

Short Communication

Serological markers for *Leishmania donovani* infection in Nepal: agreement between direct agglutination test and rK39 ELISA**Basudha Khanal¹, Suman Rijal¹, Bart Ostyn², Albert Picado^{2,3}, Kamlesh Gidwani⁴, Joris Menten², Diane Jacquet², Veerle Lejon², François Chappuis⁵ and Marleen Boelaert²**¹ B.P. Koirala Institute of Health Sciences, Dharan, Nepal² Institute of Tropical Medicine, Antwerp, Belgium³ London School of Hygiene and Tropical Medicine, London, UK⁴ Banaras Hindu University, Varanasi, India⁵ Geneva University Hospitals, Geneva, Switzerland**Summary**

Visceral leishmaniasis (VL) is an important vector-borne disease caused by *Leishmania donovani* in the Indian subcontinent. The actual incidence and role of asymptomatic infections in the region are not well known. We used the direct agglutination test (DAT) and the rK39 ELISA as *L. donovani* infection markers in 10 VL endemic villages in Nepal. DAT titre distribution showed two subgroups in the population (infected and non-infected individuals), while rK39 did not. The agreement between both tests was moderate ($\kappa = 0.53$; 95% CI 0.49–0.57). More research is needed to develop validated markers for *Leishmania* infection.

keywords visceral leishmaniasis, kala-azar, *Leishmania donovani*, infection markers

Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a systemic infection caused by an intracellular protozoan parasite of the genus *Leishmania*. Caused by *Leishmania (Leishmania) donovani* and transmitted by the sandfly *Phlebotomus argentipes*, VL is a major public health problem in the Indian subcontinent (Desjeux 2004). In Nepal, VL is endemic in 12 districts of the southeastern region bordering India (Joshi *et al.* 2008).

Infection with *L. donovani* does not always lead to clinical illness. A substantial number of infected persons remain asymptomatic and may contribute to the disease transmission and maintenance of the parasite cycle (Chappuis *et al.* 2007). Annual incidence rates and prevalence of asymptomatic infection of *L. donovani* are known to be much higher than the frequency of clinical cases (Bern *et al.* 2007), but actual figures on these rates are scanty in the Indian subcontinent (Sinha *et al.* 2008). A better estimation of the incidence of asymptomatic infection is required for a better understanding of the disease dynamics. Some of the available diagnostic methods (i.e. bone marrow aspirate) are ethically inappropriate

for use in non-symptomatic individuals. Serological tests such as the direct agglutination test (DAT) (el Harith *et al.* 1988) and the rK39 enzyme-linked immunosorbent assay (ELISA) (Burns *et al.* 1993) are less invasive tools and have so far been validated for clinical diagnosis, but not yet as markers of infection. The latter is methodologically complex as there is no gold standard for measuring infection. Nevertheless, the DAT and the rK39 ELISA have been used in large field studies (Zijlstra *et al.* 1998; Davies & Mazloumi Gavgani 1999; Koirala *et al.* 2004; Schenkel *et al.* 2006; Bern *et al.* 2007; Saha *et al.* 2009; Rijal *et al.* 2010) as they are relatively simple, cheap and in contrast to the molecular techniques currently available (Bhattarai *et al.* 2009; Moreno *et al.* 2009), they can be conducted on finger-prick samples that increases follow-up rates in healthy individuals and simplifies samples management.

In this paper, we study the agreement between DAT and rK39 ELISA used as *L. donovani* infection markers in a cross-sectional survey of all subjects older than 2 years living in 10 VL endemic villages in Nepal. This population includes non-infected, infected and current as well as past VL cases.

Materials and methods

Study site and participants

Ten VL endemic villages in the eastern districts of Nepal were selected in 2006 on the basis of their high annual VL incidence in the previous 3 years (i.e. minimum 0.8%) as detailed elsewhere (Rijal *et al.* 2010). All subjects older than 2 years were asked to provide a finger-prick blood sample in November 2006. All individuals with a valid blood sample, regardless of their age, gender and past history of VL, were included in the analyses. The blood samples were collected on Whatman no. 3 filter papers. All filter papers were dried, and samples from each household were kept in individual plastic bags with silica gel at -20°C in the B.P. Koirala Institute of Health Sciences (BPKIHS) laboratory until the samples were analysed.

Serological tests

Once removed from the freezer, filter papers were warmed up slowly in their plastic bag to room temperature to avoid condensation. Two 5-mm discs fully covered with blood were punched out from each filter paper. The two discs were eluted overnight at 4°C , one in 1000 μl of DAT dilution buffer (PBS-pH 7.2 supplemented with protein) and one in rK39 ELISA dilution buffer [0.1% BSA (Sigma, St. Louis, MO, USA), 0.1% Tween 20 in PBS] to obtain starting dilutions of 1:400.

