Pepperell, Mark Peters, Mandeep Phull, Stefania Pintus, Jagtur Singh Pooni, Frank Post, David Price, Rachel Prout, Nikolas Rae, Henrik Reschreiter, Tim Reynolds, Neil Richardson, Mark Roberts, Devender Roberts, Alistair Rose, Guy Rousseau, Brendan Ryan, Taranprit Saluja, Aarti Shah, Prad Shanmuga, Anil Sharma, Anna Shawcross, Jeremy Sizer, Manu Shankar-Hari, Richard Smith, Catherine Snelson, Nick Spittle, Nikki Staines, Tom Stambach, Richard Stewart, Pradeep Subudhi, Tamas Szakmany, Kate Tatham, Jo Thomas, Chris Thompson, Robert Thompson, Ascanio Tridente, Darell Tupper-Carey, Mary Twagira, Andrew Ustianowski, Nick Vallotton, Lisa Vincent-Smith, Shico Visuvanathan, Alan Vuylsteke, Sam Waddy, Rachel Wake, Andrew Walden, Ingeborg Welters, Tony Whitehouse, Paul Whittaker, Ashley Whittington, Meme Wijesinghe, Martin Williams, Lawrence Wilson, Sarah Wilson, Stephen Winchester, Martin Wiselka, Adam Wolverson, Daniel G Wooton, Andrew Workman, Bryan Yates, and Peter Young.

This work uses data provided by patients and collected by the NHS as part of their care and support #DataSavesLives. We are extremely grateful to the 2,648 frontline NHS clinical and research staff and volunteer medical students, who collected this data in challenging circumstances; and the generosity of the participants and their families for their individual contributions in these difficult times.

Sources of funding

This work is supported by grants from: a DFID/Wellcome Trust Epidemic Preparedness coronavirus grant (220764/Z/20/Z) to ERA and LEC, the National Institute for Health Research (NIHR; award CO-CIN-01), the Medical Research Council (MRC; grant MC_PC_19059), and by the NIHR Health Protection Research Unit (HPRU) in Emerging and Zoonotic Infections at University of Liverpool in partnership with Public Health England (PHE), in collaboration with Liverpool School of Tropical Medicine and the University of Oxford (award 200907), NIHR HPRU in Respiratory Infections at Imperial College London with PHE (award 200927), Wellcome Trust and Department for International Development (DID; 215091/Z/18/Z), the Bill and Melinda Gates Foundation (OPP1209135), Liverpool Experimental Cancer Medicine Centre (grant reference C18616/A25153), NIHR Biomedical Research Centre at Imperial College London (IS-BRC-1215-20013), and NIHR Clinical Research Network for providing infrastructure support for this research. The views expressed are those of the authors and not necessarily those of the Department of Health and Social Care, DID, NIHR, MRC, Wellcome Trust, or PHE.

References

1. World Health Organisation. 2020. Coronavirus Disease 2019 (COVID-19) Situation Report - 51.

2. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, Lau YC, Wong JY, Guan Y, Tan X, Mo X, Chen Y, Liao B, Chen W, Hu F, Zhang Q, Zhong M, Wu Y, Zhao L, Zhang F, Cowling BJ, Li F, Leung GM. 2020. Temporal dynamics in viral shedding and transmissibility of COVID-19. Nat Med 26:672–675.

3. Liu Y, Yan L-M, Wan L, Xiang T-X, Le A, Liu J-M, Peiris M, M Poon LL, Zhang W. 2020. Viral dynamics in mild and severe cases of COVID-19. Lancet Infect Dis 20:656–657.

4. FIND. 2020. GENERAL INFORMATION ON Ag-AND Ab-DETECTION RDTs FOR COVID-19.

5. Long Q-X, Liu B-Z, Deng H-J, Wu G-C, Deng K, Chen Y-K, Liao P, Qiu J-F, Lin Y, Cai X-F, Wang D-Q, Hu Y, Ren J-H, Tang N, Xu Y-Y, Yu L-H, Mo Z, Gong F, Zhang X-L, Tian W-G, Hu L, Zhang X-X, Xiang J-L, Du H-X, Liu H-W, Lang C-H, Luo X-H, Wu S-B, Cui X-P, Zhou Z, Zhu M-M, Wang J, Xue C-J, Li X-F, Wang L, Li Z-J, Wang K, Niu C-C, Yang Q-J, Tang X-J, Zhang Y, Liu X-M, Li J-J, Zhang D-C, Zhang F, Liu P, Yuan J, Li Q, Hu J-L, Chen J, Huang A-L. 2020. Antibody responses to SARS-CoV-2 in patients with COVID-19. Nat Med 1–4.

6. Staines HM, Kirwan DE, Clark DJ, Adams ER, Augustin Y, Byrne RL, Cocozza M, Cubas-Atienza AI, Cuevas LE, Cusinato M, Davies BMO, Davis M, Davis P, Duvoix A, Eckersley NM, Forton D, Fraser A, Garrod G, Hadcocks L, Hu Q, Johnson M, Kay GA, Klekotko K, Lewis Z, Mensah-Kane J, Menzies S, Monahan I, Moore C, Nebe-von-Caron G, Owen SI, Sainter C, Sall AA, Schouten J, Williams C, Wilkins J, Woolston K, Fitchett JRA, Krishna S, Planche T. 2020. Dynamics of IgG seroconversion and pathophysiology of COVID-19 infections. medRxiv 2020.06.07.20124636.

7. Ma H, Zeng W, He H, Zhao D, Jiang D, Zhou P, Cheng L, Li Y, Ma X, Jin T. 2020. Serum IgA, IgM, and IgG responses in COVID-19. Cell Mol Immunol 17:773–775.

8. Padoan A, Sciacovelli L, Basso D, Negrini D, Zuin S, Cosma C, Faggian D, Matricardi P, Plebani M. 2020. IgA-Ab response to spike glycoprotein of SARS-CoV-2 in patients with COVID-19: A longitudinal study. Clin Chim Acta 507:164–166.

9. Landis JR, Koch GG. 1977. The Measurement of Observer Agreement for Categorical Data. Biometrics 33:159.

10. Adams ER, Augustin Y, Byrne RL, Clark DJ, Cocozza M, Cubas-Atienzar AI, Cuevas LE, Cusinato M, Davies BMO, Davies M, Davies P, Duvoix A, Eckersley NM, Edwards T, Fletcher T, Fraser A j, Garrod G,

Hadcocks L, Hu Qi, johnson M, Kay GA, Keymer K, Kirwan D, Klekotko K, Lewis Z, Mason J, Mensah-Kane J, Menzies S, Monahan I, Moore CM, Nebe-von-Caron G, Owen SI, Planche T, Sainter C, Schouten J, Staines HM, Turtle L, Williams C, Wilkins J, Woolston K, Sall AA, Fitchett JRA, Krishna S. 2020. Rapid development of COVID-19 rapid diagnostics for low resource settings: accelerating delivery through transparency, responsiveness, and open collaboration. medRxiv 2020.04.29.20082099.

