Spatial analysis of *Leishmania donovani* exposure in humans and domestic animals in a recent kala azar focus in Nepal

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SUMMARY

Visceral leishmaniasis (VL) is a major public health problem in the Indian subcontinent where the *Leishmania donovani* transmission cycle is described as anthroponotic. However, the role of animals (in particular domestic animals) in the persistence and expansion of VL is still a matter of debate. We combined Direct Agglutination Test (DAT) results in humans and domestic animals with Geographic Information System technology (i.e. extraction maps and scan statistic) to evaluate the exposure to *L. donovani* on these 2 populations in a recent VL focus in Nepal. A Poisson regression model was used to assess the risk of infection in humans associated with, among other factors, the proportion of DAT-positive animals in the proximities of the household. The serological results showed that both humans and domestic animals were exposed to *L. donovani*. DAT-positive animals and humans were spatially clustered. The presence of serologically positive goats (IRR = 9·71), past VL cases (IRR = 2·62) and the proximity to a forest island dividing the study area (IRR = 3·67) increased the risk of being DAT-positive in humans. Even if they are not a reservoir, domestic animals, and specially goats, may play a role in the distribution of *L. donovani*, in particular in this new VL focus.

Key words: visceral leishmaniasis, Nepal, spatial analysis, kernel density.

INTRODUCTION

Visceral leishmaniasis (VL) also known as kala azar, is a vector-borne parasitic disease caused by *Leishmania donovani* in the Indian subcontinent and East Africa (Lukes *et al*. 2007). In the Indian subcontinent, the parasites are transmitted by the bite of infected female *Phlebotomus argentipes* and the disease is fatal if left untreated. About 90% of the estimated 500 000 annual VL cases occur in India, Nepal, Bangladesh, Sudan and Brazil (Desjeux, 1996). In Nepal, VL is endemic in the southern and central parts of the Terai region with an incidence of 184 cases per 100 000 people in the most affected districts (Joshi *et al*. 2006).

In contrast to other areas (i.e. Euro-Mediterranean, Latin America), in the Indian subcontinent VL is described as anthroponotic (Desjeux, 1996). Nevertheless, the role of domestic animals in the *L. donovani* cycle in the Indian subcontinent remains controversial. The ownership of cows and buffaloes was found to be protective for VL in Nepal (Bern *et al*. 2000) but not in Bangladesh. However, a higher density of cows in the proximity of households (not necessarily related to ownership) reduced the risk for VL in Bangladesh (Bern *et al*. 2005). The role of domestic and wild animals in *L. donovani* transmission in the Indian subcontinent has been postulated and investigated by several scientists since 1928 (Bhattacharya and Ghosh, 1983). To date, direct observation methods have failed to detect *L. donovani* bodies (amastigotes) in dogs, rodents (Srivastava and Chakarvarty, 1984), bats (Rajendran *et al*. 1985) or cattle, other than a rare cutaneous case described in an old bullock in Assam, India in 1942 (Killick-Kendrick, 1990). However, serological studies have shown some positive reactions in rodents using enzyme-linked immunosorbant assay (ELISA) in India (Srivastava and Chakarvarty, 1984) and cattle using rK39 dipstick and ELISA tests in Bangladesh (Alam *et al*. 2009). A more recent study detected *L. donovani* PCR-positive goats, cows and buffaloes in Nepal (Bhattarai *et al*. 2010). However, the implications of those results in the transmission of *L. donovani* are not yet well understood.

Elucidating the role of animals in the *Leishmania* cycle is crucial for the design of control programmes

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for VL. The current strategy of early diagnosis and treatment of humans together with vector control in the Indian Subcontinent (Guerin *et al*. 2002) may not be optimal if animals play a significant role. Serological tools allow assessment of contact between *L. donovani* and human and animal populations and, since *P. argentipes* is the sole vector in the region (Pandey *et al*. 2008), they can also be used as a proxy measure for vector exposure. In this study we combined serological data in humans and domestic animals from a recent VL focus in Nepal and Geographic Information Systems (GIS) to evaluate the association between human and domestic animal exposure to *L. donovani*.

