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PCR and direct agglutination as *Leishmania* infection markers among healthy Nepalese subjects living in areas endemic for Kala-Azar

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Summary

OBJECTIVE To compare a PCR assay and direct agglutination test (DAT) for the detection of potential markers of *Leishmania* infection in 231 healthy subjects living in a kala-azar endemic focus of Nepal.
METHODS The sample was composed of 184 (80%) persons without any known history of KA and not living in the same house as known kala-azar cases (HNK), 24 (10%) Healthy Household Contacts (HHC) and 23 (10%) past kala-azar cases which had been successfully treated (HPK).
RESULTS PCR and DAT positivity scores were, respectively: HNK, 17.6% and 5.6%; HHC, 12.5% and 20.8%; HPK, 26.1% and 95.7%. The ratio PCR-positives/DAT-positives was significantly higher in HNK (ratio = 3.1) than in HHC (ratio = 0.6, $P = 0.036$) and in HPK (ratio = 0.2, $P = 0.012$). The ratio PCR-positives/DAT-positives did not significantly differ between HHC (ratio = 0.6) and HPK (ratio = 0.2, $P = 0.473$). The positive agreement index between PCR and DAT in HNK was 5%; in HHC, 0%; in HPK, 43%.
CONCLUSIONS Our study highlights the specific character of PCR and DAT for the exploration of *Leishmania* asymptomatic infections. PCR is probably more informative for very recent infections among HNK, while DAT provides more information among HHC and HPK, a feature likely related to the power of serology to track less recent infections.

keywords Kala-azar, Nepal, PCR, DAT, asymptomatic infections

Introduction

Visceral leishmaniasis (VL) or kala-azar (KA) is a major vector-borne disease occurring in 51 countries worldwide with an estimated annual incidence of 500 000 clinical cases, 90% of whom live in India, Bangladesh, Nepal, Sudan and Brazil (Desjeux 1996). Two parasite species of the *donovani* complex constitute the etiological agents, *Leishmania donovani*, essentially encountered in the Indian sub-continent and East Africa and *L. infantum* (syn. *L. chagasi*), endemic elsewhere (Lukes *et al.* 2007). Epidemiological patterns are described as different between species, *L. donovani* being transmitted by sandflies from man to man without a known animal reservoir (anthroponotic visceral leishmaniasis, AVL) and *L. infantum* being essentially encountered in canids and occasionally humans (zoonotic visceral leishmaniasis, ZVL).

VL, the most severe form of leishmaniasis, is fatal without treatment. The number of annual deaths attributed to VL is estimated at 59 000 (Desjeux 1996). However, *Leishmania* of the *donovani* complex are no systematic killer pathogens, as asymptomatic infections appear to be very common (Chappuis *et al.* 2007). This is very well documented in the Mediterranean region, where 30–100

subclinical infections are estimated for every clinical case of VL (Pampiglione *et al*. 1975), while information is scantier in the Indian sub-continent (Bern *et al*. 2007; Sinha *et al*. 2008). So far, the VL control programs in this region do not target the asymptomatic carriers of the infection, as there is no drug safe enough to administer in this group, and as there are in fact no validated tools to detect such carriers in the population.

Detection of asymptomatic carriers requires adequate infection markers. The direct microscopic examination and/or culture of tissue aspirates from bone marrow or spleen, which are considered as a gold standard technique for diagnosis, are ethically inappropriate among asymptomatics due to the intrusive nature of the sampling procedures. Leishmania skin test represents an alternative option, but in a recent study performed in India, a serious deficit of sensitivity was observed (Gidwani *et al*. in press). Antibody detection is often used for estimating the rate of infection, but generally cannot distinguish present from past infections (Hailu 1990, De Almeida Silva *et al*. 2006). Direct detection of parasites' DNA by PCR represents an interesting alternative: because of its high sensitivity, the method may be applied to blood samples (Salotra *et al*. 2001; Riera *et al*. 2004) and used for large scale epidemiological monitoring. Furthermore, because of the short half-life of DNA in the body (24 h) (Prina *et al*. 2007), its presence indicates the current (or very recent) presence of living parasites. Sensitivity and specificity of serology and PCR are well documented in the clinical context of AVL (Chappuis *et al*. 2006; Deborggraeve *et al*. 2008), but their performance and value for detecting asymptomatic infection is less known. In the present study, we explored the contribution of a PCR assay and direct agglutination test (DAT) for the detection of potential markers of *Leishmania* infection in healthy subjects living in a Kala-Azar (KA) endemic focus of Nepal.