11. Frasier SL. 2020. False Positive Alarm. Sci Am 323:12–13.

12. Pouwels KB, House T, Robotham J V, Birrell PJ, Gelman A, Bowers N, Boreham I, Thomas H, Lewis J, Bell I, Bell JI, Newton JN, Farrar J, Diamond I, Benton P, Walker AS. 2020. Community prevalence of SARS-CoV-2 in England: Results from the ONS Coronavirus Infection Survey Pilot. medRxiv 1–13.

Table 1. Sample and condition requirements for the ten LFAs evaluated.

Test	Sample volume serum (µl)	Buffer volume	Time to read (mins)
IgG/IgM RDT 1 (ASBT Holdings Ltd)	10	80µl	10
IgG/IgM RDT 2 (ASBT Holdings Ltd)	10	100µl	10
COVID-19 Total Ab Device (Fortress Diagnostics)	5	2 drops	10
Rapid COVID-19 IgG/IgM Antibody Test (Suzhou Herui BioMed Co., Ltd, China)	10	70µl	10
NADAL® COVID-19 IgG/IgM rapid test (Nal von Minden)	10	2 drops	10
qSARS-CoV-2 IgG/IgM Rapid Test (Cellex)	10	2 drops	15-20
Covid-19 IgG/IgM Rapid Test (Nantong Egens Biotechnology)	10	2 drops	10
NG-Test® IgG-IgM COVID-19 (NG Biotech)	10	2 drops	15
Generation one RDT prototype (Mologic)	5	80µl	10
Triple Antibody RDT (Mologic)	5	80ul	10

Table 2. Sensitivity and specificity of the LFAs. Sera from RT-qPCR-positive patients and a control panel of influenza A (N=20), TB (N=5), previous flavivirus exposure through vaccination or infection (N=21), and healthy sera (N=4), collected pre-pandemic.

	Ig	RT-qPCR SARS-Cov-2 positive (N = 46)	Negative controls (N=50)	Sensitivity (%) [95% CI]	Specificity (%) [95% CI]
IgG/IgM RDT 1 (ASBT Holdings Ltd)	**G + M**	43	50	94 [82- 99]	100 [93-100]
	G	43	50	94 [82-99]	100 [93-100]
	M	17	50	37 [23- 53]	100 [93-100]
IgG/IgM RDT 2 (ASBT Holdings Ltd)	**G + M**	43	47	94 [82-99]	94 [84-99]
	G	41	50	89 [76- 96]	100 [93-100]
	M	43	47	94 [82-99]	94 [84-99]
COVID-19 Total Ab Device (Fortress Diagnostics)	**G + M**	44	48	96 [85-100]	96 [86-100]
	G	43	50	94 [82-99]	100 [93-100]
	M	44	48	96 [85-100]	96 [86-100]
Rapid COVID-19 IgG/IgM Antibody Test (Suzhou Herui BioMed Co., Ltd, China)	**G + M**	42	49	91 [79-98]	98 [89-100]
	G	42	49	91 [79- 98]	98 [89-100]
	M	12	50	26 [14- 41]	100 [93-100]
NADAL® COVID-19 IgG/IgM rapid test (Nal von Minden)	**G + M**	43	48	94 [82-99]	96 [86-100]
	G	40	50	87 [74-95]	100 [93-100]
	M	43	48	94 [82-99]	96 [86-100]
qSARS-CoV-2 IgG/IgM Rapid Test (Cellex)	**G + M**	43	49	94 [82-99]	98 [89-100]
	G	43	49	94 [82-99]	98 [89-100]

	M	11	50	24 [13-39]	100 [93- 100]
Covid-19 IgG/IgM Rapid Test (Nantong Egens Biotechnology)	**G + M**	43	46	94 [82-99]	92 [81-98]
	G	43	46	94 [82- 99]	92 [81-98]
	M	41	46	89 [76- 96]	92 [81-98]
NG-Test® IgG-IgM COVID-19 (NG Biotech)	**G + M**	41	47	89 [76-96]	94 [84-99]
	G	41	50	89 [76- 96]	100 [93-100]
	M	41	47	89 [76-96]	94 [84- 99]
Generation one RDT prototype (Mologic)	**A + G + M**	40		87 [74-95]	100 [93-100]
	A	16	50	35 [21- 50]	100 [93-100]
	G	24	50	83 [69 -92]	100 [93-100]
	M	38	50	52 [37- 67]	100 [93-100]
Triple Antibody RDT (Mologic)	**A + G + M**	42	48	94 [82-99]	96 [86-100]
	A	35	50	78 [63-89]	100 [93-100]
	G	28	48	93 [82-99]	96 [86-100]
	M	42	48	62 [47-76]	96 [86-100]

5246

Table 3. Agreement and Cohen's kappa of LFAs and ELISA IgG.

	LFA	ELISA		Agreement (%) and Kappa [95% CI]
		Positive	Negative	
IgG/IgM RDT 1 (ASBT Holdings Ltd)	Positive	42	1	93% and 0.854 [0.751 to 0.958]
	Negative	6	47	
IgG/IgM RDT 2 (ASBT Holdings Ltd)	Positive	41	0	93% and 0.854 [0.751 to 0.958]
	Negative	7	48	
COVID-19 Total Ab Device (Fortress Diagnostics)	Positive	39	1	90% and 0.792 [0.671 to 0.912]
	Negative	9	47	
Rapid COVID-19 IgG/IgM Antibody Test (Suzhou Herui BioMed Co., Ltd, China)	Positive	42	1	93% and 0.854 [0.751 to 0.958]
	Negative	6	47	
NADAL® COVID-19 IgG/IgM rapid test (Nal von Minden)	Positive	39	1	90% and 0.792 [0.671 to 0.912]
	Negative	9	47	
qSARS-CoV-2 IgG/IgM Rapid Test (Cellex)	Positive	42	2	92% and 0.833 [0.723 to 0.944]
	Negative	6	46	

Covid-19 IgG/IgM Rapid Test (Nantong Egens Biotechnology)	Positive	42	4	80% and 0.792 [0.670 to 0.914]
	Negative	6	44	
NG-Test® IgG-IgM COVID-19 (NG Biotech)	Positive	41	1	91% and 0.813 [0.698 to 0.928]
	Negative	8	46	
Generation one RDT prototype (Mologic)	Positive	38	0	90% and 0.792 [0.672 to 0.911]
	Negative	10	48	
Triple Antibody RDT (Mologic)	Positive	42	2	92% and 0.832 [0.721 to 0.943]
	Negative	6	45	