MATERIALS AND METHODS

Study site

The study was conducted in Dharan-17, a peri-urban ward of Dharan municipality in the VL endemic district of Sunsari in Nepal. Dharan-17 had 467 individuals (105 households) living in 2 populated areas physically separated by a forest island of 57 000 m². Dharan-17 had an annual VL incidence rate of 1·61% based on VL cases from 2004 to 2006 (Rijal *et al*. 2010).

Selection of participants

All individuals above 2 years of age, living in Dharan-17, were asked to provide a blood sample collected by finger prick in a Whatman #3 filter paper in November 2007. Filter papers were kept at −20 °C until the serological test was carried out.

Selection of domestic animals

In October 2007, blood from the jugular vein was collected from all goats, buffaloes and cows present in Dharan-17. In February 2008, samples from the same species were obtained in Dhankuta-3, a ward located 60 km from Dharan-17 in a non-endemic area for VL. Those samples were used as controls in the serological analyses. Blood samples were centrifuged at 3000 rpm for 10 min and the supernatant containing the serum was stored at −20 °C until the serological test was carried out.

Direct agglutination test

The direct agglutination test (DAT) was used to assess *L. donovani* infection in humans and animals. DAT was performed as described elsewhere (Jacquet *et al*. 2006) using a freeze-dried antigen suspension of trypsin-treated, fixed and stained promastigotes of *L. donovani* (Institute of Tropical Medicine, Antwerp) prepared as described by el Harith *et al*. (1988). For humans, an eluate from filter paper was processed as described elsewhere (Bhattarai *et al*. 2009). For animals, sera were thawed and homogenized by gentle mixing: 1μl of serum was used to start the serial dilutions from 1 : 200 to 1 : 12 800. Human samples that agglutinated at a dilution of 1 : 1600 or higher were considered sero-positive (Bhattarai *et al*. 2009). In animals, the cut off was determined as the mean end-titre among control animals plus 2 standard deviations (Mukhtar *et al*. 2000).

Data

The households in Dharan-17 and the limits of the forest dividing the ward were geo-referenced using a Global Positioning System (GPS) device in the field. The longitude and latitude coordinates of the households and forest limits were imported to ArcGIS 9.2 (ESRI, Redland, CA, USA) and projected (WGS_1984_UTM_Zone_45N). The area of interest, in which the analysis results are presented, was defined as a 50 meter buffer zone around the households in the ward. The total number of people and domestic animals with a DAT result per household were recorded. For the spatial analyses, only the results from goats are presented. The results on cows and buffaloes are available as additional material (available online only). Past history of VL, house structure and distance to the limits of the forest, identified as potential factors related to *L. donovani* infection, were also collected.

Spatial exploration

Extraction maps, defined as the ratio of the Gaussian kernel density surfaces of DAT-positives to the total population at risk, were used to visually explore areas of excess risk of *L. donovani* exposure in Dharan-17 (Lawson and Williams, 1993). Kernel smoothing methods allow representation of point data (i.e. households) as a continuous surface by applying a kernel structure (i.e. Gaussian) and a smoothing parameter (aka bandwidth) to locations in a space (i.e. ward) (Bailey and Gatrell, 1995). Meaningful kernel density estimates require the selection of a correct bandwidth. The normal optimal method (Bowman and Azzalini, 1997) was applied to determine the bandwidths to be used in Dharan-17 for both human and domestic animal datasets. The same bandwidth for the numerator and denominator in the extraction maps was used as suggested by Kelsall and Diggle (1995).