Materials and methods

Selection of study site

The study was conducted in the frame of community intervention trial with long lasting insecticide impregnated bednets (Kalanet project: http://www.kalanetproject.com) in Eastern districts of Nepal (Terai region), close to a tertiary care centre, B. P. Koirala Institute of Health Sciences (BPKIHS), Dharan. In Nepal, each village is administratively divided into fixed number of nine wards. For the Kalanet study, 10 wards with active VL transmission were purposely selected in villages located in the Sunsari, Morang and Saptari districts (Figure 1, Table 1) on the basis of the following criteria: (i) population between 350 and 1500 inhabitants, (ii) a minimum distance of 1 km between any two wards, (iii) VL cases being reported during each of the previous three years, showing a continuous transmission of the disease, (iv) a minimum average VL incidence rate over the past 3 years of 0.8%, and (v) amongst those wards fulfilling these criteria, those with the highest VL incidence rates over the past 3 years were selected. Ethical clearance for the

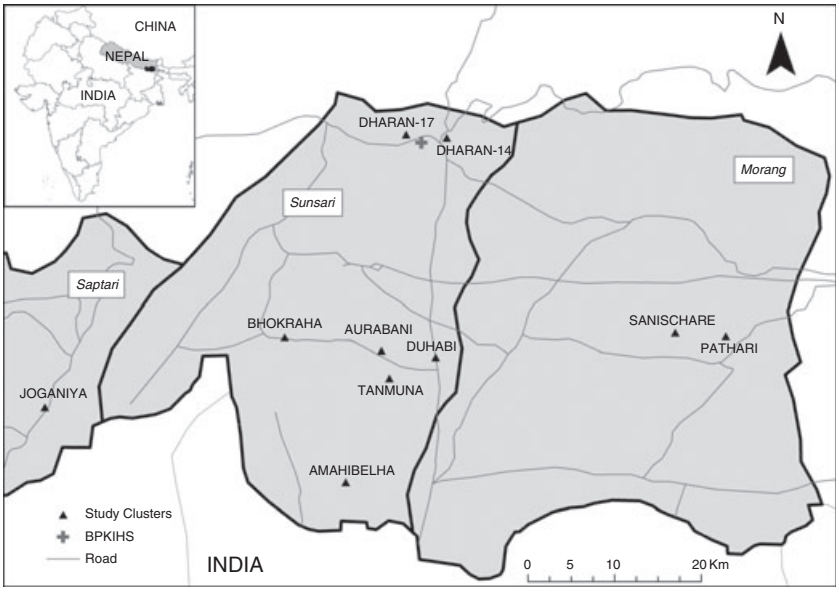

Figure 1 The 10 wards in the Terai (Nepal) where the study was conducted.

Table 1 Distribution of the PCR and DAT results belonging to each category (HNK, healthy individuals with no known past history of Kala-azar and not living in the same house as known Kala-azar cases; HHC, healthy household contacts of current or past Kala-azar cases; HPK, healthy past Kala-azar cases) in the 10 wards under the study; IR, clinical VL incidence rate by year

