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Molecular and serological markers of *Leishmania donovani* infection in healthy individuals from endemic areas of Bihar, India

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Abstract

OBJECTIVES Recent epidemiological reports indicate that asymptomatic human infections with *Leishmania donovani*, the causative agent of visceral leishmaniasis or Kala-azar (KA), occur frequently in India. We explored markers of infection.

METHODS Blood samples were collected from 286 healthy subjects from 16 villages in the Muzaffarpur district of Bihar. These individuals were classified into three groups: (i) persons with no history of KA and living in a house where no KA cases were previously reported, (ii) persons with no history of KA but living in a house where KA cases were diagnosed at the time of sampling or in the past, and (iii) successfully treated KA patients. Each sample was tested using a *Leishmania*-specific PCR to detect *Leishmania* DNA, and two serological tests to demonstrate anti-*Leishmania* antibodies: the Direct Agglutination Test and rK39 ELISA.

RESULTS PCR positivity was similar among the three groups (20–25%). In contrast, among treated patients, the percentage of serologically positive individuals was roughly five times that of healthy individuals with no KA history, as measured with either test. Living in a house where KA had been reported did not affect seropositivity.

CONCLUSION A significant proportion of asymptomatic infections of *Leishmania* exist in endemic regions. Using a combination of molecular and serological tests increases the capacity to detect infections at different stages. Further work is required to understand the kinetics of the markers.

keywords PCR, Direct Agglutination Test, rK39 ELISA, asymptomatic, visceral leishmaniasis kala-azar

Introduction

Leishmania donovani is the aetiological agent of Visceral Leishmaniasis, also known as Kala-azar (KA), on the Indian subcontinent. The state of Bihar in India is one of the most important foci in the world for KA (Alvar *et al.* 2012; Sundar & Chakravarty 2012). The transmission cycle on the Indian subcontinent is anthroponotic (Alvar *et al.* 2004). In humans, *L. donovani* causes a wide spectrum of clinical manifestations, from asymptomatic infection to full-blown disease (D'Oliveira Junior *et al.* 1997). In the last few decades, epidemiological reports from India have shown that asymptomatic infections with *L. donovani* are frequent (Chappuis *et al.* 2007). In the context of zoonotic KA, it was suggested that asymptomatic carriers of *L. infantum* (syn. *L. chagasi*) can act as reservoirs (Costa *et al.* 2002), but it is unknown if this could be the case in anthroponotic KA. Serological methods are simple and rapid for detecting KA. In many instances, serological tests may fail to detect asymptomatic *Leishmania* infections (le Fichoux *et al.* 1999; Costa *et al.* 2002; Riera *et al.* 2004). PCR may be an additional useful tool as shown in a recent study from Nepal (Bhattarai *et al.* 2009) as it can confirm the presence of parasites (Prina *et al.* 2007). In this study, in addition to PCR, two serological tests, the Direct Agglutination Test (DAT) and rK39 ELISA, were applied to document the asymptomatic population in a KA endemic focus of Bihar. Our aim was to determine the prevalence of asymptomatic human infection with *L. donovani* by

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using PCR-based assays as evidence of recent or current infection and to study agreement between the three markers of infection: PCR, DAT and rK39 ELISA.

Materials and methods

Study population and sample collection

Within the framework of the EC-Kalanet project (ClinicalTrials.gov NCT00318721), 16 rural villages (clusters) were selected in Muzaffarpur district of Bihar State in 2006 (Picado *et al.* 2010). In each cluster, blood samples were taken from approximately 20 subjects >15 years old from three groups: (i) healthy non-KA (HNK): healthy individuals with no known history of KA and not living in the same house as known KA cases, (ii) healthy household contacts (HHC): a sample of healthy household contacts of current or past KA cases (up to 10 years), and (iii) healthy past KA (HPK): individuals who had previously suffered of KA and had been successfully treated (Bhattarai *et al.* 2009). Venous blood was collected in K_2-EDTA vacutainers (BD Biosciences, USA) for PCR. Finger prick blood samples were collected on Whatmann no. 3 filter papers for serology tests (Picado *et al.* 2010). Filter papers were dried and then kept in plastic bags with silica gel and transported to the laboratory for storage at −20 °C until use.