In Dharan-17, edge-corrected kernel density maps of DAT-positive and total population at risk were created using the household locations weighted by the number of serologically positive and total individuals per household respectively. The bandwidths and the extraction maps were produced using the packages sm (Bowman and Azzalini, 2007) and spatstat (Baddeley and Turner, 2005) in R 2.9

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software (www.R-project.org) respectively. The extraction maps obtained were represented using ArcGIS 9.2. An analogous methodology was used to create the extraction maps for goats, buffaloes and cows.

Spatial clustering

The spatial scan statistic was used to assess whether DAT-positive individuals (and domestic animals) were spatially clustered in Dharan-17. A discrete Poisson model was implemented in SaTScan version 8.0 (www.SaTScan.org) considering the number of DAT-positive and total individuals per household (people and domestic animals separately) (Kulldorff, 1997). The spatial scan statistic can detect spatial clusters by using a variable circular window size while controlling for the underlying population. The size of the circular windows was limited to 50% of the population at risk and 999 Monte Carlo simulations were used to assess the statistical significance of the spatial clusters detected. The risk associated to the spatial clusters is presented as the relative risk (RR), i.e. the ratio of estimated risks inside and outside the cluster. Further technical details and references on the methodology and software used are described elsewhere (Kulldorff, 2009).

Multivariate model

A Poisson regression model was used to investigate whether the presence of DAT-positive animals was associated with increased *L. donovani* seropositiviy in humans. The number of DAT-positive individuals per household was the response variable. The explanatory variables were the proportions of DAT-positive animals (goats, cows and buffaloes) in the proximity or each household. These proportions were obtained from the corresponding goat, cow and buffalo extraction map and dichotomized taking 50% as cut-off value. Possible confounders for VL in the region where considered in the model (Bern *et al.* 2000, 2005; Schenkel *et al.* 2006; Bhattarai *et al.* 2010). The presence/absence of past VL cases per household, the type of household (i.e. mud *vs* other) and the household distance to the border of the forest island (i.e. <50 m, 50–100 m or >100 m) were included as covariates. The total number of individuals in the household (for whom a DAT result was available) was considered as the total number of persons at risk per household. Robust variance estimates were used and the interactions among the different variables in the model were assessed using the Wald test. Variables with $P<0{\cdot}1$ in the univariate analyses were included in the multivariate model. The results were presented as Incidence Rate Ratio (IRR) and their 95% Confidence intervals. Stata 10 (StataCorp LP, College Station, TX, USA) was used to fit the Poisson regression model.

Ethical aspects

Ethical clearance was obtained from the Ethical Committee of the B.P. Koirala Institute of Health Sciences (BPKIHS), Dharan, Nepal and the corresponding bodies at the Institute of Tropical Medicine Antwerp (ITM-A), Belgium and the London School of Hygiene and Tropical Medicine (LSHTM), UK. Written informed consent was obtained from individuals (or guardians for individuals less than 18 years old) and animal owners before including them in the study. International animal experimentation guidelines were followed.

RESULTS

DAT results in humans

In November 2007, 328 individuals from Dharan-17 (70% of the total population) provided a blood sample. The median age of the study population was 20 (range 2 to 73) years old and there were slightly more females (53%; $n=174$) than males. The prevalence of *L. donovani* infection was 16·1% (53/328). The individuals with a DAT titre $\geqslant 1:1600$ had a median age of 22 years old (range 2 to 72) and 60% (32/53) of them were female. In the study population, 11 people were identified as past VL cases. All of them were relatively recent cases: 3 in 2004, 6 in 2005 and 2 in 2006 affecting mainly females (63%; $n=7$). All past VL cases had a positive DAT result.

DAT results in animals

Blood was obtained from 143 goats, 22 buffaloes and 20 cows in Dharan-17; and 25 goats, 17 buffaloes and 21 cows in Dhankuta-3. The DAT results: number of animals per agglutination titre is presented in Table 1. The 63 control samples had, in general, low agglutination titres: 95·2% (60/63) of them showed an end-titre $\leqslant 1:400$ and only 1 animal – a buffalo – had a titre $\geqslant 1:1600$. All goats ($n=25$) had a titre $\leqslant 1:400$. The mean-titre + 2 standard deviations was 1:504 and 1:454 when considering all animals or only goats from the non-endemic ward. Therefore, animals with a titre $\geqslant 1:800$ were considered DAT-positive.