Twelve lateral flow immunoassays (LFAs) to detect SARS-CoV-2 antibodies

Sophie I. Owen[1*], Christopher T. Williams[1*], Gala Garrod1, Alice J. Fraser 1, Stefanie Menzies1, Lisa Baldwin1, Lottie Brown1, Rachel L. Byrne1, Andrea M. Collins2,4, Ana I. Cubas-Atienzar1, Margaretha de Vos3, Thomas Edwards1, Camille Escadafal3, Daniela M. Ferreira4, Tom Fletcher2, Angela Hyder-Wright2,4, Grant A. Kay1, Konstantina Kontogianni1, Jenifer Mason5, Elena Mitsi 4, Tim Planche6,7, Jilian A. Sacks3, Joseph Taylor5, Stacy Todd2, Caroline Tully3, Luis E. Cuevas1, Emily R. Adams1

[1]Centre for Drugs and Diagnostics Research, Liverpool School of Tropical Medicine (LSTM), Liverpool, UK

[2]Liverpool University Hospitals NHS Foundation Trust, Liverpool, UK

[3]FIND, Geneva, Switzerland

[4]Department of Clinical Sciences, Liverpool School of Tropical Medicine (LSTM), Liverpool, UK

[5]Liverpool Clinical Laboratories, Liverpool University Hospitals NHS Foundation Trust, Liverpool, UK

[6]Institute for Infection & Immunity, St George's University of London, London, UK

[7]St George's University Hospitals NHS Foundation Trust, London, UK

*Sophie I. Owen and Christopher T. Williams contributed equally to this work.

Running title: Twelve lateral flow immunoassays (LFAs) for SARS-CoV-2

Owen S.I. & Williams C.T. *et al.* Journal of Infection (2021) 6:18

*** Sophie I. Owen and Christopher T. Williams contributed equally to this work. Author order was determined alphabetically.**

Sources of funding: This work is supported by grants from DFID/Wellcome Trust Epidemic Preparedness coronavirus grant (220764/Z/20/Z) to ERA and LEC, the National Institute for Health Research (NIHR; award CO-CIN-01), the NIHR Health Protection Research Unit (HPRU) in Emerging and Zoonotic Infections at University of Liverpool in partnership with Public Health England (PHE), in collaboration with Liverpool School of Tropical Medicine and the University of Oxford (award 200907), and Pfizer (grant WI255862) to DMF, AHW, EM, and AMC. This work was also funded as part of FIND's work as co-convener of the diagnostics pillar of the Access to COVID-19 Tools (ACT) Accelerator, including support from Unitaid [grant number: 2019-32-FIND MDR], from UK Department for International Development [grant number 300341-102], and the World Health Organization.

Corresponding author: Emily R. Adams, Liverpool School of Tropical Medicine, E-mail: Emily.Adams@lstmed.ac.uk

Authors' contributions: The study design was developed by SIO, GG, AF and ERA. Clinical enrolment was coordinated by AMC, AHW, LCWT, ST and TF. Data extraction was conducted by SIO, GG, AF, SM, LB, RLB, GAK, SCM, KK, TE, AICA, and CTW. Data analysis and interpretation were conducted by SIO and CTW. The initial manuscript was prepared by SIO and CTW. Acquisition of funding by ERA, AMC, LEC, DMF, EM, and AHW. All authors edited and approved the final manuscript.

Abstract

Background: There are an abundance of commercially available lateral flow assays (LFAs) that detect antibodies to SARS-CoV-2, the aetiological agent for COVID-19. Whilst these are usually evaluated by the manufacturer, externally performed diagnostic accuracy studies to assess their performance are essential. Herein we present an evaluation of 12 LFAs manufactured by; Beijing Wantai, Bionote Co, Core Technology, CTK Biotech, Edinburgh Genetics Ltd, GenBody Inc, Jiangsu Bioperfectus Technologies, PRIME4DIA Co. Ltd., Qingdao Hightop Biotech Co Ltd., Shanghai Kehua Bio-Engineering Co. Ltd., Shenzhen Bioeasy Biotechnology Co. Ltd. and Zhuhai Livzon Diagnostics Inc.

Methods: Sera from 100 SARS-CoV-2 reverse-transcriptase polymerase chain reaction (RT-PCR) positive participants were recruited through the FASTER study. A total of 105 pre-pandemic serum samples (20 influenza A, 10 HIV, 10 TB, 10 TB-HIV, 10 dengue virus, 10 human coronavirus 229E, 2 human coronavirus OC43, 12 parasitic disease, 20 non-COVID related fever, and 1 quality assurance sample) were included as negative samples.

Results: Sensitivity against RT-PCR ranged from 37.4-79% for IgM/IgG, 30.3-74% for IgG alone, and from 21.2-67% for IgM alone. Sensitivity improved over 21 days post symptom onset for 10/12 tests by a mean of 15% for IgG/IgM, and by a mean of 16.5% for IgG. For IgM sensitivity was higher before 21 days post symptom onset for 6 tests, and after 21 days post symptom onset for the other 6 tests. Specificity ranged from 74.3-99.1% for IgM/IgG, 82.9-100% for IgG alone, and 75.2-98% for IgM alone. Compared to the EuroImmun IgG enzyme-linked immunosorbent assay (ELISA), sensitivity ranged from 44.6-95.4% and specificity ranged from 85.4-100%.

Conclusion: There are many LFAs currently available, with varied sensitivity and specificity. Understanding the diagnostic accuracy of these tests will be vital as we come to rely more on the antibody status of a person moving forward, and as such manufacturer-independent evaluations are crucial.

Introduction

As of May 2021, there have been over 100,000,000 confirmed cases of COVID-19 worldwide, however the total number of cases is much higher (1). This is due to, amongst other reasons, the lack of diagnostic testing worldwide in the first wave of the pandemic, the continued difficulties in testing in some lower-middle income countries and the number of asymptomatic infections that continue to go undetected (2,3). Detecting antibody responses to SARS-CoV-2 therefore could prove vital, both for understanding previous exposure on an individual level, but also at community and regional levels.

During SARS-CoV-2 infection, IgM and IgG titres begin to increase from around 10 days post-symptom onset with IgM titres generally declining earlier than IgG (4,5). The reference standard for detecting an antibody response, either IgM, IgG, or both, to SARS-CoV-2 is the enzyme-linked immunosorbent assay (ELISA). Many ELISAs have been developed and commercialised for SARS-CoV-2 which are highly sensitive and specific (6). The process however is time-consuming, expensive and requires specialist laboratory equipment and trained professionals to perform.

To this end, large numbers of lateral-flow immunoassays (LFAs) have been developed that detect IgM and IgG responses. These are simple tests which require only a drop of blood and the addition of buffer to give a result in 10-15 minutes. Results are easy to interpret; if a test line appears the participant is considered positive, if no test line appears the participant is considered negative. LFAs are easy to mass-produce and are affordable making them ideal for mass-testing of populations, rapid identification of antibody response in travel situations, or for home-testing. Those that detect both IgM and IgG are also able to give an indication of when that person was likely infected, due to the dynamics of the antibody response.