The DAT was performed following standard procedures (el Harith *et al.* 1988) and using a freeze-dried version of fixed, trypsin-treated and stained promastigotes of *L. donovani* prepared in ITM-A (Jacquet *et al.* 2006). Hundred microlitres of the eluate was transferred to a well of the first column of a V-shaped microtitre plate (8 \times 12 wells; Greiner, Frickenhausen, Germany). The rest of the wells were then dispensed with 50 μl of DAT diluents with 2-Mercapto-Ethanol (preparation: 0.24 ml 2-ME per vial of 30 ml of DAT-diluent). Using a multichannel micropipette, a serial dilution of 1:400 up to 1:25 600 was obtained by mixing and transferring 50 μl from the first well to the subsequent wells. One positive and one negative control were run every 5th plate. Wells in the last row were kept for antigen control. Fifty microlitres of properly mixed and re-suspended DAT antigen was dispensed to every well. Plates were then sealed, shaken gently and incubated overnight in a horizontal position at ambient temperature.

The DAT results (i.e. agglutinating titre) were read against a white background by two independent readers. In case of discrepancy, the reading of a third reader was decisive. Samples with a titre $\geq 1:1600$ were considered positive. This titre, which is lower than the one used for VL diagnosis in clinical suspects (1:3200), was chosen to

increase the sensitivity to detect *L. donovani* infection (Davies & Mazloumi Gavvani 1999; Saha *et al.* 2009).

The filter paper eluates were tested for antibodies against rK39 antigen based on the ELISA protocols described by Burns *et al.* (1993) and Zijlstra *et al.* (1998). The rK39 antigen was kindly provided by S. G. Reed, Infectious Disease Research Institute (IDRI), Seattle, USA. Flat-bottomed 96-well microtitre plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 25 ng/well of rK39, in a 0.1 M carbonate-bicarbonate buffer pH 9.6. After overnight incubation at 4°C and subsequent washes with PBS with 0.1% of Tween 20 (washing buffer), excess protein binding sites were blocked at room temperature for 2 h with 150 μl /well of blocking buffer (1% BSA in PBS). Plates were washed five times with washing buffer. Hundred microlitres of the filter paper eluates was added to the duplicate wells and incubated for 1 h at room temperature. After five washes, 100 μl of protein-A horse radish peroxidase (Genei, Bangalore, India) diluted 1:16 000 in rK39 dilution buffer were added and incubated for 30 min at room temperature. After six washes with washing buffer, 100 μl of tetra methyl benzidine (TMB- H_2O_2) (Genei, Bangalore, India) was added to each well and left for 15 min at dark. To stop the enzymatic reaction, 100 μl /well of 1N H_2SO_4 was added.

The optical density (OD) was measured at 450 nm using a microtitre plate ELISA reader (Human, Wiesbaden, Germany). Positive (serum diluted at 1:400 from a parasitologically confirmed VL case) and four negative controls (filter paper eluate from non-endemic healthy individuals processed as above) were run in each plate, and the positive control was used as a reference to calculate a relative value of positivity of each sample, expressed as percentage positivity (PP) (Wright *et al.* 1993). The ELISA results (PP) were log-transformed. The Westgard rule was applied for quality control (Westgard *et al.* 1981).

For determining the cut-off, finger-prick blood in filter paper (processed as above) from 37 healthy individuals from a non-endemic VL area (NEH) in Nepal was analysed as described earlier. The 37 samples were run 17 times, and the threshold for *L. donovani* infection was set up as the maximum log PP + mean 3 Standard Deviations (SD) within all re-runs to account for remaining plate to plate variation and for maximum specificity.

Data analyses

Data were double entered in an EPI INFO 2000 database (CDC, Atlanta, GA, USA). The frequency of DAT and rK39 ELISA results per agglutinating titre and log PP value, respectively, were plotted. A box plot and Spearman's correlation coefficient were used to plot and test the

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relationship between DAT titres and log rK39 PP values, respectively. The strength of agreement between the DAT and the rK39 ELISA results, dichotomised as positive-negative, was determined by the Kappa coefficient. All tests and graphics were produced using R 10.1.2 (<http://www.r-project.org>).

Ethical considerations

The data presented here were collected as part of the KALANET community intervention trial (Clinicaltrials.gov CT-2005-015374). Ethical clearance for the study was granted from the ethical committees of BPKIHS, University of Antwerp and London School of Hygiene and Tropical Medicine. Written informed consent was obtained from all participants, or guardians for individuals under 18 years old, before enrolment.