In Dharan-17, 21·6% (40/185) of the total number of animal samples were DAT-positive. A higher proportion of goats were serologically positive (23·1%; 33/143) than buffaloes (22·7%; 5/22) and cows (10·0%; 2/20) (Table 1).

Spatial analysis

When DAT results for individuals and domestic animals were grouped per household ($n=105$), 33 and 23 of them had at least 1 person or animal with a DAT-positive result respectively. Eight households

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Table 1. Agglutination of *Leishmania donovani* antigen by sera of buffaloes, cows, goats and all domestic animals from Dharan-17 and Dhankuta-3 (control samples) using the direct agglutination test (DAT)

(The cut off used as serological *Leishmania* infection marker ($\geqslant 1:800$) is identified with a dotted line in the table.)

	Buffalo		Cow		Goat		Total animals	
DAT Titre	Dharan ($n=22$)	Control ($n=17$)	Dharan ($n=20$)	Control ($n=21$)	Dharan ($n=143$)	Control ($n=25$)	Dharan ($n=185$)	Control ($n=63$)
<1 : 200	3	16	0	0	20	0	23	0
1 : 200	10	0	15	15	61	17	86	48
1 : 400	4	0	3	4	29	8	36	12
1 : 800[a]	5	0	0	2	15	0	20	2
1 : 1600	0	1	1	0	15	0	16	1
1 : 3200	0	0	1	0	3	0	4	0
1 : 6400	0	0	0	0	0	0	0	0
1 : 12 800	0	0	0	0	0	0	0	0

[a] Cut off for DAT in animals.