Sample preparation

For the serology tests (DAT and rK39 ELISA), two 5-mm discs fully covered with blood were punched out from each filter paper. To obtain dilutions of 1:400, one disc was diluted in 1000 μl of DAT dilution buffer (PBS-pH 7.2 supplemented with protein) and one in 1000 μl rK39 ELISA dilution buffer (0.1% BSA (Sigma, St. Louis, USA), 0.1% Tween 20 in PBS), and they were kept overnight at 4 °C.

For the molecular test, DNA was extracted from 200 μl blood using the DNA Blood Minikit following the manufacturer's protocol (Qiagen, Hilden, Germany) and stored at −20 °C. Elution buffer was included in each series as DNA extraction control.

Laboratory tests

For the PCR amplification assay, the primer pair sense 18S-L-F 5′-CGTAGTTGAACTGTGGGCTGTGC- 3′ and the antisense 18S-L-R 5′-ACTCCCGTGTTTCTTGTTTC TTTGAA-3′ was used for amplification of a 115-bp fragment of the 18S rRNA gene using ABI 9700 thermo cycler (Applied Biosystems, Foster city, USA) as described elsewhere (Deborggraeve *et al.* 2008). This very sensitive assay (sensitivity-92.1% and specificity-99.64%, Deborggraeve *et al.*) is able to detect one parasite in 200 μl of blood and 10 fg purified DNA (equivalent of 0.05 parasite genomes). The PCR products were run on a 2% agarose gel and visualised in the gel documentation system (Alpha-Innotech, USA). Samples were considered positive after visualising 115 bp bands in the gel. Negative samples were subjected to the same PCR assay in a second round, using 1/10 dilution of template DNA to reduce potential inhibitory effects. Samples that remained negative after this second round were submitted to an inhibition control by co-amplifying the corresponding DNA sample together with 0.1 pg of DNA (this DNA should always amplify) from a cultured reference *L. donovani* strain MHOM/NP/02/BPK043/0, described by Rijal *et al.* (2007). Positive and negative controls were included in each batch of PCR. The two positive controls consisted of 1 ng and 0.1 pg of *L. donovani* MHOM/NP/02/ BPK043/0 DNA, while milli-Q water was included as negative control. Amplicons were sequenced on an ABI3130 sequencer (Applied Biosystems, Foster city, USA), and BLAST analysis (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) revealed that the obtained sequences correspond to the 18S rRNA gene of *Leishmania*.

For the Direct Agglutination Test, commercially available freeze dried DAT antigen was procured from the Institute of Tropical Medicine, Belgium. DAT was performed following standard procedures (el Harith *et al.* 1998). Briefly 100 μl of 1:400 eluates was serially diluted down to 1:25 600 in V-shaped microtitre plates in DAT diluents. One positive control serum obtained from a parasitologically confirmed patients with a DAT titre of 1:102 400, and one negative control from a subject from non-endemic area for KA with a DAT titre of less than 1:200, was run every fifth plate. Wells in the last row were kept for antigen control. 50 μl of DAT antigen was dispensed to every well (Jacquet *et al.* 2006). Plates were then covered, shaken gently and incubated overnight at room temperature. The DAT results were read against a white background. Samples with a titre $\geq$1:1600 were considered positive (Davies and Mazloumi Gavgani 1999; Saha *et al.* 2009).

The rK39 ELISA was performed as described elsewhere (Burns *et al.* 1993; Zijlstra *et al.* 1998; Khanal *et al.* 2010). Briefly, flat-bottom 96-well microtitre plates were coated with 25 ng/well of rK39 antigen in coating buffer and incubated overnight at 4 °C. The plates were then blocked with blocking buffer (1% BSA in 0.05 M phosphate buffer) for 2 h at room temperature. Plates were loaded with serum sample diluted 1:400 and incubated at room temperature for 1 h. The plates were washed five

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times with PBS containing 0.1% Tween-20 (pH 7.4) and incubated with peroxidase-conjugated goat antihuman IgG (1:32 000 dilution in serum dilution buffer) at 37 °C for 1 h. Then, the plates were incubated with TMB substrate (GeneI, Bangalore, India) for 5 min at room temperature in the dark. Finally, the reaction was stopped with 0.1 N H_2SO_4. The optical density was measured at 450 nm. Each sample was assayed in duplicate (Vaish *et al.* 2011). The optical density (OD) was measured at 450 nm using a microtitre plate ELISA reader (Molecular Devices, USA). A positive (parasitologically confirmed KA case) and a negative controls [filter paper eluate from non-endemic healthy control, (NEHC)] 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 cut-off was set up as the maximum log PP + mean 3 standard deviations (SD) from 37 NEHC run 15 times. The cut-off for rK39 ELISA was set at 3.1 log PP. For quality control, the Westgard rule was applied (Westgard *et al.* 1981).

