Detection of IgG1 against rK39 improves monitoring of treatment outcome in visceral leishmaniasis

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Forty word summary of article's main point

IgG1 ELISAs (versus IgG) and a novel IgG1-based rapid diagnostic test (RDT) using rK39 antigen provide greatly enhanced discrimination between post-treatment cure versus relapse in visceral leishmaniasis ($p < 0.0001$). This RDT may have a significant role in targeted disease control.

Abstract

Background. Visceral leishmaniasis (VL), caused by the *Leishmania donovani* complex, is a fatal neglected tropical disease that is targeted for elimination in India, Nepal and Bangladesh. Improved diagnostic tests are required for early case detection and for monitoring outcome of treatment. Previous investigations using *Leishmania* lysate antigen demonstrated that IgG1 response is a potential indicator of clinical status after chemotherapy.

Methods. IgG1 or IgG ELISAs with rK39 or lysate antigens, and novel IgG1 rK39 rapid diagnostic tests (RDTs) were assessed with Indian VL serum samples from the following clinical groups: paired pre- and post-chemotherapy (deemed cured); relapsed; other infectious diseases, and endemic healthy controls.

Results. With paired pre- and post-treatment samples (n = 37 pairs), ELISAs with rK39 and IgG1-specific conjugate gave a far more discriminative decrease in post-treatment antibody response when compared to IgG ($p < 0.0001$). Novel IgG1 rK39 RDTs provided strong evidence for decreased IgG1 response in patients who had successful treatment ($p < 0.0001$). Furthermore, both IgG1 rK39 RDTs (n = 38) and ELISAs showed a highly significant difference in test outcome between cured patients and those who relapsed (n = 23) ($p < 0.0001$). RDTs were more sensitive than corresponding ELISAs.

***Conclusions*.** We present here strong evidence for the use of IgG1 in monitoring treatment outcome in VL, and the first use of an IgG1-based RDT using rK39 antigen for the discrimination of post-treatment cure versus relapse in VL. Such an RDT may have a significant role in monitoring patients and in targeted control and elimination of this devastating disease.

Introduction

In 2012 the World Health Organization (WHO) estimated the global burden of visceral leishmaniasis (VL) to be 200,000-400,000 cases annually with 20,000-40,000 deaths. The vast majority of cases occur within the Indian subcontinent (ISC), eastern Africa and Brazil, with India accounting for an estimated 70% of global cases but with a recent significant decline [1,2]. In India, VL is caused solely by *Leishmania donovani*, spread by the vector *Phlebotomus argentipes*, and the disease is considered anthroponotic, with no proven animal reservoirs. Post kala-azar dermal leishmaniasis (PKDL) is a non-life threatening potential sequela of treated VL, and patients with PKDL have been shown to be readily infectious to biting sand flies of the appropriate vector species [3, 4].

Since 2005 India, Bangladesh and Nepal have been pursuing the elimination of VL as a public health problem (<1 case per 10,000) [5]; highly endemic blocks persist in the Indian states of Bihar, Jharkhand and West Bengal [6]. In 2016 approximately 6,250 total cases were reported, representing a fall of over 50% since 2013 [6]. The elimination programme focuses on: early case detection, with successful treatment; improved surveillance; and integrated vector control [5]. Thus, a successful VL control programme requires the implementation of specific and early diagnosis.

Clinical features of VL are prolonged fever (>14 days), hepatosplenomegaly, anemia, pancytopenia and weight loss, non-specific symptoms that prevent definitive clinical diagnosis. Parasitological diagnosis of *Leishmania* amastigotes is by microscopy of bone marrow or spleen aspirates, which are high risk procedures. The detection of IgG against rK39, a fragment of the *Leishmania* kinesin-like gene [7], has been used with clinical

presentation to diagnose VL cases; however IgG levels may remain detectable even years after successful cure and disease clearance, as reported from India [8, 9], Brazil [10] and Sudan [11]. Furthermore, asymptomatic individuals who are serologically positive far outnumber clinical cases [12, 13], with only a small proportion of asymptomatics progressing to active disease, thereby reducing the positive predictive value of the current rK39 rapid diagnostic test (RDT).