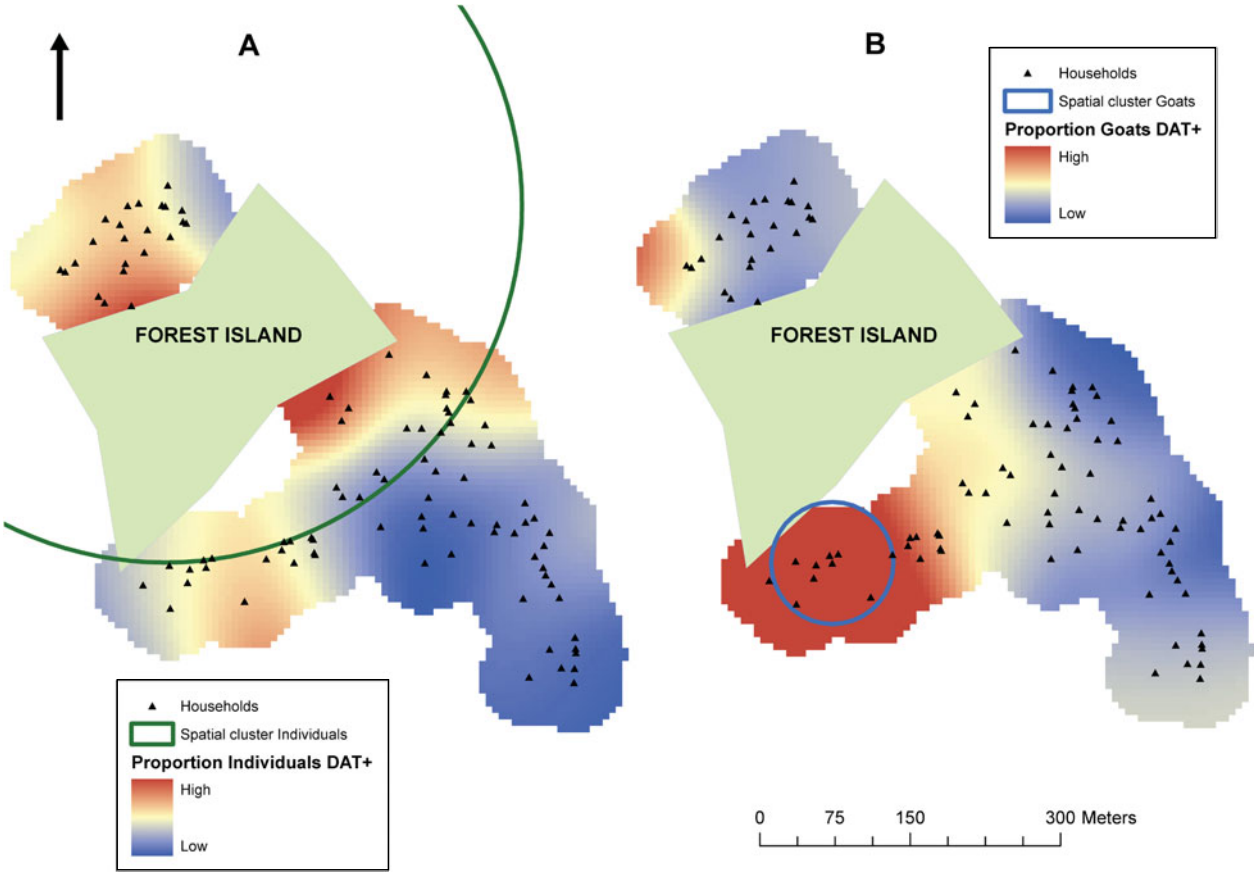

Fig. 1. Map of households in Dharan-17 and the extraction maps presenting the excess risk areas (colour gradient) of DAT-positive individuals (A) and DAT-positive goats (B) using a bandwidth of 46 and 44 meters respectively. The spatial clusters of DAT-positive individuals and goats detected by SaTScan are also presented as green and blue circles in maps A and B respectively.

had both serologically positive individuals and animals and 52 households, 8 of them owning domestic animals, have only negative results. The extraction maps used to spatially extrapolate the household data were built using bandwidth values of 46 meters for individuals (Fig. 1A), 44 for goats (Fig. 1B) and 38 meters for cows and buffaloes respectively as determined by the normal optimal method. The excess risk areas (in red) for DAT-positive individuals were mainly located around the forest island that

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divides the ward. A high proportion of DAT-positive goats were localized in the southern part of the forest. It was in that same area where SaTScan identified a spatial cluster (circle in blue) involving 8 households which had a higher proportion (RR = 4·1) of serologically positive goats compared to the households outside the cluster. The cluster was borderline statistically significant ($P = 0{\cdot}054$). The spatial cluster identified for humans (circle in green) was much larger and involved 38 households from both sides of the forest with higher risk (RR = 3·0 $P = 0{\cdot}028$) of having DAT-positive individuals. See the additional material presented in the Online version only for the results of cows and buffaloes.

Based on the univariate analyses, the presence of past VL cases in the household ($P < 0{\cdot}001$), the proportion of DAT-positive buffaloes ($P = 0{\cdot}010$) and goats ($P = 0{\cdot}063$) and the distance to the forest ($P = 0{\cdot}034$) were included as covariates in the multivariate mode. The final Poisson regression model showed that being close to serologically positive goats (proportion of DAT-positive goats > 50%) was strongly associated to an increased number of DAT-positive individuals in the household (IRR = 9·71, 95% CI: 4·99 18·92). Similarly, but with a lower IRR, the presence of past VL cases in the house (IRR = 2·62, 95% CI: 1·46; 4·70) and being less than 50 meters (IRR = 3·67, 95% CI: 1·54; 8·73) or between 50 and 100 meters (IRR = 2·74, 95% CI: 1·09; 6·90) to the forest were also associated to a higher number of DAT-positive individuals. The interaction term between proportion of DAT-positive goats and distance to the forest was statistically significant ($P < 0{\cdot}05$ by Wald test) and was kept in the final model. The variable on DAT-positive buffaloes was not statistically significant and therefore removed from the final multivariate model.