Name of wards	IR/Year (%)	HNK		HHC		HPK		Total	
		PCR	DAT	PCR	DAT	PCR	DAT	PCR	DAT
Amahibelha	0.81	2+/15	1+/15	0+/3	2+/3	0+/0	0+/0	2+/18	3+/18
Aurabani	2.34	4+/22	2+/22	0+/1	0+/1	1+/4	4+/4	5+/27	6+/27
Bhokraha	1.58	0+/12	0+/12	0+/8	0+/8	0+/3	2+/3	0+/23	2+/23
Dharan-14	2.49	3+/18	0+/18	0+/1	0+/1	0+/5	5+/5	3+/ 24	5+/24
Dharan-17	1.61	9+/19	0+/19	0+/1	1+/1	2+/2	2+/2	11+/22	3+/22
Duhabi	2.08	2+/21	4+/21	0+/0	0+/0	2+/4	4+/4	4+/25	8+/25
Joganiya	1.00	3+/23	1+/23	1+/1	0+/1	0+/0	0+/0	4+/24	1+/24
Patahri	1.36	4+/15	1+/15	0+/4	0+/4	0+/1	1+/1	4+/20	2+/20
Sanischare	0.92	4+/18	0+/18	2+/2	0+/2	1+/4	4+/4	7+/24	4+/24
Tanmuna†	0.82	1+/19	1+/19	0+/3	2+/3	0+/0	0+/0	1+/22	3+/22
Total		32+/182	10+/182	3+/24	5+/24	6+/23	22+/23	41+/229	37+/229

†Out of the 24 samples available from this ward, two gave invalid PCR results (inhibition); hence PCR/DAT comparison was only made on 22 samples.

conduct of the Kalanet study was obtained from the Ethical Committee of the BPKIHS, Dharan, Nepal and the corresponding body at the Institute of Tropical Medicine Antwerp (ITM-A), Belgium and the London School of Hygiene and Tropical Medicine (LSHTM), UK.

Selection of study population

The study population consisted of three groups of healthy individuals (all above 14 years). Firstly, in each ward we identified in the Kalanet census register all healthy individuals with no known history of kala-azar and not living in the same house as known kala-azar cases (further called Healthy No-kala-azar; HNK). A random sample was taken among these HNK, by a random selection process performed in the trial census database. Secondly, a sample of healthy household contacts (HHC) of current or past VL cases was selected. Thirdly, we recruited individuals who had previously suffered of kala-azar and had been successfully treated (Healthy Past kala-azar, HPK). Altogether 231 individuals were recruited: 184 (80%) HNK, 24 (10%) HHC and 23 (10%) HPK. Written informed consent from the study subjects was obtained prior to their enrolment in this study and for persons under 18, the informed consent was obtained from adult relatives.

Sample collection and processing

Blood samples were collected during November–December 2006 from the 231 individuals by vein puncture method and divided in two: (i) 1 ml of blood was brought in a tube containing Na_2EDTA (240 μg/ml of blood, Sigma, molecular biology grade) solution for PCR; (ii) a few drops were impregnated on filter paper (Whatmann 3.3 mm Chr, VWR international) for DAT. The blood on EDTA was mixed and immediately stored in a chilled icebox. The filter papers were air dried and kept in plastic bags containing beads of silica gel. All chilled iceboxes containing blood samples and filter papers were transferred on the same day to the laboratory at BPKIHS. A volume of 180 μl of the EDTA-blood samples was transferred to a test tube containing 180 μl of AS1 buffer (Cat no 1006243, Qiagen, Benelux), mixed well and stored at room temperature. All blood samples with AS1 buffer were dispatched at ambient temperature to the ITM-A for DNA extraction and PCR analysis. The filter papers were stored at –20 °C for DAT analysis at BPKIHS.

Preparation of DNA samples

The QIAamp DNA mini kit (Cat no: 56301, Qiagen, Benelux) was used to extract DNA from the blood samples containing AS1 buffer. Briefly, the whole sample (360 μl) was treated with proteinase K solution and AS2 buffer (Qiagen, Benelux) and incubated at 56 °C for 10 min. After the digestion of the blood samples, manufacturer's instructions were followed in order to elute the DNA in 50 μl AE elution buffer (Qiagen, Benelux). A blood sample from a healthy Belgian volunteer, who had never visited any endemic region of visceral Leishmaniasis, was included in each series as DNA extraction control. All the extracted DNA samples were kept at 4 °C until used.