Many LFAs are commercially available, and externally performed diagnostic accuracy studies, independent of the manufacturer, are required to generate robust performance data. Here, we present the evaluation of twelve LFAs and describe their diagnostic accuracy in a cohort of 100 confirmed SARS-CoV-2 positive participants with varying disease severity and 105 samples from participants confirmed as negative or collected pre-pandemic. This study forms part of a larger initiative to generate and share independent performance data on COVID-19 tests coordinated by FIND, the global diagnostics alliance.

Methods

Ethics statement

Research samples were provided with informed written consent. Participants were recruited through the Facilitating A SARS CoV-2 TEst for Rapid triage (FASTER) study, approved by the National Health Service Research Ethics Committee (20/SC/0169) under the Integrated Research Application System no. 282147 and the Flavimmune study (16/NW/0160).

Participants

A total of 142 serum/plasma samples from 125 SARS-CoV-2 RT-qPCR -positive participants were used in this study to assess the sensitivity of the LFAs. One hundred serum/plasma samples from RT-qPCR -positive participants were used for each LFA due to the limited quantity of some serum samples. Full details are given in Table S1. Briefly, 24 patients presenting at the Liverpool University Hospitals NHS Foundation Trust (Liverpool, UK) were recruited as part of the FASTER study and provided 41 serum samples collectively at different timepoints (D0, D2, D7, D28 post-admission). Sera from patients with RT-qPCR confirmed SARS-CoV-2 infection (n=84) were provided by Liverpool Clinical Laboratories (LCL) as leftover diagnostic samples. Participants with RT-qPCR confirmed SARS-CoV-2 infection who did not result in hospital attendance (n=12) were also recruited (7). In addition, the NIBSC COVID-19 convalescent plasma panel, human (20/118), as well as NIBSC 20/130 plasma positive control were used for the evaluation. The COVID-19 convalescent plasma panel (NIBSC 20/118) and NIBSC 20/130 were obtained from the National Institute for Biological Standards and Control, UK.

A total of 105 SARS-CoV-2 negative serum samples were used to assess specificity of the LFAs. See Table S2 for full details. These consisted of 84 pre-pandemic sera collected from individuals diagnosed with influenza A (n=20), tuberculosis (TB) (n=10), human immunodeficiency virus (HIV) (n=10), TB/HIV (n=10), dengue virus (n=10), parasitic diseases (n=12), human coronavirus 229E (n=10) and human coronavirus OC43 (n=2). A panel of pre-pandemic plasma from participants with non-COVID-19-related fever (n=20) were provided by FIND and an additional quality assurance sample (n=1).

Enzyme-linked immunosorbent assay (ELISA) to detect IgG

The Anti-SARS-CoV-2 ELISA (IgG) kit (EI 2606-9601 G) (EuroImmun, Germany) was used to screen all serum samples for the presence of anti-SARS-CoV-2 IgG, as per the manufacturer's instructions. Samples

with an OD value greater than the calibrator were considered positive, samples with OD value lower than the calibrator were considered negative.

Lateral flow immunoassays

Twelve LFAs (Table 1) were evaluated according to manufacturer's instructions. Briefly, 10-20 µl serum was required depending on the test, followed by 2-3 drops of buffer. Results were read independently by two people; if there was any disagreement a third person acted as a tiebreaker. Full details are given in Table 1. Of the 12 tests, 11 detected IgM and IgG separately, with only Beijing Wantai giving a 'total antibody' result. All tests were CE-IVD marked.

Data analysis

Sensitivity was calculated against RT-qPCR confirmed SARS-CoV-2 infections including sensitivity when stratified by days post-symptom onset. Specificity was calculated against RT-qPCR confirmed SARS-CoV-2 negative samples or samples collected pre-pandemic. Sensitivity was then calculated against RT-qPCR confirmed SARS-CoV-2 infections also positive by IgG ELISA. Specificity was calculated against IgG ELISA negative samples. Percentage agreement and Cohen's Kappa statistic against IgG ELISA were calculated (8). Data analyses were carried out in MedCalc for Windows, version 19.8 (MedCalc Software, Ostend, Belgium).

Results

Sensitivity and specificity against RT-qPCR

Sensitivity of the LFAs against RT-qPCR ranged from 37.4-79.0% for IgM/IgG, 30.3-74.0% for IgG only, and 21.2-67.0% for IgM only (Table 2, Figure 1). The sensitivity for an IgM/IgG response increased in 10 out of 12 tests at >21 days post-symptom onset, with a mean increase of 15.0% (Table 2, Figure 1). The sensitivity for IgG increased >21 days post-symptom onset, with a mean increase of 16.5% (Table 2, Figure 1). For IgM, sensitivity was higher ≤21 days post-symptom onset in six of the LFAs and higher at >21 days post-symptom onset for five tests (Table 2, Figure 1).

Specificity ranged from 74.3-99.1% for IgM/IgG, 82.9-100% for IgG only, and 75.2-98.0% for IgM only (Table 2, Figure 1). For all tests, except for Beijing Wantai which is a combined IgM/IgG only, and Shenzhen Bioeasy where specificity for IgM/IgG and IgG were identical, IgG alone had a higher specificity

than IgM/IgG (Table 2, Figure 1). Four tests reported 100% specificity for IgG (Table 2). Nine LFAs had a higher IgG specificity than IgM (Table 2, Figure 1).

Diagnostic accuracy of LFAs against IgG ELISA

Of the 142 participants positive by RT-qPCR, 90 (63.4%) were positive by IgG ELISA. Of the 105 pre-pandemic samples, 85 were tested by IgG ELISA due to sample availability, of which 82 (96.5%) were negative by IgG ELISA. Compared to IgG ELISA, LFA sensitivity was found to range between 44.6% and 95.4% (Table 3). Shanghai Kehua had the highest sensitivity at 95.4% (Table 3). Compared to IgG ELISA, LFA specificity was found to range between 85.4% and 100% (Table 3). The highest agreement between IgG measured by LFA and IgG measured by ELISA was seen with Shanghai Kehua (Table 4).

Discussion

There is a plethora of LFAs available on the market today, all purporting to offer high sensitivity and specificity, but often without rigorous, manufacturer-independent evaluations. In this study, we evaluated 12 LFAs on serum samples collected from RT-qPCR -positive individuals and individuals with a wide range of diagnosed diseases pre-pandemic. We demonstrate the differences in sensitivity and specificity of the responses of combined IgM/IgG, IgG and IgM against a RT-qPCR and an IgG ELISA, in patients presenting with both acute and convalescent SARS-CoV-2 infections.