Studies from India and Nepal have reported post-chemotherapy relapse of VL up to and beyond 12 months following the end of treatment [14, 15]. With liposomal amphotericin B, a new first line treatment in India, the relapse rate is an estimated 6.7%, with a significant proportion of patients relapsing between 6 and 12 months after treatment [14, 16]. To improve the monitoring of treatment outcome of VL, and for control of the disease, WHO has identified the vital need for a marker of post-chemotherapeutic cure, and the high priority incorporation of such a biomarker into a point-of-care RDT [17].

Here, we investigated whether IgG1 detection in combination with rK39 antigen could improve serological assessment of treatment outcome in VL, particularly to discriminate cure from relapse.

Methods

Ethics statement

In India, sample collection was approved by the Ethics Committee of Banaras Hindu University, Varanasi. In Sudan approval was by the Ethical Research Committee, the Medical and Health Sciences Campus, University of Khartoum and the National Health Research Ethics Committee, Federal Ministry of Health, Sudan. Written informed consent was obtained from adult subjects or from the parents or guardians of individuals less than

18 years of age (who also gave verbal consent). This research was also approved, as part of the NIDIAG (Syndromic approach to Neglected Infectious Diseases (NID) at primary health care level) research consortium (https://cordis.europa.eu/project/rcn/97322_en.html), by the London School of Hygiene and Tropical Medicine Ethics Committee.

Samples

We selected Indian sera or plasma from archived samples that were collected after 2007 from male and female adults and children in the endemic region of Muzaffarpur, Bihar, India (Table 1). Indian VL cases had been diagnosed by positive rK39 serology and parasitologically confirmed by microscopy of splenic or bone marrow aspirates. Indian paired samples were from parasitologically confirmed VL patients at day of diagnosis (day 0) and when deemed cured (6 months; n = 40 pairs). Unpaired relapsed sera were from VL patients who had been treated but sampled at relapse (n = 23). As described below, not all cure pair and relapse samples were used in every assay. Control samples were from clinically confirmed tuberculosis cases (n = 10), and from people living in regions endemic and non-endemic for VL, with no clinical symptoms (EHC and NEHC respectively, n = 10 in each group). We also used Sudanese serum samples collected in 2011 and 2013, from Gedaref, Sudan. In Sudan, cases of VL had been diagnosed by microscopy of bone marrow or lymph node aspirates in conjunction with serological assays. These diagnoses were made according to their respective national procedures, prior to the present study. Sera/plasma were stored at -80°C until use. Samples were previously assayed against culture-adapted promastigote lysate (Marlais et al, manuscript submitted). All patients were HIV negative.

Antigens

Recombinant rK39 protein was obtained commercially (RAG0061, Rekom Biotech, Spain). *L. donovani* whole cell lysates were derived from two strains: culture-adapted MHOM/IN/80/DD8 promastigote, and MHOM/IN/00/BHU1 that had been cryopreserved as amastigotes. Both strains were cultured in αMEM (M0644, Sigma Aldrich, UK) supplemented as previously described [18]. For strain BHU1, the cryopreserved amastigotes were recovered into αMEM and then passaged once into fresh medium prior to harvesting as amastigote-derived promastigotes for lysate preparation. The whole cell lysates were prepared and sonicated as previously described [19]. Sonicates were centrifuged at 14000 x g for 10 minutes at 4°C, and the supernatants containing lysate antigens stored at -80°C with protease inhibitor cocktail (P8340, Sigma Aldrich). Protein concentrations of these antigens were determined using the BCA Protein Assay kit against bovine serum antigen standards (23227, ThermoFisher Scientific, UK) according to manufacturer's instructions.

ELISAs

For optimisation, we used six Sudanese sera (3 high titre, 1 low and 2 negative) with titrated rK39 antigen; in subsequent assays we used rK39 resuspended at 0.25 µg/ml in coating buffer (15 mM Na_2CO_3, 34 mM $NaHCO_3$, pH 9.6).