PCR amplification assay

We used a PCR assay targeting the small subunit ribosomal RNA genes of *Leishmania*, previously shown (Deborggraeve *et al*. 2008) to be able to detect one parasite in 180 μl of blood and 10 fg purified DNA (equivalent of 0.05 parasite genomes). The 115-bp amplicon was detected on a 2% agarose gel. Negative samples were submitted to a new amplification, using 1/10 dilution of template DNA to decrease potential inhibitor. Samples that remained negative were redone, but the PCR was spiked with 0.1 pg of DNA from a cultured *L. donovani* strain (MHOM/NP/03/BPK206/0) to reveal possible PCR inhibition, as this DNA should always amplify. Positive and negative controls were included in each PCR run. The two positive controls consisted of 1 ng and 0.1 pg of *L. donovani* (MHOM/NP/03/BPK206/0) DNA, while DNA free milli-Q water was included as negative control. In order to confirm that the amplified DNA corresponded well to *Leishmania*, amplicons were sequenced by a commercial service of capillary sequencers (Applied Biosystems 3730 DNA Analyzer) using the forward and reverse primer. The results were compared with *Leishmania* sequences from GenBank.

Direct agglutination test

Direct agglutination test was performed as described elsewhere (Jacquet *et al*. 2006) using freeze dried antigen suspension of trypsin-treated *Leishmania* promastigotes (purchased from ITG-Belgium), as described by Harith *et al*. (1988). A 5-mm filter paper disc fully covered with blood was punched out (which corresponds to 5 μl blood, thus in average 2.5 μl serum) and eluted in 1000 μL DAT buffer (PBS-PH 7.2 supplemented with protein) in a sealable tube, to obtain a 1:400 dilution. After overnight incubation at 4 °C, 100 μl of the 1:400 dilution was transferred into the first well of a V-shaped microtiter plate (8 × 12 wells, Greiner, Germany). Using a multi-channel pipette, 50 μl of this dilution was then mixed with 50 μl of DAT-diluent including 2-Mercapto-Ethanol (preparation: 0.24 ml 2-ME per vial of 30 ml DAT-diluent) in the subsequent wells, to obtain serial dilutions from 1:400 in the first well to 1:25 600 (eight steps). In the next step, 50 μl of DAT antigen was added into each well; the plate was sealed, shaken gently and incubated overnight at ambient temperature according to standard DAT procedures (Jacquet *et al*. 2006). One positive and one negative control were run every fifth plate. Samples that agglutinated at a dilution of 1:1600 or higher (threshold chosen as *Leishmania* infection marker in the Kalanet project) were considered seropositive. Quality control (QC) was done on 10% of all filter papers collected. The selection of filter papers for QC included, apart from a balanced random sample of positive and negative results, all borderline results (1:1600, 1:3200 and undetermined) in order to obtain a distinct definitive result. In case of discriminate results in QC, a third analysis was done at ITM-A, Belgium.

Statistical analysis

A Poisson regression followed by using the delta method described by Oehlert (1992) was used to assess differences between the HNK, the HHC and the HPK groups in the PCR-positives/DAT-positives ratios. The agreement between the two tests within each group category was assessed by calculating positive and negative agreement indices with credibility intervals according to the method described by Graham and Bull (1998). Considering the outcome of two tests in general, the values *a, b, c* and *d* denote the observed frequencies for each possible combination of ratings by tests 1 and 2: *a*, being the number of samples positive with both 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 and *d*, the number of samples negatives with both tests. The proportion of specific agreement for the positive ratings (pr^+) (the positive agreement index), and for the negative ratings (pr^-) (the negative agreement index) were calculated as follows:

$$pr^+ = \frac{2a}{2a+b+c}$$

and

$$pr^- = \frac{2d}{2d+b+c}$$

pr^+, for example, estimates the conditional probability, given that one of the test results, randomly selected, is positive, the other will also be positive. These specific agreement indices do not have the limitations of the kappa statistic (K), like the influence of trait prevalence. Credibility intervals (95%) were calculated using the Bayesian method proposed by Graham and Bull (1998). In this analysis a non-informative prior distribution was used because no specific prior knowledge about the long-run cell probabilities was available. The relation between the PCR and DAT positivity in different clusters in the HNK population and the corresponding incidence rate in each cluster was tested through a survey logistic regression model, accounting for the clustering effects at village level.

