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Biomarkers for intracellular pathogens: establishing tools as vaccine and therapeutic endpoints for visceral leishmaniasis

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Abstract

Visceral leishmaniasis in South Asia is a serious disease affecting children and adults. Acute visceral leishmaniasis develops in only a fraction of those infected individuals, the majority being asymptomatic with the potential to transmit infection and develop disease. We followed 56 individuals characterized as being asymptomatic by seropositivity with rk39 rapid diagnostic test in a hyperendemic district of Bangladesh to define the utility of *Leishmania*-specific antibodies and DNA in identifying infection. At baseline, 54 of the individuals were seropositive with one or more quantitative antibody assays and antibody levels persisted at follow up. Most seropositive individuals (47/54) tested positive by quantitative PCR at baseline, but only 16 tested positive at follow up. The discrepancies among the different tests may shed light on the dynamics of asymptomatic infections of *Leishmania donovani*, as well as underscore the need for standard diagnostic tools for active surveillance as well as assessing the effectiveness of prophylactic and therapeutic interventions.

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Introduction

One of the characteristics of neglected diseases is the lack of tools for successful disease management or prevention. As an example, it has only been recently that tools to detect infected individuals have begun to be applied in regions where *Leishmania donovani* is endemic. *Leishmania* are vector-borne protozoan parasites spread by the bite of the infected phlebotomine sand-fly [1,2]. Two *Leishmania* spp. cause visceral leishmaniasis (VL; also known as kala-azar), which is the most severe form, often fatal if not treated [3]. Half a million new VL cases are diagnosed every year and, with a mortality rate of 10%, VL is second only to malaria in terms of fatality [4].

In the Indian subcontinent VL is caused by *L. donovani*. An estimated 100 000 VL cases are reported annually in the endemic foci of northeastern India, Nepal and Bangladesh, costing 400 000 disability-adjusted life-years and putting 150 million people at risk for infection[5] The situation in this region, where humans are the only reservoir of the parasite, makes elimination feasible [3]. In 2005 these three countries committed to a kala-azar elimination programme, with the intent of decreasing incidence to 1 in 10 000 per year by 2015 [6]. Goals of the programme include early diagnosis and treatment, coordinated vector control and effective disease surveillance through passive and active case detection [7]. A pressing concern that could interfere with this goal is the presence of large populations who harbour infection with no overt signs of disease but who can potentially develop VL and probably spread infection [8,9]. Considerably more asymptomatic individuals than those with VL disease are identified in areas of endemicity [10,11].

Traditionally, both direct (detection of parasites) and indirect (detection of antibodies) tests have been used in the diagnosis of VL. The standard diagnostic method for VL has been microscopic visualization of amastigotes in splenic, lymph node or bone marrow aspirates, ethically and technically unsuitable for asymptomatic individuals [12]. The kala-azar elimination programme does not evaluate asymptomatic individuals because defined parameters to identify low levels of infection are lacking. A limited number of antibody detection tests, including the direct agglutination test (DAT), ELISA and immunochromatography-based rapid tests, have been standardized [12]. Given the endemic nature of *L. donovani* infection, serological tests lack the ability to discern between uninfected, antibody-positive individuals and asymptomatic infected individuals [13,14]. Unlike the situation in Africa and Brazil, *Leishmania*-specific antibodies persist for extended periods of time in the Indian subcontinent, emphasizing the importance of defining biomarkers that reflect the dynamics of asymptomatic infections [14]. PCR-based tests directly demonstrating the presence of parasite nucleic acids probably represent a more accurate tool for the assessment of asymptomatic individuals, with the advantage of being sensitive enough to detect very low levels of parasite DNA [15]. Serological and PCR-based techniques can complement each other to establish how these biomarkers reflect asymptomatic *L. donovani* infection. Given the potential availability of vaccines against *L. donovani*, these tests could be used to identify individuals or populations that will benefit most from vaccination.

To determine the utility of various tests, a group of asymptomatic infected individuals in a region where *L. donovani* is hyperendemic were examined over a period of 24 months with serological tests, DAT and ELISA, as well as a sensitive quantitative PCR test to define the dynamics of these biomarkers.

Patients and Methods

Study design, ethics and parameters

We defined an asymptomatic infected individual as a person from a VL endemic area with no past history of VL or post-kala-azar dermal leishmaniasis (PKDL), clinically healthy and positive by the rK39 rapid test (Kalazar Detect™; Inbios, Seattle, WA, USA) in the field using finger-prick blood. We recruited 3849 clinically healthy people in the Harirampur Union of subdistrict Trishal, Mymensingh district, where VL is hyperendemic with a reported incidence of 65 per 10 000 people in 2007 (Trishal Hospital). Initial consent was obtained from the head of the household to screen household members based on past VL or PKDL history, then individual written consent was obtained before enrolment in the study. Initial screening was conducted using the Kalazar Detect™ rk39 rapid diagnostic test (RDT) and 332 were found positive, with 200 deemed fit to participate in the study based on no prior history of VL or PKDL (50% were female and 35.5% were under 15 years). Fifty-six were then enrolled as study subjects based on the availability of matching serum and DNA samples when the study was initiated (baseline) and 12 months after initiation (follow up) (Fig. 1a). All serological and PCR tests were conducted on these 56 serum and DNA samples collected at baseline and 12-month follow up (Fig. 1b). The enrolees were monitored each month for clinical symptoms of VL during household visits up to 24 months after study initiation, with three of the 56 enrolees developing VL disease by 24 months. They were referred to the study clinic where a qualified medical officer examined them following the diagnostic criteria for VL of the National Guideline before referring them to the Trishal Hospital for treatment (Fig. 1b) [16].

Sample collection and storage

Blood specimens were collected at enrolment and then at 12 months. Blood specimens were collected by venepuncture. For DNA extraction, an aliquot of 2 mL was placed in an EDTA-containing vacutainer and centrifuged at 2200 g for 20 min for separation of buffy coat. Collected buffy coat was transported to the ICDDRB Parasitology Laboratory maintaining the cold chain and DNA was isolated using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. For serum preparation, 2 mL of blood was placed in red-top vacutainers and allowed to clot at room temperature for an hour and centrifuged for 5 min for separation of serum. Collected serum was transported as above.

Serological tests

DAT was performed according to the manufacturer's instructions (KIT Biomedical Research, Amsterdam, the Netherlands) with minor modifications [17]. Briefly, in V-bottom 96-well plates, healthy US control and test serum samples were serially diluted two-fold starting at 1 : 400 in 0.9% sodium chloride added 0.8% β-mercaptoethanol at a final volume of 50 μL per well. Antigen re-suspended in 50 μL of 0.9% sodium chloride was then added per well. Each plate included at least two blank wells containing only sample diluent. After brief mixing, plates were covered and left undisturbed at room temperature for 18 h before reading. Samples were run in singlet per plate and duplicated by a second researcher. Results were independently scored by three readers. Each reader recorded the titre as the last sample dilution at which agglutination was apparent. The