Results & Discussion

Blood samples were obtained from 5397 individuals. The study population included 277 individuals reporting a past episode of VL. Demographic and epidemiological characteristics of the study population are presented elsewhere (Rijal *et al.* 2010). Using a titre $\geq 1:1600$ as a cut-off, 9.1%

(491/5397) of the individuals were considered DAT-positive. This prevalence is similar to levels reported in previous surveys using DAT as a marker of infection in Nepal (Koirala *et al.* 2004; Schenkel *et al.* 2006). The distribution of DAT results was clearly bi-modal and discriminated between individuals infected, currently or in the past, with *L. donovani* (titres $\geq 1:1600$) and the non-infected population (titres $< 1:1600$) (Figure 1a).

In contrast to the DAT distribution, the distribution of rK39 results from the study population was more continuous and presented as a mixture of two normal curves with a broad overlap between the two subpopulations (median: 2.8 log PP, SD: 0.5) (Figure 1b). The rK39 ELISA results of the non-endemic individuals ranged from 1.7 to 2.6 log PP (mean = 2.2 log PP, SD = 0.24), giving a cut-off value of 3.4 log PP. Of 5221 samples analysed – 176 did not have enough blood for a second test – 12.6% ($n = 657$) were considered positive (Figure 1b). The proportion of infected individuals in ELISA was higher than that obtained by DAT despite the use of the maximum log PP as threshold. Previous studies have reported difficulties to determine a cut-off for rK39 ELISA (Kurkjian *et al.* 2005). A study using rK39 ELISA in Bangladesh used a similar approach to set up the infection threshold by taking the 99th percentile of readings from 38 NEH (Kurkjian *et al.* 2005).

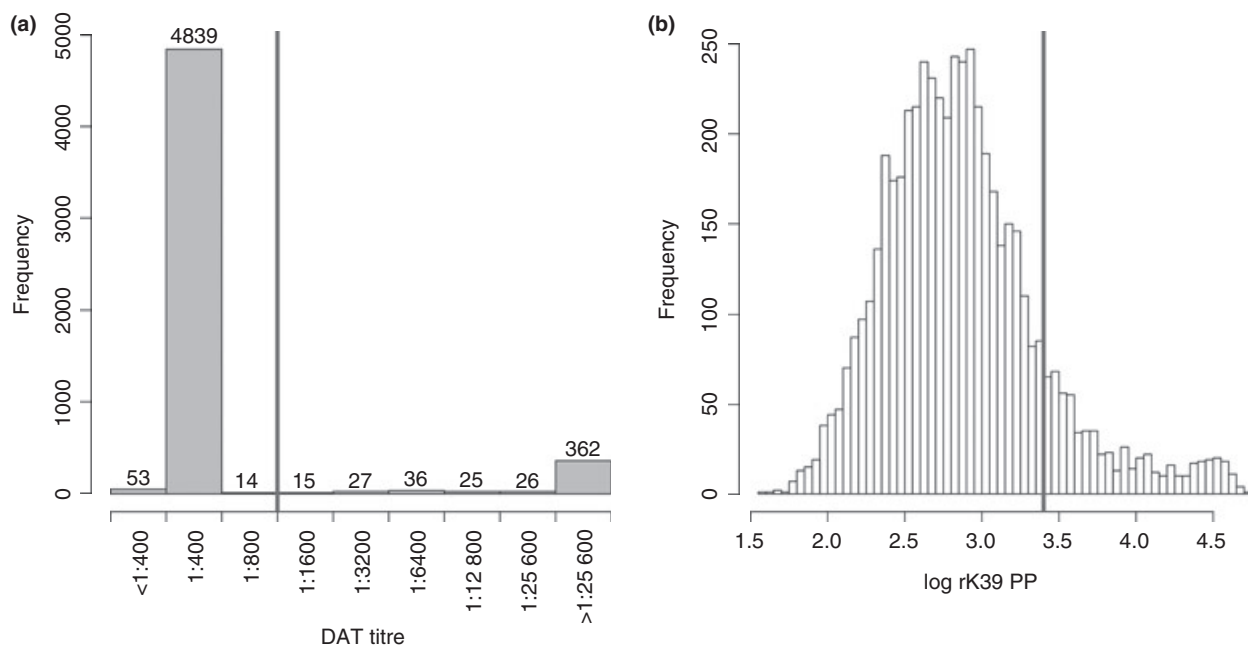


Figure 1 (a) Histogram presenting the distribution of the direct agglutination test (DAT) results per agglutinating titre with number of samples per titre on the top of the bars. The cut-off to identify *Leishmania donovani* infections ($\geq 1:1600$) is noted with a vertical line. (b) Distribution of the rK39 ELISA results presented as frequency of percent positivity (PP). The cut-off value used (3.4 log PP) is noted with a vertical line.