Bionote had the highest overall sensitivity (79.0%), with a sensitivity of 88.2% at >21 days post-symptom onset for an IgM/IgG response. Genbody Inc. had the lowest sensitivity with an overall sensitivity of 37.4% for an IgM/IgG response. Sensitivity of IgM/IgG and IgG improved for the majority of LFAs over 21 days post-symptom onset in agreement with other LFA evaluation studies (9). In this study, no test met the clinical sensitivity requirements of >98% (95% CI: 96-100%) on samples collected ≥20 days post-symptom onset laid out in the target product profile (TPP) published by the UK government (10). However, our data are calculated on fewer than 200 confirmed positive cases as specified by the TPP (10). As expected with an earlier decline in IgM titres, fewer LFAs had improved sensitivity for IgM >21 days post-symptom onset. It is important to note that the samples used in this study were collected before the roll out of any COVID-19 vaccine.

The large variation in performance in LFAs reported here is in accordance with other evaluations (9). The variations in diagnostic accuracy may in part be due to the antigen used to detect SARS-CoV-2 antibodies. The two main immunogenic antigens of SARS-CoV-2 are the nucleocapsid and the surface

spike protein, split into domains S1 and S2, with the receptor-binding domain in S1. S1 is thought to be the most specific, with low-level cross-reactivity demonstrated for S2 and nucleocapsid (11). It is one or a combination of these antigens that are used for serological testing.

Not all manufacturers included in this evaluation disclose the antigen(s) used in their test. This information is key to testing during vaccine-rollout, with two vaccines, Pfizer-BioNTech and Moderna, containing mRNA encoding spike proteins to elicit an anti-spike immune response. LFAs that detect a response to spike antigens should prove useful for detecting both prior exposure to SARS-CoV-2, but also vaccinated individuals. Those that do not detect the relevant spike antigen may not prove as useful in detecting an immune response within vaccinated individuals but may have a role in identifying immune responses to breakout infections in vaccinated populations. Further studies should look at the use of LFAs in vaccinated individuals.

Our pre-pandemic negative panel consisted of serum from individuals diagnosed with a wide range of diseases, and in general, the false-positive results were found not to be linked to one disease. However, one sample from a returning traveller with malaria in 2005 gave a false positive result for 7 out of the 12 LFAs, which warrants further study. Of the 20 Influenza A samples, only 3 false positives were reported across all 12 LFAs. One sample with previous human coronavirus 229E exposure gave a false positive result for 4/12 LFAs as well as the EuroImmun IgG ELISA, and another gave a false positive result in 3/12 LFAs. Seven out of the twelve human coronavirus 229E and OC43 samples reported no false positives for any LFAs, whist 1 sample reported 2 false positives and the remaining 2 samples reported 1 false positive. This suggests cross-reactivity with immune responses to other human coronaviruses is possible but likely to be minimal, additionally pre-pandemic coronavirus patients often presented with other syndromes and coronavirus diagnosis has only been made due to multiplex molecular panels; this indicates samples used here may have unusual properties which may initiate cross-reaction as opposed to the coronaviruses present.

There is still no established ‘gold-standard’ serological test for SARS-CoV-2. The EuroImmun IgG ELISA was chosen at the time as it was one of the only CE-marked ELISA assays. Previous studies have reported false positives with the EuroImmun IgG ELISA, and we report here 3/105; one from a HIV patient in Nigeria in 2018, one from a patient with dengue virus in Brazil in 2015 and one with an individual with human coronavirus 229E in the UK in 2019. These were excluded from the ELISA/LFA specificity analysis. Cross-reactivity was seen in an evaluation of the EuroImmun Anti-SARS-CoV-2 NCP ELISA carried out in

Nigeria in a negative control panel in which 50.2% of participants had the P. falciparum HRP2 antigen (12). The specificity of the EuroImmun ELISA in this study was 96.5%.

LFAs can be helpful in measuring exposure of a community to SARS-CoV-2, particularly in areas where testing of symptomatic individuals was not and is not readily available. Manufacturer-independent evaluations provide helpful data as to the accuracy of LFAs. Further evaluations are needed following the commencement of vaccination campaigns to evaluate the use of LFAs in vaccinated individuals.

References

1. WHO. WHO Coronavirus (COVID-19) Dashboard [Internet]. 2021 [cited 2021 Mar 22]. Available from: https://covid19.who.int/

2. Giri AK, RSJB Rana D. Charting the challenges behind the testing of COVID-19 in developing countries: Nepal as a case study. Biosaf Heal [Internet]. 2020 [cited 2021 Mar 23];2:53–6. Available from: http://dx.doi.org/10.1016/j.bsheal.2020.05.002

3. Gao Z, Xu Y, Sun C, Wang X, Guo Y, Qiu S, et al. A systematic review of asymptomatic infections with COVID-19. Vol. 54, Journal of Microbiology, Immunology and Infection. Elsevier Ltd; 2020. p. 12–6.

4. To KKW, Tsang OTY, Leung WS, Tam AR, Wu TC, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect Dis [Internet]. 2020 May 1 [cited 2021 Mar 23];20(5):565–74. Available from: www.thelancet.com/infection

5. Ma H, Zeng W, He H, Zhao D, Jiang D, Zhou P, et al. Serum IgA, IgM, and IgG responses in COVID-19. Cell Mol Immunol [Internet]. 2020 [cited 2020 Oct 20];17:773–5. Available from: https://doi.org/10.1038/s41423-020-0474-z

6. Pickering S, Betancor G, Galão RP, Merrick B, Signell AW, Wilson HD, et al. Comparative assessment of multiple COVID-19 serological technologies supports continued evaluation of point-of-care lateral flow assays in hospital and community healthcare settings. Fouchier RAM, editor. PLOS Pathog [Internet]. 2020 Sep 24 [cited 2021 Mar 23];16(9):e1008817. Available from: https://dx.plos.org/10.1371/journal.ppat.1008817

7. Brown L, Byrne RL, Fraser A, Owen SI, Cubas Atienzar AI, Williams C, et al. Self-sampling of capillary blood for serological testing of SARS-CoV-2 by COVID-19 IgG ELISA. medRxiv [Internet]. 2020 Jan 1;2020.09.25.20183459. Available from: http://medrxiv.org/content/early/2020/09/27/2020.09.25.20183459.abstract

8. Landis JR, Koch GG. The Measurement of Observer Agreement for Categorical Data. Biometrics. 1977 Mar;33(1):159.

9. Whitman JD, Hiatt J, Mowery CT, Shy BR, Yu R, Yamamoto TN, et al. Evaluation of SARS-CoV-2 serology assays reveals a range of test performance. Nat Biotechnol [Internet]. 2020 Oct 1 [cited 2021 Apr 26];38(10):1174–83. Available from: https://doi.org/10.1038/s41587-020-0659-0

10. Target Product Profile: antibody tests to help determine if people have recent infection to SARS-CoV-2: Version 2 - GOV.UK [Internet]. [cited 2021 May 26]. Available from: https://www.gov.uk/government/publications/how-tests-and-testing-kits-for-coronavirus-covid-19-work/target-product-profile-antibody-tests-to-help-determine-if-people-have-recent-infection-to-sars-cov-2-version-2

11. Li D, Li J. Immunologic Testing for SARS-CoV-2 Infection from the Antigen Perspective. J Clin Microbiol [Internet]. 2020 Dec 14 [cited 2021 Apr 26];59(5). Available from: https://doi.org/10.1128/JCM.02160-20.