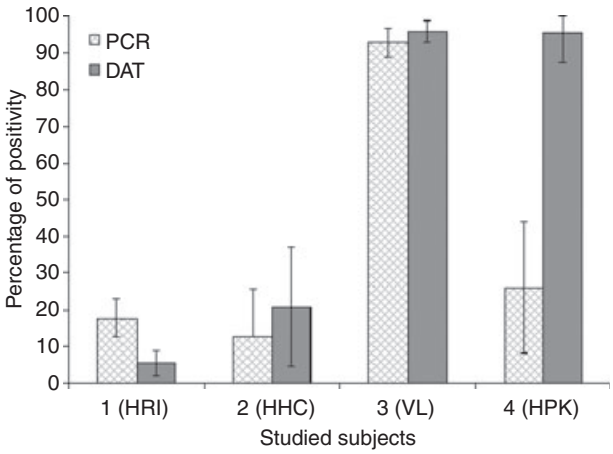

Figure 2 Distribution of PCR and DAT positive samples in (i) healthy individuals (HNK, HHC and HPK; see legend of Table 1 for definition) and (ii) VL patients [data from Harith *et al.* (1988)] from the VL endemic region of Terai, Nepal. The error bars show the 95% confidence intervals (CI) for the prevalence of each test.

We did the analysis with and without controlling for age and sex.

Results

PCR and DAT positivity in the whole sample

Out of the 231 samples, 41 were positive by PCR (seven with undiluted DNA and 34 with 1/10 dilution), 188 were negative (no inhibition observed in inhibition control) and two were classified as undetermined (inhibition observed in inhibition control); all sequenced amplicons revealed a *Leishmania* genus-specific sequence. PCR positivity rate was thus 41/229, i.e. 18%. A positive DAT titre (cut off ≥1:1600) was encountered in 37 samples (16.2%). Comparison between the three categories of healthy subjects revealed 17.6% of PCR positivity among HNK, 12.5% among HHC and 26.1% among HPK (Figure 2). With respect to DAT, there was 5.5% of positivity among HNK; 20.8% among HHC and 95.6% among HPK (Figure 2). For comparison, we included in Figure 2 data reported elsewhere (Deborggraeve *et al.* 2008) from the analysis of a sample of clinical VL cases: 92.1% PCR positivity (on blood samples, using the same PCR assay as in current study) *vs.* 96.9% DAT positivity rate (in this case, the VL diagnostics cut off was used: ≥1:3200). In order to compare the relative differences between the two markers in each of the human categories here considered, we calculated ratios between PCR- and DAT-positivity. The Poisson regression indicated that the ratio

Table 2 Positive and negative agreement indices (AI) between PCR and DAT results and 95% credibility interval estimates

Groups	Positive AI (%)	95% Credibility interval	Negative AI (%)	95% Credibility interval
Total healthy	18	8–29	83	79–87
HNK	5	0–18	88	84–91
HHC	0	0–3	80	62–91
HPK	43	20–63	11	0–37
VL*	96	93–98	32	11–58

See legend of Table 1 for definition of HNK, HHC and HPK.
*VL on the base of data published elsewhere Harith et al. (1988), we also calculated the AIs between the same two tests in clinical VL cases (VL).

PCR-positives/DAT-positives was significantly higher in HNK (ratio = 3.1) than in HHC (ratio = 0.6), $P = 0.036$, and in HPK (ratio = 0.2), $P = 0.012$. The ratio PCR-positives/DAT-positives was not significantly different between HHC (ratio = 0.6) and HPK (ratio = 0.2), $P = 0.473$.

Agreement between PCR and DAT

The agreement between the results obtained by PCR and DAT (at a 1:1600 cut off for healthy individuals) was estimated calculating positive and negative agreement indices. These indices in Table 2 show that among HNK and HHC, the agreement is better in the negative rather than the positive direction, but that trend is not verified for HPK. In comparison, a high positive agreement index was observed among clinical VL cases (calculated from data reported elsewhere (Deborggraeve *et al.* 2008).

