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

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36 **Forty word summary of article's main point**

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

40

41 **Abstract**

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

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

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

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

64

65 **Introduction**

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

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

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

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

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

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

103 **Methods**

104 **Ethics statement**

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

110 18 years of age (who also gave verbal consent). This research was also approved, as part of
111 the NIDIAG (Syndromic approach to Neglected Infectious Diseases (NID) at primary health
112 care level) research consortium (https://cordis.europa.eu/project/rcn/97322_en.html), by
113 the London School of Hygiene and Tropical Medicine Ethics Committee.

114

115 **Samples**

116 We selected Indian sera or plasma from archived samples that were collected after 2007
117 from male and female adults and children in the endemic region of Muzaffarpur, Bihar, India
118 (Table 1). Indian VL cases had been diagnosed by positive rK39 serology and parasitologically
119 confirmed by microscopy of splenic or bone marrow aspirates. Indian paired samples were
120 from parasitologically confirmed VL patients at day of diagnosis (day 0) and when deemed
121 cured (6 months; n = 40 pairs). Unpaired relapsed sera were from VL patients who had been
122 treated but sampled at relapse (n = 23). As described below, not all cure pair and relapse
123 samples were used in every assay. Control samples were from clinically confirmed
124 tuberculosis cases (n = 10), and from people living in regions endemic and non-endemic for
125 VL, with no clinical symptoms (EHC and NEHC respectively, n = 10 in each group). We also
126 used Sudanese serum samples collected in 2011 and 2013, from Gedaref, Sudan. In Sudan,
127 cases of VL had been diagnosed by microscopy of bone marrow or lymph node aspirates in
128 conjunction with serological assays. These diagnoses were made according to their
129 respective national procedures, prior to the present study. Sera/plasma were stored at -
130 80°C until use. Samples were previously assayed against culture-adapted promastigote
131 lysate (Marlais et al, manuscript submitted). All patients were HIV negative.

132

133 **Antigens**

134 Recombinant rK39 protein was obtained commercially (RAG0061, Rekom Biotech, Spain). *L.*
135 *donovani* whole cell lysates were derived from two strains: culture-adapted
136 MHOM/IN/80/DD8 promastigote, and MHOM/IN/00/BHU1 that had been cryopreserved as
137 amastigotes. Both strains were cultured in α MEM (M0644, Sigma Aldrich, UK) supplemented
138 as previously described [18]. For strain BHU1, the cryopreserved amastigotes were
139 recovered into α MEM and then passaged once into fresh medium prior to harvesting as
140 amastigote-derived promastigotes for lysate preparation. The whole cell lysates were
141 prepared and sonicated as previously described [19]. Sonicates were centrifuged at 14000 x
142 g for 10 minutes at 4°C, and the supernatants containing lysate antigens stored at -80°C with
143 protease inhibitor cocktail (P8340, Sigma Aldrich). Protein concentrations of these antigens
144 were determined using the BCA Protein Assay kit against bovine serum antigen standards
145 (23227, ThermoFisher Scientific, UK) according to manufacturer's instructions.

146

147 **ELISAs**

148 For optimisation, we used six Sudanese sera (3 high titre, 1 low and 2 negative) with titrated
149 rK39 antigen; in subsequent assays we used rK39 resuspended at 0.25 μ g/ml in coating
150 buffer (15 mM Na₂CO₃, 34 mM NaHCO₃, pH 9.6).

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

156 PBS / 0.05% Tween 20 (PBST), 200 µl/well of blocking buffer (PBS / 2% skimmed milk
157 powder (Premier Foods, UK)) was applied to the whole plate and incubated for 2 hours at
158 37°C. Following three washes, 100 µl/well of serum/plasma diluted 1:200 in PBST / 2% milk
159 (PBSTM) was added, such that the same samples were arranged identically in each
160 quadrant. Following incubation at 37°C for 1 hour and six washes in PBST, 100 µl/well of
161 1:5,000 dilution in PBSTM of horse-radish peroxidase (HRP) labelled anti-human IgG1
162 (ab99774, Abcam, UK) or anti-human IgG (709-035-149, Jackson ImmunoResearch, USA)
163 were added to the left and right halves of the plate respectively. Following incubation at
164 37°C for 1 hour and six PBST washes, 100 µl/well of substrate solution (50 mM
165 phosphate/citrate buffer (pH 5.0) containing 2 mM σ -phenylenediamine HCl (P1526, Sigma
166 Aldrich) and 0.009% H₂O₂ (216763, Sigma Aldrich) was added to the entire plate and
167 incubated in the dark. Reactions were stopped by the addition of 100 µl/well of 1M H₂SO₄
168 and absorbance was read at 490 nm. Samples were assayed on duplicate plates
169 simultaneously.