Statistical analysis

The proportions of positive samples among the different study groups and tests were compared using the chi-square test. Similarly, the proportions of DAT and PCR-positive samples per study group were compared with those obtained in a similar study in Nepal (Bhattarai *et al.* 2009) using the chi-square or fisher exact tests. $P < 0.05$ were considered statistically significant.

Positive (Pr+) and negative (Pr-) agreement indices between the two tests (PCR *vs.* DAT, PCR *vs.* rK39 ELISA and DAT *vs.* rK39 ELISA) within each group were calculated as described by Graham and Bull (1998). The following formula was used:

$$\text{Pr+} = 2a/(2a + b + c) \textit{ and } \text{Pr-} = 2d/(2d + b + c)$$

where a, b, c and d in the formula are the variables that represent the observed frequencies for each possible combination of ratings by test 1 and test 2: a, representing the number of samples positive with both the tests; b, the number of samples negative with test 1 and positive with test 2; c, the number of samples positive with test 1 and negative with test 2; d, the number of samples negative with both tests. The positive agreement index estimates the conditional probability, given that one of the test results is positive, the other will also be positive. The negative agreement index estimates the conditional probability, given that one of the test results is negative, the other will also be negative. Credibility intervals (95%) were estimated as described by Samsa (1996) using the formula Pr+ (Positive agreement index)−z critical * standard error (SE) for lower CI and Pr+ + z critical * SE for upper CI (Samsa, 1996). Similarly we put Pr-value in formula for calculating 95% CI of negative agreement index (Pr-).The value of Z critical for 95% CI is 1.96.

SE was calculated as follows, with n standing for the total number of samples:

$$[\text{SE}(P+) = (\text{Pr} + *(1 - \text{Pr+})/(a + b)) * *.5/(n) * *.5]\text{and}$$

$$[\text{SE}(P-) = (\text{Pr-} * (1 - \text{Pr-})/(c + d)) * *.5/(n) * *.5]$$

Here n = Total number of samples.

Ethical considerations

Ethics Committees of the Institute of Medical Sciences (Banaras Hindu University) and the University of Antwerp approved the study. Written informed consent was obtained from the study participants. For persons under 18, the informed consent was obtained from adult relatives.

Results

Laboratory tests results

Blood samples were provided by 286 individuals, ranging from 6 to 26 per study cluster. The mean age of the participants was 28.7 years old and 39.2% were male. Demographic and epidemiological characteristics of the study population are described in detail elsewhere (Singh *et al.* 2010). Participants were divided as follows in the three study groups: 150 (52.4%) HNK, 97 (33.9%) HHC and 39 (13.6%) HPK. One participant (1/286) only had 2 test results (i.e. DAT and PCR) as there was not enough blood to conduct the rK39 ELISA. The laboratory results are summarised in Table 1. Overall, 23.1% (n = 66) of the subjects were PCR positive, and none of them presented symptoms of KA when blood samples were collected. The amplicon was sequenced and was 100% identical with *Leishamia* SSU gene. The primer used is highly specific and very well validated in many countries. (Deborggraeve *et al.* 2008; Bhattarai *et al.* 2009).

Serology revealed that 24.5% (n = 70) and 26.3% (n = 75) of participants were DAT and rK39 ELISA positive, respectively. The differences between tests were not statistically significant. The proportions of PCR-positive samples per study group were similar: 24.6% in HNK 20.6% in HHC and 23.0% in HPK. The proportion of

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Table 1 Comparative results of PCR, DAT and rK39 ELISA from India and Nepal Proportion (%) of samples positive per test: PCR, Direct Agglutination Test (DAT) and rK39 ELISA and per study group: (i) HNK – persons with no history of kala-azar (KA) and not living in the same house as known KA cases, (ii) HHC – healthy household contacts, and (iii) HPK – successfully treated past KA cases. The PCR and DAT results are compared with those obtained in a similar study in Nepal (Bhattarai *et al.* 2009)