12. Steinhardt LC, Ige F, Iriemenam NC, Greby SM, Hamada Y, Uwandu M, et al. Cross-reactivity of two SARS-CoV-2 serological assays in a malaria-endemic setting. J Clin Microbiol [Internet]. 2021 Apr 14 [cited 2021 Apr 27]; Available from: http://jcm.asm.org/lookup/doi/10.1128/JCM.00514-21

Acknowledgements

We would like to thank all those who participated in the FASTER study for their involvement in this research. We would also like to thank the teams from Liverpool University Hospitals National Health Services Foundation Trust, National Institute for Health Research and the Liverpool School of Tropical Medicine team for their assistance with the sample collection and processing: Kostas Liatsikos, Farah Shiham, Jesus Reine, Carla Solorzano Gonzalez, Elissavet Nikolaou, Ashleigh Howard, Lisa Hitchins, Katerina Cheliotis, Esther German, Sherin Pojar, Christopher Myerscough, Madi Farrar, Sharon Glynn.

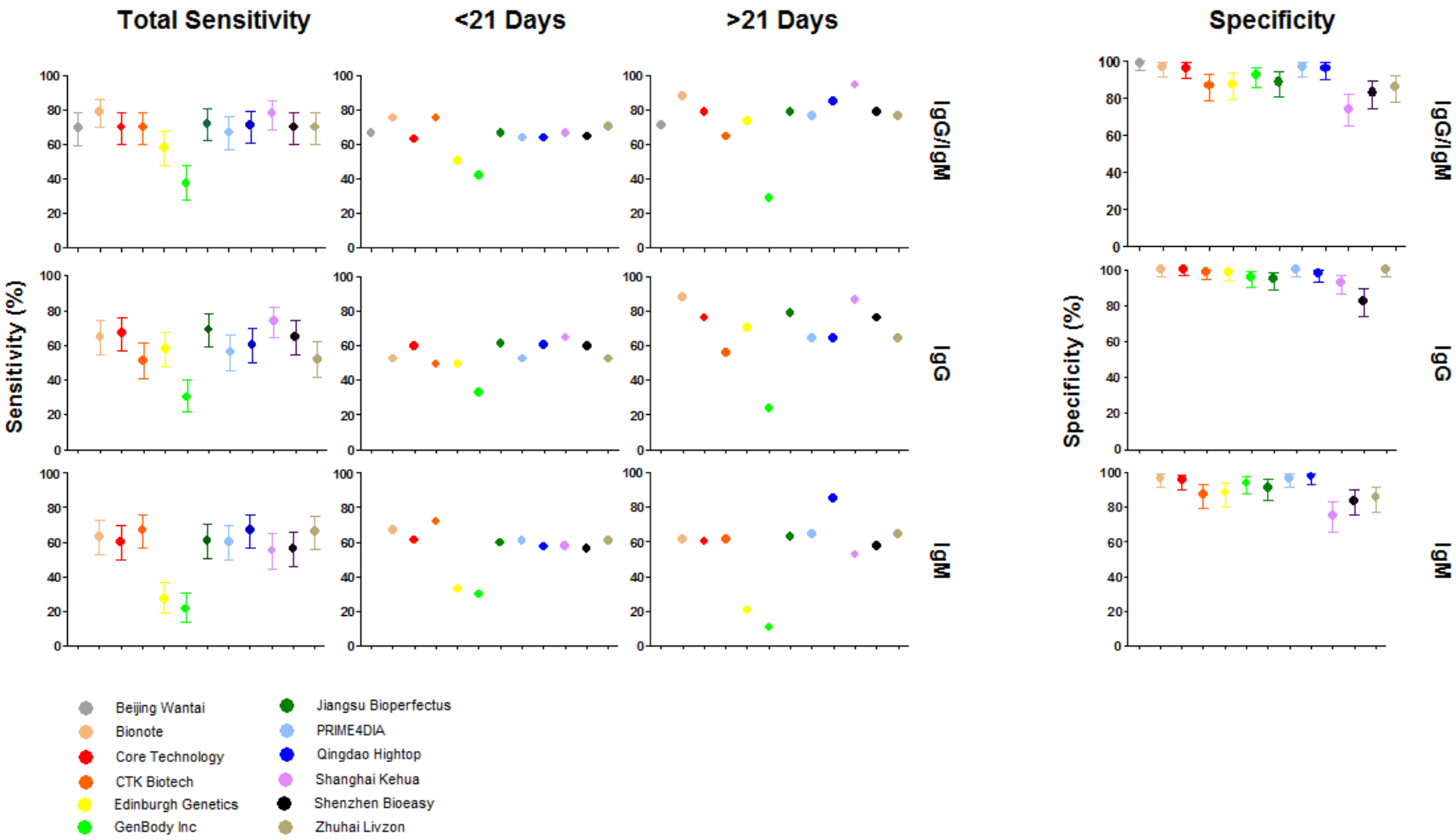


Figure 6. Sensitivity (total, ≤ 21 and >21 days post symptom onset) and specificity of the antibody response (IgM/IgG, IgG, IgM) to each LFA.

Note, sensitivity was calculated against RT-PCR results and specificity was calculated using a pre-pandemic panel.

Table 1. Details of LFAs evaluated.

Manufacturer	Test name (manufacturer)	Referred to herein as	Product Code	Lot Number	Volume of sera (µl)	Drops of buffer	Time to result (minutes)
Beijing Wantai Biological Pharmacy Enterprise Co., Ltd	WANTAI SARS-CoV-2 Ab Rapid Test	Beijing Wantai	WJ-2750	JNB20200408	10	2	15
Bionote Co., LTD.	NowCheck COVID-19 IgM/IgG Test	Bionote	RB2901DG	2901D002	10	3	10
Core Technology Co., Ltd	COVID-19 IgM/IgG Ab Test	Core Technology	B290-21	20200406	10	2	10
CTK Biotech	Onsite COVID-19 IgM/IgG Rapid Test	CTK Biotech	R0180C	F0507R1C00	10	2	10
Edinburgh Genetics Limited	Edinburgh Genetics COVID-19 Colloidal Gold Immunoassay Testing Kit, IgM/IgG Combined	Edinburgh Genetics	TIL225AEGCV0055	2000555A	20 into 2ml buffer	2-3	10
GenBody Inc.	COVID-19 IgM/IgG	GenBody	COVI040, PQGB021 (reader)	FJF029201	10	3	10
Jiangsu Bioperfectus Technologies Co., Ltd	Novel Corona Virus（SARS-CoV-2） IgM/IgG Rapid Test Kit	Jiangsu Bioperfectus	SC30201W	20200401	10	3	10
PRIME4DIA Co., Ltd	P4DETECT COVID-19 IgM/IgG	PRIME4DIA		CMG200701	10	3	10
Qingdao HIGHTOP Biotech Co., Ltd.	SARS-CoV-2 IgM/IgG Ab Rapid Test	Qingdao HIGHTOP	H100	COV1252004C	10	2	15
Shanghai Kehua Bio-Engineering Co., Ltd	Diagnostic Kit for SARS-CoV-2 IgM/IgG Antibody (Colloidal Gold)	Shanghai Kehua	R-423-20-C-CE	423200334	10	3	15
Shenzhen Bioeasy Biotechnology Co., Ltd	2019-Novel Coronavirus (2019-nCoV) IgM/IgG GICA Rapid Test Kit	Shenzhen Bioeasy	YRLG22301025	2003N104	10	2	10