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

173

174 **Prototype Rapid Diagnostic Tests**

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

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

188

189 **Statistical analysis**

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

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

202

203 **RESULTS**

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

205 Figure 1 compares IgG1 and total IgG recognition of rK39 antigen in ELISAs, with 37 paired
206 samples at day 0 and at 6 months (when deemed to be cured), and with 20 relapsed
207 samples. With the same group of patients, the IgG1 titres with cured sera (at 6 months)
208 were more discriminative of clinical status, compared to total IgG. Comparing cure and
209 relapse data, IgG1 provided better discrimination than IgG, even when the changes from day
210 0 samples were not considered. With the rK39 antigen, the ELISA readings of cured sera (6
211 months) were clustered more towards low values when developed with anti-IgG1 (Figure 1):
212 81.2% (30/37) were below the cut-off value ($A_{490} = 0.214$) compared with only 9% (4/37) for
213 their total IgG (cut-off $A_{490} = 0.413$). There was very strong evidence for a difference (p
214 <0.0001) between IgG1 and total IgG for 6 month cured readings. The ELISAs using the rK39
215 developed more rapidly than those with promastigote antigen on the same plate and
216 therefore the times for stopping the reactions across the entire plates were based on their
217 anti-rK39 reaction intensities (Figure 2). We did not observe any significant differences in the
218 ELISA performances using the amastigote-derived or culture-adapted promastigote lysates
219 (Pearson correlation coefficient 0.98, $p <0.0001$) (Supplementary Figure S1).

220

221 **IgG1 rapid diagnostic tests discriminate relapse from cure**

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

225 RDT sensitivity for VL (Day 0) was 94.7% (82.3-99.4) and 100% (90.7-100), for 0.1rK RDT and
226 0.6rK RDT, respectively. Of the 21 samples from patients at relapse, 19 were positive with
227 0.6rK RDT, and 18 positive with 0.1rK RDT. With both 0.6rK and 0.1rK RDTs, there was very
228 strong evidence ($p < 0.0001$) for a difference in test positivity between 6 month samples
229 from individuals who relapsed versus 6 month samples from individuals who were cured.

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

239

240 **DISCUSSION**

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

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

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

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

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

270 In terms of future application within a clinical environment, an optimum rK39 concentration
271 will be required. The 0.1 mg/ml concentration produced some negative results and on visual
272 inspection positive test bands were less clear than with the 0.6rK test; the 0.6 mg/ml
273 concentration did not cause increased background or false positives with controls. However,
274 considering the greater cost of manufacture involved it would be appropriate to evaluate
275 intermediate concentrations. Pilot trials indicate that the IgG1 RDT is directly applicable to
276 3.5 µl of finger-prick whole blood in the field (unpublished observations).

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

285

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295

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303

304 **Potential conflicts of interest**

305 All authors: no reported conflicts.

306

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368

369 **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.

370 ^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 (n = 38)	Day 0	6 months ^a	0.1rK	0.6rK
	Positive	Negative	22/38 (57.9%)	26/38 (68.4%)
		Decrease	8/38 (21.1%)	7/38 (18.4%)
		No decrease	6/38 (15.8%)	5/38 (13.2%)
	Negative	Negative	2/38 (5.2%)	0/38 (0%)
		Positive	0/38 (0%)	0/38 (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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386 **Figure 1. Decrease in IgG1 levels of cured patients was more evident and consistent than**
387 **the decline in total IgG, by ELISA.** ELISA results for the rK39 antigen with cured VL paired
388 samples (n = 37 pairs) and relapse samples (n = 20). * indicates very strong evidence for a
389 difference (paired t-test $p < 0.0001$). Strong evidence was also seen between IgG1 and IgG in
390 6 month cured samples ($p < 0.0001$, not depicted).

391

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

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

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