To compare antigenicity of rK39 and promastigote antigens using Indian sera, and with separate detection of IgG1 and IgG, each ELISA plate (735–0465, VWR, UK) was divided into quadrants. The rK39 antigen at 0.25 µg/ml, and culture-adapted promastigote lysates at 2 µg/ml diluted in coating buffer, were used to coat the top and bottom halves respectively of the same plate at 100 µl/well and incubated at 4°C overnight. Following three washes with

PBS / 0.05% Tween 20 (PBST), 200 µl/well of blocking buffer (PBS / 2% skimmed milk powder (Premier Foods, UK)) was applied to the whole plate and incubated for 2 hours at 37°C. Following three washes, 100 µl/well of serum/plasma diluted 1:200 in PBST / 2% milk (PBSTM) was added, such that the same samples were arranged identically in each quadrant. Following incubation at 37°C for 1 hour and six washes in PBST, 100 µl/well of 1:5,000 dilution in PBSTM of horse-radish peroxidase (HRP) labelled anti-human IgG1 (ab99774, Abcam, UK) or anti-human IgG (709-035-149, Jackson Immunoresearch, USA) were added to the left and right halves of the plate respectively. Following incubation at 37°C for 1 hour and six PBST washes, 100 µl/well of substrate solution (50 mM phosphate/citrate buffer (pH 5.0) containing 2 mM σ-phenylenediamine HCl (P1526, Sigma Aldrich) and 0.009% H_2O_2 (216763, Sigma Aldrich) was added to the entire plate and incubated in the dark. Reactions were stopped by the addition of 100 µl/well of 1M H_2SO_4 and absorbance was read at 490 nm. Samples were assayed on duplicate plates simultaneously.

To compare lysates, 2 µg/ml of amastigote-derived promastigote antigen was coated onto the top half of the plate at 100 µl/well, in place of rK39, and otherwise the assay was performed as described above.

Prototype Rapid Diagnostic Tests

Co-authors at Coris BioConcept manufactured the novel IgG1 rK39 rapid diagnostic tests described here. The RDT is composed of a nitrocellulose strip sensitised with antigen and containing anti-human IgG1-specific antibody conjugated with colloidal gold, housed within a plastic cassette with a buffer application well and a test/reading window. The antigen used

was rK39 at two different concentrations, namely 0.1 mg/ml (0.1rK) and 0.6 mg/ml (0.6rK), on separate cassettes. Serum/plasma at volumes of 3.5 µl were pipetted onto the sample application zone in the test/reading window, then 120 µl of buffer solution was dispensed into the buffer application well. After 15 minutes, a test was deemed valid if a red control band was present in line with the 'C' on the cassette, and deemed positive if a second band was present in line with the 'T'. If no band was visible at the 'T', then the test was deemed negative. Change in test line intensity between paired day 0 and 6 month samples (becomes negative, decreased, no decrease) was assessed visually. The RDTs were read blind without reference to the ELISA results.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016 (Microsoft Corporation, USA), Stata 14 (StataCorp, USA) and for ELISA data (2-tailed, paired t-test with 95% confidence interval) using R [20]. Serum from the same endemic healthy control was included in each quadrant of each ELISA plate, from which the cut-off was established for each antigen/detection antibody combination by a mean of the EHC readings plus 3 standard deviations. Mean ELISA result for each sample was determined from the duplicate assays. Paired t-tests were used to determine the significance of differences between day 0 and 6 months.

RDT results were compared with defined clinical status to establish sensitivity with exact confidence intervals calculated with the Clopper-Pearson exact method. A two-sided Fisher's exact test was used to compare relapse versus 6 month post-treatment samples with both RDT types.