Zhuhai Livzon Diagnostics Inc.	Diagnostic Kit for IgM/IgG Antibody to Coronavirus (SARS-CoV-2) (Lateral Flow)	Zhuhai Livzon	CK2004240410	10	2	10	

Table 2. Sensitivity and specificity of the 12 LFAs. Sensitivity was calculated using SARS-CoV-2 RT-qPCR positive sera/plasma and specificity determined on pre-pandemic sera/plasma.

Test	Ig	All samples		≤ 21 DAYS POST SYMPTOM ONSET Sensitivity vs RT-qPCR (%)	> 21 DAYS POST SYMPTOM ONSET Sensitivity vs RT-qPCR (%)	More sensitive > 21 days?
		Sensitivity vs RT-qPCR (%) [95% CI]	Specificity vs pre-Pandemic panel (%) [95% CI]			
Beijing Wantai	IgG + IgM	69.7 (69/99 TP) [59.7-78.5]	99.1 (104/105 TN) [94.8-100]	66.7 (38/57)	71.1 (27/38)	Yes
	IgG + IgM	79.0 (79/100 TP) [69.7-86.5]	97.0 (97/100 TN) [91.5-99.4]	75.4 (46/61)	88.2 (30/34)	Yes
Bionote	IgG	65.0 (65/100 TP) [54.8-74.3]	100 (100/100 TN) [96.4-100]	52.5 (32/61)	88.2 (30/34)	Yes
	IgM	63.0 (63/100 TP) [52.8-72.4]	97.0 (97/100 TN) [91.5-99.4]	67.2 (41/61)	61.8 (21/34)	No
	IgG + IgM	70.0 (70/100 TP) [60.0-78.8]	96.2 (101/105 TN) [90.5-99.0]	63.2 (36/57)	79.0 (30/38)	Yes
Core Technology	IgG	67.0 (67/100 TP) [56.9-76.1]	100 (105/105 TN) [96.6-100]	59.7 (34/57)	76.3 (29/38)	Yes
	IgM	60.0 (60/100 TP) [49.7-69.7]	96.2 (101/105 TN) [90.5-99.0]	61.4 (35/57)	60.5 (23/38)	No
	IgG + IgM	70.0 (70/100 TP) [60.0-78.8]	86.7 (85/98 TN) [78.4-92.7]	75.4 (46/61)	64.7 (22/34)	No
CTK Biotech	IgG	51.0 (51/100 TP) [40.8-61.1]	99.0 (97/98 TN) [94.5-100]	49.2 (30/61)	55.9 (19/34)	Yes
	IgM	67.0 (67/100 TP) [56.9-76.1]	87.8 (86/98 TN) [79.6-93.5]	72.1 (44/61)	61.8 (21/34)	No
Edinburgh Genetics	IgG + IgM	58.0 (58/100 TP) [47.7-67.8]	87.6 (85/97 TN) [79.4-93.4]	50.8 (31/61)	73.5 (25/34)	Yes

	IgG	56.0 (56/100 TP) [45.7-65.9]	99.0 96/97 TN) [94.4-100]	49.2 (30/61)	70.6 (24/34)	Yes
	IgM	27.0 (27/100 TP) [18.6-36.8]	88.7 (86/97 TN) [80.6-94.2]	32.8 (20/61)	20.6 (7/34)	No
	IgG + IgM	37.4 (37/99 TP) [27.9-47.7]	92.4 (97/105 TN) [85.5-96.7]	42.1 (24/57)	29.0 (11/38)	No
GenBody Inc.	IgG	30.3 (30/99 TP) [21.5-40.4]	96.2 (101/105 TN) [90.5-99.0]	33.3 (19/57)	23.7 (9/38)	No
	IgM	21.2 (21/99 TP) [13.6-30.6]	94.3 (99/105 TN) [88.0-97.9]	29.8 (17/57)	10.5 (4/38)	No
	IgG + IgM	72.0 (72/100 TP) [62.1-80.5]	88.6 (93/105 TN) [80.9-94.0]	66.7 (38/57)	79.0 (30/38)	Yes
Jiangsu Bioperfectus	IgG	69.0 (69/100 TP) [59.0-77.9]	95.2 (100/105 TN) [89.2-98.4]	61.4 (35/57)	79.0 (30/38)	Yes
	IgM	61.0 (61/100 TP) [50.7-70.6]	91.4 (96/105 TN) [84.4-96.0]	59.7 (34/57)	63.2 (24/38)	Yes
	IgG + IgM	67.0 (67/100 TP) [56.9-76.1]	97.0 (98/101 TN) [91.6-99.4]	63.9 (39/61)	76.5 (26/34)	Yes
PRIME4DIA	IgG	56.0 (56/100 TP) [45.7-65.9]	100 (101/101 TN) [96.4-100]	52.5 (32/61)	64.7 (22/34)	Yes
	IgM	60.0 (60/100 TP) [49.7-69.7]	97.0 (98/101 TN) [91.6-99.4]	60.7 (37/61)	64.7 (22/34)	Yes
	IgG + IgM	71.0 (71/100 TP) [61.1-79.6]	96.0 (97/101 TN) [90.2-98.9]	63.9 (39/61)	85.3 (29/34)	Yes
Qingdao HIGHTOP	IgG	60.0 (60/100 TP) [49.7-69.7]	98.0 (99/101 TN) [93.0-99.8]	60.7 (37/61)	64.7 (22/34)	Yes
	IgM	67.0 (67/100 TP) [56.9-76.1]	98.0 (99/101 TN) [93.0-99.8]	57.4 (35/61)	85.3 (29/34)	Yes
Shanghai Kehua	IgG + IgM	78.0 (78/100 TP) [68.6-85.7]	74.3 (78/105 TN) [64.8-82.3]	66.7 (38/57)	94.7 (36/38)	Yes