DISCUSSION

Almost 22% of the domestic animals sampled in Dharan-17 had a significant level of *L. donovani* antibodies as their serum agglutinated at a titre of 1 : 800 or above. All 3 species tested positive to DAT: buffaloes, cows – which were also found to be *L. donovani* positive in Bangladesh (Alam *et al.* 2009) – and specially goats – which have been linked to *Leishmania* transmission in Kenya (Williams *et al.* 1991). The cut off used in animals was lower than that (1 : 3200) applied in a similar study in Sudan (Mukhtar *et al.* 2000) and to detect *L. donovani* infection in humans in a previous study in Nepal (Schenkel *et al.* 2006). However, it was based on animal samples from a non-VL area in Nepal and was higher than that used to diagnose canine leishmaniasis (Oskam *et al.* 1996). Even if a more stringent cut-off had been applied (i.e. 1 : 1600) 10·8% (20/185) of animals would be considered positive which is similar to the prevalence found in people living in VL endemic villages in Nepal: 9·1% (Rijal *et al.* 2010) but 6 points below the prevalence in humans in Dharan-17. The fact that a significant number of animals – 16% of the goats – from the same group were PCR positive to a *Leishmania* specific test (Bhattarai *et al.* 2010) reduces the risk that the DAT-positive results reported here were due to cross-reactivity with other trypanosomatids (Mahmoud and Elmalik, 1977).

The spatial distribution of DAT-positive individuals and goats did not overlap perfectly but were spatially clustered at the border of the forest island dividing the study area as shown by the extraction maps and the SaTScan statistic results. This spatial aggregation was already observed by Bhattarai *et al.* (2010), when PCR results were analysed in the same population, and was confirmed by the Poisson regression model that identified proximity to the forest island as a factor increasing the proportion of *L. donovani*-infected individuals. Interestingly, proximity to the forest interacted with the proportion of DAT-positive goats suggesting that there is a 'synergy' between positive animals and proximity to the forest, which increases the risk of having DAT-positive individuals by a factor of 9·71 (compared to an IRR of 2·62 associated to past VL cases). Our results contrast to some extent with the protection effect associated with the high density of cows in the proximity of households described in Bangladesh (Bern *et al.* 2000). However, our study used a different end-point (i.e. infection in humans and animals *vs* VL) and analysed the *L. donovani* infection epidemiology in a particular setting (i.e. recent peri-urban VL focus). The increased risk of VL related to goats, compared to the rest of the domestic animals, was also reported by Bhattarai *et al.* (2010) when the same samples were analysed by PCR. However, any interpretation of the results should take into account that the number of samples collected per species was unbalanced. Goats represented 77% (143/185) of the total animal samples in Dharan-17.

The serological results presented in this study are insufficient to infer any claims about infection establishment, let alone infectivity, and the possible role of domestic animals as reservoirs of *L. donovani*. However, they indicate that, at least in Dharan-17, domestic animals are in contact with *L. donovani* and have a significant role in the spatial distribution of the parasite and thus on the risk of infection in humans, by attracting infected *P. argentipes*. The role of domestic animals, especially goats, in the *L. donovani* cycle and distribution should be re-assessed as it could have an impact on the effectiveness of the current VL control strategy in the region.

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REFERENCES

Alam, M., Khan, G. M., Ghosh, D., Mondal, D., Jamlil, K. M. and Haque, R. (2009). A pilot study to investigate animal reservoir of Kala-azar – are cattle a reservoir for Kala-azar in Bangladesh?, *Fourth World Congress on Leishmaniasis, Lucknow, India, p. 269*.