	PCR positives: % (N)	DAT positives: % (N)	rK39 ELISA positives: % (N)‡
India			
HNK	24.6% (37/150)	14.0% (21/150)	18.1% (27/149†)
HHC	20.6% (20/97)	14.4% (14/97)	19.5% (19/97)
HPK	23.0% (9/39)	89.0% (35/39)	74.3% (29/39)
Total	23.1% (66/286)	24.5% (70/286)	26.3% (75/285)
Nepal*			
HNK	17.6% (32/182)	5.4% (10/182)	NA
HHC	12.5% (3/24)	20.8% (5/24)	NA
HPK	26.1% (6/23)	95.0% (22/23)	NA
Total	18.0% (41/229)	16% (37/229)	NA

NA, not applicable; HHC, healthy household contacts; HNK, healthy non-kala-azar; HPK, healthy past kala-azar.
*PCR and DAT results reported in a similar study in Nepal (Bhattarai *et al.* 2009).
†rK39 ELISA result of 1 sample is not available.
‡Results of rK39 ELISA are only from India.

serologically positive individuals was higher in the HPK group (89.0% and 74.3% for DAT and rK39 ELISA respectively) than in the HHC and HNK groups. The differences in positivity among the 3 study groups (HNK, HHC and HPK) were statistically significant for DAT ($P < 0.001$) and rK39 ELISA ($P < 0.001$). When the PCR results were compared with a similar study in Nepal (Bhattarai *et al.* 2009), the proportion of PCR positives was not significantly different between countries in HNK, HHC or HPK groups. Similar results were obtained in HHC and HPK groups when the proportion of DAT-positive samples was compared between countries.

The agreement indices and their 95% credibility intervals between tests are presented in Table 2. The positive agreement indices between the tests (PCR, DAT and rK39 ELISA) were consistently higher in the HPK group than those obtained in other groups. Among HNK and HHC, the agreement was negative between PCR DAT and between DAT-rK39 ELISA ranging from 27.6% to 36.0%; in other words, a large proportion of the HNK and HHC subjects positive with one of the methods were negative with the other one.

We compared PCR with DAT and ELISA positivity in each of the 16 clusters, and a broad range of positivity

Table 2 Percentage agreement indices: Positive and negative agreement indices (AI) between PCR, Direct Agglutination Test (DAT) and ELISA in the three study groups: (i) HNK: persons with no history of kala-azar (KA) and not living in the same house as known KA cases, (ii) HHC: healthy household contacts, and (iii) HPK: successfully treated past KA cases

Groups		% Positive AI	% Negative AI
HNK	PCR *vs*. DAT	17.9 (5.5–30.2)	36.0 (27.1–44.8)
	PCR *vs*. ELISA	20.9 (5.5–36.2)	25.0 (17.3–32.6)
	DAT *vs*. ELISA	15.8 (2.0–29.5)	27.6 (19.6–35.5)
HHC	PCR *vs*. DAT	17.0 (0.5–33.4)	30.8 (20.4–41.0)
	PCR *vs*. ELISA	20.1 (2.5–37.6)	29.5 (19.2–39.6)
	DAT *vs*. ELISA	16.6 (0.0–33.3)	29.4 (19.3–39.5)
HPK	PCR *vs*. DAT	36.7 (5.2–68.2)	5.8 (0.0–14.1)
	PCR *vs*. ELISA	35.2 (4.0–66.4)	13.3 (1.1–25.4)
	DAT *vs*. ELISA	81.3 (67.1–95.5)	37.3 (7.3–67.2)

HHC, healthy household contacts; HNK, healthy non-kala-azar; HPK, healthy past kala-azar.

variation was observed (Table 3). However, small sample size in some clusters might influence the result.

Discussion

In this study, molecular and serological methods were combined to detect asymptomatic *L. donovani* infections in KA endemic villages in Bihar, India. In South Asia, the serological tests, used in this study, have high sensitivity and specificity, and for DAT, it is 97.1% and 85.6%, respectively (Chappuis *et al.* 2006). In a large study from our lab, the sensitivity of rK39 ELISA was 99.6%, and the specificity was 100%, 92.23% and 96.59% for non-endemic and endemic healthy controls and different diseases, respectively (Vaish *et al.* 2011). Instead of the field friendly rK39 rapid test, we used ELISA to detect anti-K39 antibodies for lack of data and manufacturer's recommendations for using blood eluted from filter papers.