PCR and DAT positivity in the different wards

In a further analysis, we compared PCR and DAT positivity in each of the 10 wards here considered (only among HNK). PCR positivity varied from 0% (Bhokraha) to 47.4% (Dharan-17), while DAT positivity (cut-off 1:1600) ranged from 0% (Bhokraha, Dharan-14 and -17, Sanischare) to 19% (Duhabi); see Table 1. The 10 wards being characterized by different VL incidence rates in the last years, we also compared the PCR and DAT positivity rates among HNK with the VL incidence. The comparisons were made by the survey logistic regression model and did not show the evidence of a relation between the PCR and DAT positivity (when not controlling for age and sex) and the corresponding incidence rate in different clusters in the HNK population. (Figure 3a (PCR) and b (DAT) shows the observed prevalences and incidence rates for the

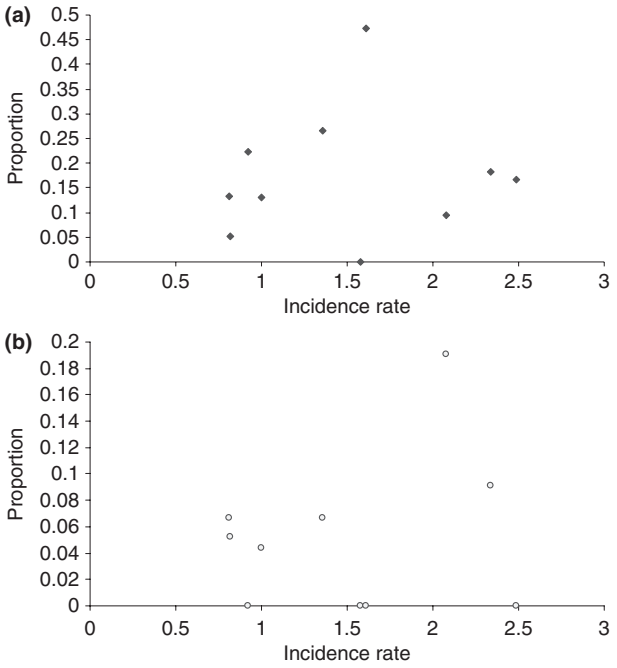

Figure 3 (a) Relationship between PCR positivity among HNK sampled in a given cluster and the VL incidence observed in the same cluster during the previous years (b) Relationship between DAT positivity among HNK sampled in a given cluster and the VL incidence observed in the same cluster during the previous years.

different clusters). However, when controlling for age, the relation between DAT positivity and incidence rate became significant ($P = 0.018$). Furthermore, we also saw the significant effect of age on DAT positivity ($P = 0.0137$), which was not the case for PCR positivity.

Discussion

We aimed to explore *Leishmania* infection markers among asymptomatic carriers in the context of anthroponotic visceral leishmaniasis. Therefore, we collected over a short period of time samples from healthy individuals living in 10 wards with active transmission of VL, in the Terai region, Nepal. The ratio PCR-positives/DAT-positives was significantly higher in HNK than in HHC and HPK, while the ratios observed in HHC and HPK did not differ significantly. This highlighted the need to treat our results separately according to the three categories of asymptomatic carriers here considered.

When considering HNK alone (about 80% of our sample), we encountered a PCR positivity rate of 17.6%, while the DAT positivity (cut-off value 1:1600) was 5.6%. Positive agreement between both methods (5%) was poor, but negative agreement was 88%. Without any known reference point for PCR (on asymptomatics) in the Indian sub-continent, we compared our results with observations made in the context of zoonotic visceral leishmaniasis. In the Mediterranean region, PCR positivity rates among asymptomatic blood donors from endemic regions reach 5.9% (Riera *et al.* 2008). The higher value we observed might be explained by (i) technical factors (such as the analytical sensitivity of the PCR assays used in the respective studies), (ii) possible sampling bias (e.g. in Riera's study, blood donors not representing the whole population from an endemic area.), (iii) epidemiological features (e.g. transmission intensity in the respective epidemiological settings) or (iv) biological features (higher parasitaemias might be expected in AVL, where humans are considered as the parasite reservoir). We found more information in the Indian sub-continent on serology. Bimal *et al.* (2005) determined 12.8% of DAT positivity in a VL-endemic area of Bihar (India) among members of control households with no cases or history of the disease, but in this case, a less stringent cut-off was used (1:800). Using a higher cut-off value (1:3200), Schenkel *et al.* (2006) reported 7.5% of positivity in a population living in a VL-endemic region of Nepal. Our results might indicate that in our experimental conditions the sensitivity of DAT for detecting asymptomatic infections among HNK might be lower than that of PCR. This could be related to the performances of the respective methods, but also to a difference in the kinetics of the respective bio-markers. In an experimental model of canine visceral leishmaniasis, parasites were already encountered in the blood of infected dogs 4 months post-infection, while *Leishmania* specific antibodies were recently detected by ELISA from month 6 after infection (Rodríguez-Cortés *et al.* 2007). We do not know if the situation would be the same in humans and if using DAT for detection of antibodies, but considering a time frame of about 6 months between the transmission peak in Terai (estimated in April–May) (Bhandari 2008) and our sampling (November–December), it is possible that antibody levels among HNK were still too low at sampling time. We may also not exclude a priori that some of these infections would be transient: in the canine experimental study mentioned above, 33% of the dogs showed a positive PCR only at 4 months post infection and *Leishmania*-specific antibodies were hardly detectable over the 13-months follow-up (Rodríguez-Cortés *et al.* 2007). A follow-up study (in progress) should allow further documenting these different hypotheses.