Baddeley, A. and Turner, R. (2005). Spatstat: an R package for analyzing spatial point patterns. *Journal of Statistical Software* **12**, 1–42.

Bailey, T. C. and Gatrell, A. C. (1995). *Interactive Spatial Data Analysis*. John Wiley & Sons, New York, USA.

Bern, C., Joshi, A. B., Jha, S. N., Das, M. L., Hightower, A., Thakur, G. D. and Bista, M. B. (2000). Factors associated with visceral leishmaniasis in Nepal: bed-net use is strongly protective. *American Journal of Tropical Medicine and Hygiene* **63**, 184–188.

Bern, C., A. Hightower, W., Chowdhury, R., Ali, M., Amann, J., Wagatsuma, Y., Haque, R., Kurkjian, K., Vaz, L. E., Begum, M., Akter, T., Cetre-Sossah, C. B., Ahluwalia, I. B., Dotson, E., Secor, W. E., Breiman, R. F. and Maguire, J. H. (2005). Risk factors for kala-azar in Bangladesh. *Emerging Infectious Diseases* **11**, 655–662.

Bhattacharya, A. and Ghosh, T. N. (1983). A search for Leishmania in vertebrates from kala-azar-affected areas of Bihar, India. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **77**, 874–875.

Bhattarai, N. R., Van der Auwera, G., Khanal, B., De Doncker, S., Rijal, S., Das, M. L., Uranw, S., Ostyn, B., Praet, N., Speybroeck, N., Picado, A., Davies, C., Boelaert, M. and Dujardin, J. C. (2009). PCR and direct agglutination as *Leishmania* infection markers among healthy Nepalese subjects living in areas endemic for Kala-Azar. *Tropical Medicine & International Health* **14**, 404–411.

Bhattarai, N. R., Van der Auwera, G., Rijal, S., Picado, A., Speybroeck, N., Khanal, B., De Doncker, S., Das, M. L., Ostyn, B., Davies, C., Coosemans, M., Berkvens, D., Boelaert, M. and Dujardin, J. C. (2010). Domestic animals and epidemiology of visceral leishmaniasis, Nepal. *Emerging Infectious Diseases* **16**, 231–237.

Bowman, A. and Azzalini, A. (1997). *Applied Smoothing Techniques for Data Analysis: the Kernel Approach with S-PLUS Illustrations*. Oxford University Press Inc., New York, USA.

Bowman, A. and Azzalini, A. (2007). R package 'sm': nonparametric smoothing methods (version 2.2) computer program, version By Bowman, A. and Azzalini, A. http://www.stats.gla.ac.uk/~adrian/sm

Desjeux, P. (1996). Leishmaniasis. Public health aspects and control. *Clinics in Dermatology* **14**, 417–423.

el Harith, A., Kolk, A. H., Leeuwenburg, J., Muigai, R., Huigen, E., Jelsma, T. and Kager, P. A. (1988). Improvement of a direct agglutination test for field studies of visceral leishmaniasis. *Journal of Clinical Microbiology* **26**, 1321–1325.

Guerin, P. J., Olliaro, P., Sundar, S., Boelaert, M., Croft, S. L., Desjeux, P., Wasunna, M. K. and Bryceson, A. D. (2002). Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infectious Diseases* **2**, 494–501.

Jacquet, D., Boelaert, M., Seaman, J., Rijal, S., Sundar, S., Menten, J. and Magnus, E. (2006). Comparative evaluation of freeze-dried and liquid antigens in the direct agglutination test for serodiagnosis of visceral leishmaniasis (ITMA-DAT/VL). *Tropical Medicine & International Health* **11**, 1777–1784.

Joshi, D. D., Sharma, M. and Bhandari, S. (2006). Visceral leishmaniasis in Nepal during 1980-2006. *Journal of Communicable Diseases* **38**, 139–148.