	IgG	74.0 (74/100 TP) [64.3-82.3]	93.3 (98/105 TN) [86.8-97.3]	64.9 (37/57)	86.8 (33/38)	Yes
	IgM	55.0 (55/100 TP) [44.7-65.0]	75.2 (79/105 TN) [65.9-83.1]	57.9 (33/57)	52.6 (20/38)	No
	IgG + IgM	70.0 (70/100 TP) [60.0-78.8]	82.9 (87/105 TN) [74.3-89.5]	64.9 (37/57)	79.0 (30/38)	Yes
Shenzhen Bioeasy	IgG	65.0 (65/100 TP) [54.8-74.3]	82.9 (87/105 TN) [74.3-89.5]	59.7 (34/57)	76.3 (29/38)	Yes
	IgM	56.0 (56/100 TP) [45.7-65.9]	83.8 (88/105 TN) [75.4-90.3]	56.1 (32/57)	57.9 (22/38)	Yes
	IgG + IgM	70.0 (70/100 TP) [60.0-78.8]	86.0 (86/100 TN) [77.6-92.1]	70.5 (43/61)	76.5 (26/34)	Yes
Zhuhai Livzon	IgG	52.0 (52/100 TP) [41.8-62.1]	100 (100/100 TN) [96.4-100]	52.5 (32/61)	64.7 (22/34)	Yes
	IgM	66.0 (66/100 TP) [55.9-75.2]	86.0 (86/100 TN) [77.6-92.1]	60.7 (37/61)	64.7 (23/34)	Yes

Table 3. Sensitivity and specificity of LFAs against IgG ELISA.

Manufacturer	Sensitivity (%) [95% CI]	Specificity (%) [95% CI]
Beijing Wantai	92.3 (60/65 TP) [83.0-97.5]	98.8 (81/82 TN) [93.4-100]
Bionote	90.0 (54/60 TP) [79.5-96.2]	100 (78/78 TN) [95.4-100]
Core Technology	92.3 (60/65 TP) [83.0-97.5]	100 (82/82 TN) [95.6-100]
CTK Biotech	81.7 (49/60 TP) [69.6-90.5]	100 (76/76 TN) [95.3-100]
Edinburgh Genetics	80.0 (48/60 TP) [67.7-89.2]	100 (75/75 TN) [95.2-100]
GenBody	44.6 (29/65 TP) [32.3-57.5]	100 (76/76 TN) [95.6-100]
Jiangsu Bioperfectus	93.9 (61/65 TP) [85.0-98.3]	96.3 (79/82 TN) [89.7-99.2]
PRIME4DIA	86.7 (52/60 TP) [75.4-94.1]	100 (79/79 TN) [95.4-100]
Qingdao HIGHTOP	85.0 (51/60 TP) [73.4-92.9]	98.7 (78/79 TN) [93.2-100]
Shanghai Kehua	95.4 (62/65 TP) [87.1-99.0]	97.6 (80/82 TN) [91.5-99.7]
Shenzhen Bioeasy	83.1 (54/65 TP) [71.7-91.2]	85.4 (70/82 TN) [75.8-92.2]
Zhuhai Livzon	78.3 (47/60 TP) [65.8-87.9]	100 (78/78 TN) [95.4-100]

Table 4. Agreement and Cohen's Kappa between IgG measured by LFA and IgG ELISA.

	LFA	ELISA		Kappa [95% CI]
		Positive	Negative	
Beijing Wantai	Positive	60	1	0.9167 [0.8514-0.982]
	Negative	5	81	
Bionote	Positive	54	0	0.9105 [0.8405-0.9805]
	Negative	6	78	
Core Technology	Positive	60	0	0.9305 [0.8706-0.9904]
	Negative	5	82	
CTK Biotech	Positive	49	0	0.8327 [0.7379-0.9275]
	Negative	11	76	
Edinburgh Genetics	Positive	48	0	0.8163 [0.7171-0.9155]
	Negative	12	75	
GenBody	Positive	29	0	0.4733 [0.3238-0.6228]
	Negative	36	82	
Jiangsu Bioperfectus	Positive	61	3	0.9033 [0.8334-0.9732]
	Negative	4	79	
PRIME4DIA	Positive	52	0	0.8808 [0.8006-0.961]
	Negative	8	79	
Qingdao HIGHTOP	Positive	51	1	0.8510 [0.7620- 0.9400]
	Negative	9	78	
Shanghai Kehua	Positive	62	2	0.9309 [0.8714-0.9904]
	Negative	3	80	
Shenzhen Bioeasy	Positive	54	12	0.6833 [0.5644-0.8022]
	Negative	11	70	
Zhuhai Livzon	Positive	47	0	0.8034 [0.7017-0.9051]
	Negative	13	78	

Table S1. Positive sera/plasma used to calculate diagnostic accuracy of LFAs.

Confirmed Pathogen/Disease	N	Years collected	Sample Type	Country of collection	Collection site
SARS-CoV-2/COVID-19	41	2020	Sera	UK	RLUTH and Aintree University Hospital recruited through FASTER
SARS-CoV-2/COVID-19	84	2020	Sera	UK	RLUTH and Aintree University Hospital provided by LCL
SARS-CoV-2/COVID-19	12	2020	Sera	UK	Liverpool School of Tropical Medicine
NIBSC 20/118	4	2020	Plasma	UK	National Institute for Biological Standards and Control, UK
NIBSC 20/130	1	2020	Plasma	UK	National Institute for Biological Standards and Control, UK

Table S2. Negative controls used to calculate diagnostic accuracy of LFAs.

Confirmed Pathogen/Disease	N	Participant population	Dates collected	Sample Type	Country of collection	Collection site
Influenza A	20	Confirmed respiratory disease	First half 2019	Sera	UK	RLUTH NHS trust
Tuberculosis	10	Confirmed respiratory disease	July-October 2018	Sera	Nigeria	Zankli Research Centre, Abuja
HIV	10	Sick with non-respiratory disease	May-June 2018	Sera	Nigeria	Zankli Research Centre, Abuja
Tuberculosis/HIV	10	Confirmed respiratory disease	June-October 2018	Sera	Nigeria	Zankli Research Centre, Abuja
Dengue virus	10	Sick with non-respiratory disease	Jul-15	Sera	Brazil	Aracaju
Human coronavirus 229E	10	Confirmed respiratory disease	May 2018-January 2020	Sera	UK	St Georges NHS trust
Human coronavirus OC43	2	Confirmed respiratory disease	May 2018-October 2019	Sera	UK	St Georges NHS trust
Plasmodium falciparum	3	Sick with non-respiratory disease	1988-2005	Sera	UK	RLUTH NHS trust
Schistosoma mansoni, S. haematobium	4	Sick with non-respiratory disease	1999-2003	Sera	UK	RLUTH NHS trust
Entamoeba histolytica	3	Sick with non-respiratory disease	1992-1996	Sera	UK	Shrewsbury NHS, Manchester NHS, Stepping Hill NHS
Strongyloides stercoralis	2	Sick with non-respiratory disease	1973-1991	Sera	UK	RLUTH NHS trust, HTD London
FIND Fever Panel	20	Sick with non-respiratory disease	2017-2018	Plasma	Malawi	NA
FIND QA PANEL NTC	1			Plasma		