Among HHC, PCR positivity was 12.5%, which is very similar to values reported in the same category of subjects

in India (10%) (Salotra *et al*. 2001). DAT positivity was 20.8%, which is slightly less than 37.2% observed among HHC in the nearby state of Bihar (Bimal *et al*. 2005). In comparison to HNK, the ratio PCR-positives/DAT-positives was inverted, as a consequence of a decrease of PCR positives and an increase of DAT positives. In another study performed in Bihar, DAT positivity was also higher in HHC than in other healthy subjects (Bimal *et al*. 2005). These authors concluded therefore that compared with their neighbours, individuals who shared households with active or cured cases of VL appeared at greater risk of *L. donovani* infection. Such a result is expected in the context of anthroponotic visceral leishmaniasis, in which humans are considered as a reservoir of parasites so that HHC are at higher risk of acquiring the infection by peridomestic sand fly vector. Why this effect was only shown here by DAT and not by PCR remains to be understood, but it is possible that as a consequence of repeated boosting, antibody titers might be higher in this category of individuals.

Among HPK, a third scenario was evidenced by PCR and DAT. The molecular method showed 26.1% of positive cases, while a positivity rate of 95.6% was encountered with DAT. Similar features (persistence of *Leishmania* DNA and DAT antibodies) were reported just at the end of VL treatment in India and Sudan (Zijlstra *et al*. 2001; Maurya *et al*. 2005), whereas during follow-up DAT remained positive but PCR became negative in the majority of the past kala-azar individuals. Considering the time frame since the end of treatment (2–4 years), the PCR positive cases might represent re-infections: positivity rate was very similar to the 'background' one reported among HNK. Relapses should also be considered, and these patients were all treated with antimonials and the Terai region is characterized by a rate of treatment failure ranging between 11.4% (Rijal *et al*. 2003) and 24% (Rijal, unpublished data). Follow-up of these individuals should allow determining if these parasites present in the blood could lead to new clinical episodes.

Comparison between the wards evidenced a broad range of variation of PCR and DAT positivity among HNK, independently from the VL incidence (except for DAT, when controlling for age) observed in the different wards in the previous years. Our sample size in each ward was relatively small (about 20 individuals) and this might have influenced the results. However, the case of Dharan-17 deserves particular attention: this was the ward where our HNK sample showed the highest PCR positivity rate (47.4%) and the lowest DAT positivity (0%). Interestingly, this is a recent peri-urban focus of VL, while the other wards are classical rural foci. This might explain the low serological reactivity of tested samples, while recent and new infections would be detected so far by PCR only. Follow-up of this new focus at whole population level might bring relevant information on the evolution of transmission dynamics.

In conclusion, our study highlights the specific character of PCR and DAT for the exploration of *Leishmania* asymptomatic infections. Both methods seem to tell us a different story in the different category of healthy individuals here analysed, PCR being probably more informative for very recent infections among HNK, while DAT provides more information among HHC and HPK, a feature likely related to the power of serology to track less recent infections. These observations should be verified by longitudinal studies on larger samples. Our study highlights the need to structure the population when studying infection among asymptomatic individuals.

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