Kelsall, J. E. and Diggle, P. J. (1995). Non-parametric estimation of spatial variation in relative risk. *Statistics in Medicine* **14**, 2335–2342.

Killick-Kendrick, R. (1990). Are cattle a reservoir host of kala-azar in India? *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 754.

Kulldorff, M. A. (1997). Spatial scan statistic. *Communications in Statistics: Theory and Methods* **26**, 1481–1496.

Kulldorff, M. A. (2009). *SaTScan User Guide Computer Program*, version By Kulldorff, M. A. http://www.satscan.org/techdoc.html

Lawson, A. B. and Williams, F. L. (1993). Applications of extraction mapping in environmental epidemiology. *Statistics in Medicine* **12**, 1249–1258.

Lukes, J., Mauricio, I. L., Schonian, G., Dujardin, J. C., Soteriadou, K., Dedet, J. P., Kuhls, K., Tintaya, K. W., Jirku, M., Chocholova, E., Haralambous, C., Pratlong, F., Obornik, M., Horak, A., Ayala, F. J. and Miles, M. A. (2007). Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proceedings of the National Academy of Sciences, USA* **104**, 9375–9380.

Mahmoud, M. M. and Elmalik, K. H. (1977). Trypanosomiasis:goats as a possible reservoir of *Trypanosoma congolense* in the Republic of the Sudan. *Tropical Animal Health and Production* **9**, 167–170.

Mukhtar, M. M., Sharief, A. H., el Saffi, S. H., Harith, A. E., Higazzi, T. B., Adam, A. M. and Abdalla, H. S. (2000). Detection of antibodies to *Leishmania donovani* in animals in a kala-azar endemic region in eastern Sudan: a preliminary report. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**, 33–36.

Oskam, L., Slappendel, R. J., Beijer, E. G., Kroon, N. C., van Ingen, C. W., Ozensoy, S., Ozbel, Y. and Terpstra, W. J. (1996). Dog-DAT: a direct agglutination test using stabilized, freeze-dried antigen for the serodiagnosis of canine visceral leishmaniasis.

FEMS Immunology and Medical Microbiology **16**, 235–239.

Pandey, K., Pant, S., Kanbara, H., Shuaibu, M. N., Mallik, A. K., Pandey, B. D., Kaneko, O. and Yanagi, T. (2008). Molecular detection of *Leishmania* parasites from whole bodies of sandflies collected in Nepal. *Parasitology Research* **103**, 293–297.

Rajendran, P., Chatterjee, S. N., Dhanda, V. and Dhiman, R. C. (1985). Observations on the role of vespertilionid bats in relation to non-human vertebrate reservoir in Indian kala-azar. *Indian Journal of Pathology and Microbiology* **28**, 153–158.

Rijal, S., Uranw, S., Chappuis, F., Picado, P., Khanal, B., Paudel, I. P., Andersen, E. W., Meheus, F., Ostyn, B., Das, M. L., Davies, C. and Boelaert, M. (2010). Epidemiology of *Leishmania donovani* infection in high-transmission foci in Nepal. *Tropical Medicine & International Health* **15** (Suppl. 2) (in the Press).

Schenkel, K., Rijal, S., Koirala, S., Koirala, S., Vanlerberghe, V., Van der Stuyft, P., Gramiccia, M. and Boelaert, M. (2006). Visceral leishmaniasis in southeastern Nepal: a cross-sectional survey on *Leishmania donovani* infection and its risk factors. *Tropical Medicine & International Health* **11**, 1792–1799.

Srivastava, L. and Chakarvarty, A. K. (1984). Investigation of possible zoonotic reservoirs of Indian kala-azar. *Annals of Tropical Medicine and Parasitology* **78**, 501–504.

Williams, A. O., Mutinga, J. and Rodgers, M. (1991). Leishmaniasis in a domestic goat in Kenya. *Molecular and Cellular Probes* **5**, 319–325.