Despite obtaining similar overall results for PCR, DAT and rK39 ELISA, differences were observed among study groups and tests, both in the proportion of positives and the agreement between tests. Serological tests had an analogous behaviour: (i) similar proportion of positives in HNK and HHC groups, and (ii) high percentage of positives in past KA cases. However, the agreement between serological tests was in general poor except in the HPK group. Those results would confirm that the sensitivity to detect asymptomatic *L. donovani* infections differs between PCR and serological tests (Bhattarai *et al.* 2009) as well as between DAT and rK39 ELISA (Khanal *et al.* 2010). These differences may be related to the

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Table 3 Cluster wise PCR, DAT and rK39 Elisa positivity in the three study groups

	HNK			HHC			HPK			Total		
Cluster	PCR	DAT	rK39	PCR	DAT	rK39	PCR	DAT	rK39	PCR	DAT	rK39
C01	1+/12	0+/12	3+/12	2+/4	1+/4	1+/4	0+/4	4+/4	2+/4	3+/20	5+/20	6+/20
C02	4+/8	1+/8	1+/8	2+/9	3+/9	4+/9	2+/4	4+/4	4+/4	8+/21	8+/21	9+/21
C03	3+/12	0+/12	1+/12	4+/8	1+/8	0+/8	0+/3	3+/3	3+/3	7+/23	4+/23	4+/23
C04	2+/12	1+/12	1+/12	1+/7	0+/7	0+/7	0+/1	1+/1	0+/1	3+/20	2+/20	1+/20
C05	4+/11	2+/11	4+/11	0+/8	1+/8	2+/8	1+/1	1+/1	1+/1	5+/20	4+/20	7+/20
C06	3+/12	1+/12	0+/12	4+/11	4+/11	1+/11	1+/3	2+/3	2+/3	8+/26	7+/26	3+/26
C08	1+/2	1+/2	0+/2	0+/3	0+/3	0+/3	0+/1	1+/1	1+/1	1+/6	2+/6	1+/6
C09	6+/9	3+/9	3+/9	2+/8	0+/8	0+/8	2+/6	4+/6	4+/6	10+/23	7+/23	7+/23
C10	1+/12	5+/12	3+/11	1+/2	0+/2	0+/2	0+/0	0+/0	0+/0	2+/14	5+/14	3+/13
C11	3+/14	1+/14	0+/14	1+/8	1+/8	2+/8	0+/0	0+/0	0+/0	4+/22	2+/22	2+/22
C12	0+/3	0+/3	0+/3	0+/9	1+/9	3+/9	0+/5	5+/5	2+/5	0+/17	6+/17	5+/17
C13	2+/15	2+/15	8+/15	0+/5	0+/5	2+/5	0+/2	2+/2	2+/2	2+/22	4+/22	12+/22
C14	1+/10	2+/10	1+/10	0+/4	1+/4	1+/4	0+/2	2+/2	2+/2	1+/16	5+/16	4+/16
C15	2+/6	1+/6	1+/6	2+/4	1+/4	2+/4	1+/3	3+/3	3+/3	5+/13	5+/13	6+/13
C16	4+/12	1+/12	1+/12	1+/7	0+/7	1+/7	2+/4	3+/4	3+/4	7+/23	4+/23	5+/23
Total	37+/150	21+/150	27+/149	20+/97	14+/97	19+/97	09+/39	35+/39	29+/39	66+/286	70+/286	75+/285

DAT, Direct Agglutination Test; HHC, healthy household contacts; HNK, healthy non-kala-azar; HPK, healthy past kala-azar. Result of one sample (C10) for rK39 Elisa is not available.

techniques as well as the kinetics of the makers targeted (i.e. antibodies *vs*. antigens) and have practical implications.