RESULTS

IgG1 in ELISA is more discriminative than total IgG as an indicator of cure

Figure 1 compares IgG1 and total IgG recognition of rK39 antigen in ELISAs, with 37 paired samples at day 0 and at 6 months (when deemed to be cured), and with 20 relapsed samples. With the same group of patients, the IgG1 titres with cured sera (at 6 months) were more discriminative of clinical status, compared to total IgG. Comparing cure and relapse data, IgG1 provided better discrimination than IgG, even when the changes from day 0 samples were not considered. With the rK39 antigen, the ELISA readings of cured sera (6 months) were clustered more towards low values when developed with anti-IgG1 (Figure 1): 81.2% (30/37) were below the cut-off value (A_{490} = 0.214) compared with only 9% (4/37) for their total IgG (cut-off A_{490} = 0.413). There was very strong evidence for a difference ($p < 0.0001$) between IgG1 and total IgG for 6 month cured readings. The ELISAs using the rK39 developed more rapidly than those with promastigote antigen on the same plate and therefore the times for stopping the reactions across the entire plates were based on their anti-rK39 reaction intensities (Figure 2). We did not observe any significant differences in the ELISA performances using the amastigote-derived or culture-adapted promastigote lysates (Pearson correlation coefficient 0.98, $p < 0.0001$) (Supplementary Figure S1).

IgG1 rapid diagnostic tests discriminate relapse from cure

In total 254 RDTs were performed, on 89 individual patients (Table 2). Ten endemic healthy controls, 10 non-endemic healthy controls and 10 confirmed tuberculosis patients were negative with both the 0.1rK and 0.6rK RDTs.

RDT sensitivity for VL (Day 0) was 94.7% (82.3-99.4) and 100% (90.7-100), for 0.1rK RDT and 0.6rK RDT, respectively. Of the 21 samples from patients at relapse, 19 were positive with 0.6rK RDT, and 18 positive with 0.1rK RDT. With both 0.6rK and 0.1rK RDTs, there was very strong evidence ($p < 0.0001$) for a difference in test positivity between 6 month samples from individuals who relapsed versus 6 month samples from individuals who were cured.

In comparison with the IgG1 rK39 ELISA, the 0.6rK IgG1 RDT gave the same positive result for 17/18 (94.4% sensitive) samples. For the remaining sample, this RDT was positive, and the ELISA reading was just below the cut-off. For the cure pairs (day 0 and 6 month sample pairs), 20 of the 26 patients were positive (day 0) by both IgG1 rK39 ELISA and the 0.6rK IgG1 RDT, and decreased to negative at 6 months. Four of the other patients were negative by ELISA at both time points but were positive by the RDT at day 0 and negative at 6 months; the remainder were positive by the RDT at both time points. Thus, the RDTs, which use more concentrated sample, were overall somewhat more sensitive than the corresponding ELISAs.

DISCUSSION

Improved diagnostics for VL are required to discriminate between post-treatment cure versus relapse, and to predict progression from asymptomatic carrier to active VL. There is also a need for diagnostics to distinguish PKDL from other dermatological conditions, and to detect VL in HIV co-infected patients who are immunocompromised [21].

Since its early validation for VL diagnosis [22], rK39 antigen used in either ELISA or RDT format has been used with IgG detection. However, IgG levels can remain elevated for

several years after successful treatment [8], whereas IgG1 may decline rapidly in the absence of sustained and appropriate antigenic stimulus [23, 24]. Here, we describe the capacity of rK39 with IgG1 level detection to characterise the post-treatment clinical status of Indian VL. We demonstrated the greater discriminatory potential of IgG1 compared to IgG, as an indicator of post-chemotherapeutic outcome in VL. We have adapted the IgG1 rK39 assay to an easy to manufacture, point-of-care, reproducible, rapid and inexpensive test of cure for VL.

ELISA comparison between IgG and IgG1 against rK39 demonstrated that with IgG1 there was a significantly greater decrease in response following cure ($p < 0.0001$, Figure 1), supporting the continued development of IgG1-based diagnostics [19]. Paired samples from cured patients and non-paired samples from patients who relapsed allowed evaluation of the IgG1 rK39 RDTs. In support of previous observations [19], the majority of 6 month cured samples were negative, with a significant difference between cured and relapsed individuals ($p < 0.0001$). Thus, the IgG1 rK39 RDT provides a potential point-of-care means of serological assessment of treatment success [25]. However, this does not obviate the need for concomitant clinical evaluation.