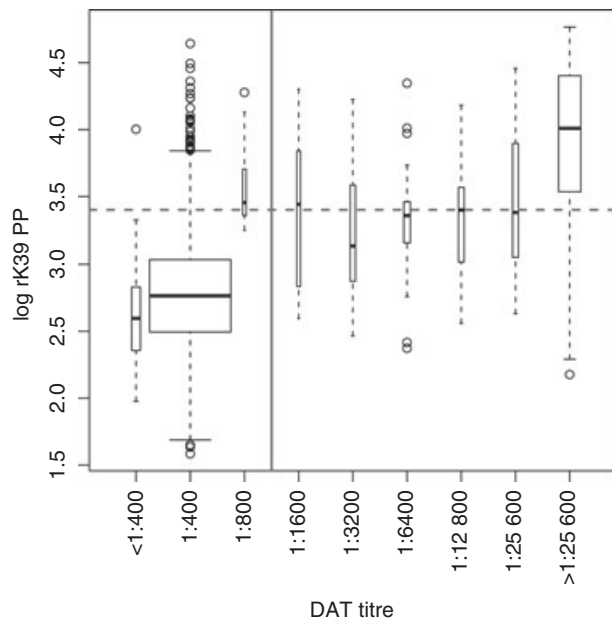
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Figure 2 Boxplot of rK39 ELISA results in function of direct agglutination test (DAT) titre. Boxes' widths are proportional to the square roots of the number of observations. The cut-off values used for rK39 ELISA (3.4 log PP) and direct agglutination test (DAT) ($\geq 1:1600$) are noted as horizontal (dotted) and vertical lines, respectively.

Table 1 Two-by-two table showing the number of positive and negative samples using the direct agglutination test (DAT) and rK39 ELISA and applying titre $\geq 1:1600$ and 3.4 log Percent Positivity (PP) as cut-off values for DAT and ELISA, respectively

		rK39 ELISA Cut-off		Total
		3.4 log PP		
		Negative	Positive	
DAT Cut-off	Negative	4426	335	4761
	Positive	138	322	460
	Total	4808	413	5221

The plot of rK39 ELISA results in function of DAT titre shows some agreement (Figure 2) but had a low correlation coefficient (Spearman's $\rho = 0.40$ P -value < 0.001). When the results were dichotomised, the agreement between tests was moderate ($\kappa = 0.53$; 95% CI 0.49–0.57) (Table 1). The Kappa value was not significantly modified when 1:3200 was used as cut-off for DAT ($\kappa = 0.52$; 95% CI 0.48–0.56 – data not shown). The differences observed between tests may be caused by the different nature of antigen used: whole-cell promastigote in the DAT and recombinant antigen predominantly found in amastigote, the tissue form of *L. donovani* in rK39 ELISA. DAT and

rK39 ELISA may detect different antibodies which may be expressed in different levels or have different kinetics (Hailu 1990) in asymptomatic individuals and past VL cases. Moreover, a cross-sectional survey will include a mix of infected persons: those with recent infections (incident) and others who have been infected at varying time-points in the past. DAT and rK39 ELISA may not necessarily measure those in the same way. Variable cross-reactivity with malaria has been reported for rK39 ELISA: ranging from 0% to 83% (Kumar *et al.* 2001; Carvalho *et al.* 2003; Pedras *et al.* 2008; Romero *et al.* 2009) and for DAT: ranging from 0% to 10% (Sundar *et al.* 2006; Pedras *et al.* 2008). However, its effect in this study's outcomes should be limited by the low malaria incidence reported in the study population (estimated annual incidence 0.03%, Picado *et al.* unpublished data). Molecular methods to detect asymptomatic leishmanial infections, which are effective in Brazil (Moreno *et al.* 2009) and Nepal (Bhattarai *et al.* 2009), may overcome this pitfall and seem to detect more recent infections than DAT (Bhattarai *et al.* 2009). However, their current technical requirements limit their use in large-scale field studies.

Even if rK39 ELISA was inaccurate for diagnosing asymptomatic *L. infantum* infections in Brazil (Moreno *et al.* 2009), it was successfully used to report *L. donovani* infections in Sudan (Zijlstra *et al.* 1998) and Bangladesh (Bern *et al.* 2007). Similarly, the DAT has been used in epidemiological studies assessing *L. donovani* infection in endemic populations (Davies & Mazloumi Gavvani 1999; Koirala *et al.* 2004; Schenkel *et al.* 2006; Saha *et al.* 2009; Rijal *et al.* 2010). This study was not designed to evaluate the accuracy (validity) of these two tests to demonstrate infection but to study their level of agreement when they are used cross-sectionally in endemic populations. The results of this paper have implications for trial design and interpretation of published data. Nevertheless, all current and potential *L. donovani* infection markers need validation in prospective studies.

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