The role of PCR-positive asymptomatic individuals in *L. donovani* transmission has not been established in the Indian subcontinent, but a recent mathematical model suggests a major role of asymptomatics in driving transmission (Stauch *et al.* 2011). Nevertheless, the percentage of PCR-positive individuals was also significant in household contacts and past KA cases. The cross-sectional design of this study does not allow establishing if these results are due to incident or past infections. Prospective studies should allow evaluating the use of PCR as a sensitive marker for recent or ongoing infections (Bhattarai *et al.* 2009). The PCR results for all three studied groups are similar to those reported in Nepal (Bhattarai *et al.* 2009). The PCR-positive prevalence was higher in our study than in previous reports from India: 7.9% (Topno *et al.* 2010) and 10% (Maurya *et al.* 2005). The differences may be due to differences in the study population (i.e. high endemic KA villages in our study), sample size (n = 20 in Maurya *et al.* 2005) or the sensitivity of the PCR used.

The serological results confirm that a large proportion of past KA patients (HPK) remain serologically positive after cure, 89% and 74.3% for DAT and rK39 ELISA, respectively. Similar results were observed in previous studies in the Indian subcontinent, Brazil or Africa (Gidwani *et al.* 2011). As expected, the proportion of serologically positive individuals in the other two groups was lower than for past KA (HPK). But surprisingly, it was similar for people living in houses where KA cases had been reported and asymptomatics from houses with no KA cases (Table 1). Those results contrast with those obtained in Nepal, where the proportion of DAT positive was 4 times higher in HHC than HNK (Table 1). Those results may be partially explained by the differences in *L. donovani* infection and KA dynamics in both countries: India has higher incidence of KA cases and prevalence of DAT-positive individuals than Nepal (Picado *et al.* 2010).

To our knowledge, this is the first report on the agreement of DAT and rK39 ELISA results from asymptomatic individuals in India. In Nepal, when both tests results were applied in a large population (n = 5397) from KA endemic populations, the agreement was moderate (Kappa= 0.53) (Khanal *et al.* 2010). In our study, the sample size was smaller (n = 286), and the population was divided into three groups based on the KA history of individuals and households. The agreement between DAT and rK39 varied among those groups (Table 2). In general, both tests were able to identify as serologically positive the same individuals with KA history (agreement 81.3% in HPK). However, the agreement between DAT and rK39 ELISA positives in the other two groups (i.e. HHC and HNK) was poor (Table 2). The differences between those serological tests may be due to differences in sensitivity of DAT and rK39 ELISA related to differences in the nature of the antibody used or in the type of antigen detected by both tests (Khanal *et al.* 2010).

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The variations in positivity among clusters could be due to different rates of infection in classical rural and periurban villages at the time of sampling. However, the sample size (20) in each cluster is relatively low.

We observed no PCR positivity in cluster 12 (Table 3), which excludes the possibility of the current/new infection in this focus. However, in the same cluster 33% seropositives indicate the past infection. Similarly, in cluster 13, seropositivity was 2–5 times higher than PCR positive. In cluster 03, both HHC and HNK groups have significantly higher PCR positives. These results suggest that thorough screening of clusters, especially cluster 03, 12 and 13 should be pursued as it may shed light on the better understanding of transmission dynamics which still remains poorly understood.

The results of this study would suggest that applying DAT and rK39 ELISA in parallel would increase the capacity to detect *L. donovani* infected individuals from HHC and HNK groups. The agreement between PCR and the serological tests (DAT or rK39 ELISA) was consistently poor in the positive results in HHC and HNK groups but not for past KA cases (HPK). These results were analogous to those obtained in a similar study in Nepal (Bhattarai *et al.* 2009). Nevertheless, serological tests may also be useful as marker of recent infections as shown in a recent study in India and Nepal where DAT seroconversion was associated to an increased risk of developing KA (Ostyn *et al.* 2011). The poor agreement between molecular and serological tests to detect asymptomatic infections has also been reported in Brazil (Moreno *et al.* 2009). Based on their results, we suggest that PCR should be used as a marker of *L. infantum* infection in asymptomatic individuals in epidemiological studies in Brazil.

The fact that this study is based on a single cross-sectional survey does not allow concluding which is the best test to identify new infections. The DAT seroconversion has been successfully used as epidemiological tool in longitudinal studies (Picado *et al.* 2010) but the use of PCR is still limited. Prospective studies will allow determining the value of molecular techniques as a marker of incident *L. donovani* infection. Nevertheless, the results of this study highlight the added value of using a combination of tests (molecular and serological) to increase the capacity to detect asymptomatic infections at different stages. Further work should also consider the added value of assays probing cellular immunity.

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