It is not known whether the individuals deemed to be cured at 6 months remained free from relapse thereafter. In one study, most patients who relapsed did so between 6 and 12 months post-treatment [14]. Therefore, as 14 (0.1rK) and 12 (0.6rK) of 38 patients deemed cured at 6 months were positive by IgG1, albeit the majority with decreased signal strength (Table 2), further validation of the IgG1 rK39 RDT, at 12 or 18 month clinical and serological follow-up would be required to determine the relapse rate in comparison with the rate among the RDT negative patients deemed cured.

In terms of future application within a clinical environment, an optimum rK39 concentration will be required. The 0.1 mg/ml concentration produced some negative results and on visual inspection positive test bands were less clear than with the 0.6rK test; the 0.6 mg/ml concentration did not cause increased background or false positives with controls. However, considering the greater cost of manufacture involved it would be appropriate to evaluate intermediate concentrations. Pilot trials indicate that the IgG1 RDT is directly applicable to 3.5 µl of finger-prick whole blood in the field (unpublished observations).

This is the first report of the use of rK39 with detection of IgG1. We show that this combination gives a better discrimination between cure and relapse than using IgG, and that this assay can be adapted into a low cost, point-of-care (POC) RDT format. Similarly, POC RDTs are required to identify those asymptomatic serologically positive individuals who are most likely to progress to active disease, and PKDL patients with non-specific dermatological clinical presentations. The implementation of such POC RDTs within discriminative case finding initiatives would be of significant benefit in the ISC as it prepares for a post-elimination environment, in which effective diagnostic surveillance is critical.

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Potential conflicts of interest

All authors: no reported conflicts.

References

1. World Health Organization. Control of the Leishmaniases WHO Technical Report Series 949: World Health Organization, **2010**.
2. Alvar J, Velez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One **2012**; 7(5): e35671.
3. Napier L, Smith R, Das Gupta C, Mukerji S. The infection of *Phlebotomus argentipes* from dermal leishmanial lesions. Ind J Med Res **1933**; 21(1): 173-8.

4. Molina R, Ghosh D, Carrillo E, 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(1): 150-3.

5. World Health Organization. Regional Strategy Framework for Elimination of Kala-azar from the South-East Asia Region 2011-2015 SEA-CD-239. **2012**.

6. National Vector Borne Disease Control Programme. Available at: http://nvbdcp.gov.in/.

7. Burns JM, Jr., 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(2): 775-9.

8. Gidwani K, Picado A, Ostyn B, et al. Persistence of *Leishmania donovani* antibodies in past visceral leishmaniasis cases in India. Clin Vaccine Immunol **2011**; 18(2): 346-8.

9. Patil RR, Muliyil JP, Nandy A, Addy A, Maji AK, Chatterjee P. Dynamics of the antibodies in cohorts of cured cases of visceral leishmaniasis: its implication on the validity of serological test, value in prognosis and in post therapeutic assessment. Hum Vaccin Immunother **2012**; 8(6): 725-30.

10. De Almeida Silva L, Romero HD, Prata A, et al. Immunologic tests in patients after clinical cure of visceral leishmaniasis. Am J Trop Med Hyg **2006**; 75(4): 739-43.

11. Zijlstra EE, Nur Y, Desjeux P, Khalil EA, El-Hassan AM, Groen J. Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. Trop Med Int Health **2001**; 6(2): 108-13.

12. Hasker E, Kansal S, Malaviya P, et al. Latent infection with *Leishmania donovani* in highly endemic villages in Bihar, India. PLoS Negl Trop Dis **2013**; 7(2): e2053.

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

14. Burza S, Sinha PK, Mahajan R, et al. Risk factors for visceral leishmaniasis relapse in immunocompetent patients following treatment with 20 mg/kg liposomal amphotericin B (Ambisome) in Bihar, India. PLoS Negl Trop Dis **2014**; 8(1): e2536.
15. Rijal S, Ostyn B, Uranw S, et al. Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. Clin Infect Dis **2013**; 56(11): 1530-8.
16. Sundar S, Singh A, Rai M, Chakravarty J. Single-dose indigenous liposomal amphotericin B in the treatment of Indian visceral leishmaniasis: a phase 2 study. Am J Trop Med Hyg **2015**; 92(3): 513-7.
17. World Health Organization. Research priorities for Chagas disease, human African trypanosomiasis and leishmaniasis. Technical Report Series 975, **2012**.
18. Nolder D, Roncal N, Davies CR, Llanos-Cuentas A, Miles MA. Multiple hybrid genotypes of *Leishmania* (*Viannia*) in a focus of mucocutaneous leishmaniasis. Am J Trop Med Hyg **2007**; 76(3): 573-8.
19. Bhattacharyya T, Ayandeh A, Falconar AK, et al. IgG1 as a potential biomarker of post-chemotherapeutic relapse in visceral leishmaniasis, and adaptation to a rapid diagnostic test. PLoS Negl Trop Dis **2014**; 8(10): e3273.
20. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/, **2013**.
21. Singh OP, Sundar S. Developments in diagnosis of visceral leishmaniasis in the elimination era. J Parasitol Res **2015**; 2015: 239469.
22. Sundar S, Reed SG, Singh VP, Kumar PCK, Murray HW. Rapid accurate field diagnosis of Indian visceral leishmaniasis. The Lancet **1998**; 351(9102): 563-5.
23. Pan Q, Hammarstrom L. Molecular basis of IgG subclass deficiency. Immunol Rev **2000**; 178: 99-110.

24. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. Front Immunol **2014**; 5: 520.

25. Singh DP, Sundar S, Mohapatra TM. The rK39 strip test is non-predictor of clinical status for kala-azar. BMC Res Notes **2009**; 2: 187.

Table 1: Indian samples used in ELISAs and/or RDTs.

Sample	**n** [a]	**Description**
Cured, paired samples	40 pairs	From parasitologically confirmed VL patients at day of diagnosis (day 0) and when deemed cured (6 months).
Relapsed	23	VL treated and subsequently relapsed. Sampled at the time of relapse diagnosis.
Endemic healthy controls	10	Serum from patients living in regions endemic for VL, with no clinical symptoms.
Non-endemic healthy controls	10	Serum from individuals living in regions non-endemic for VL, with no clinical symptoms.
TB	10	Serum from patients with clinically confirmed tuberculosis.

[a] Not all samples were used with all assays (see Results).

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379 **Table 2: Results of Indian VL and control sera with IgG1 rK39 RDT.** rK39 was used at 0.1mg/ml

380 (0.1rK) and 0.6mg/ml (0.6rK)

Sample types			**Positive/total (%)**	
Cured VL paired samples	**Day 0**	**6 months[a]**	**0.1rK**	**0.6rK**
(n = 38)	Positive	Negative	22/38	26/38
			(57.9%)	(68.4%)
		Decrease	8/38	7/38
			(21.1%)	(18.4%)
		No decrease	6/38	5/38
			(15.8%)	(13.2%)
	Negative	Negative	2/38	0/38
			(5.2%)	(0%)
		Positive	0/38	0/38
			(0%)	(0%)
Unpaired samples				
Relapse VL samples (n = 21)			18/21 (85.7%)	19/21 (90.5%)
Endemic Healthy Control (n = 10)			0/10	0/10
Non-Endemic Healthy Control (n = 10)			0/10	0/10
Tuberculosis patients' samples (n =10)			0/10	0/10

381 [a] 6 month reading is test line intensity assessed visually compared to day 0.

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Figure 1. Decrease in IgG1 levels of cured patients was more evident and consistent than the decline in total IgG, by ELISA. ELISA results for the rK39 antigen with cured VL paired samples (n = 37 pairs) and relapse samples (n = 20). * indicates very strong evidence for a difference (paired t-test $p<0.0001$). Strong evidence was also seen between IgG1 and IgG in 6 month cured samples ($p<0.0001$, not depicted).

Figure 2. Example of ELISA plate quadrants. CP, cured paired serum samples at day 0 (pre-treatment) and at 6 months after treatment (patients deemed cured); EHC, endemic healthy control; R, patient deemed relapsed.

Supplementary Figure S1. Comparative IgG1 ELISA absorbance values obtained using active VL, cured VL and relapsed VL patients' serum samples against amastigote-derived and culture-adapted promastigotes lysate antigens. Pearson $r = 0.98$; $p = <0.0001$, for lack of significant difference.