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Note: Persistence of *Leishmania donovani* antibodies in past Visceral Leishmaniasis cases in India

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Abstract

Anti-*Leishmania donovani* antibodies persistence in past Visceral Leishmaniasis (VL) cases was retrospectively assessed by means of Direct Agglutination Test (DAT) and rK39 ELISA. Antibody titres remained high for an extended period of time in past VL. These results highlight the need to carefully elicit the history of patients with VL symptoms.

Key words: *Leishmania donovani*, DAT, rK39 ELISA, kala azar

Visceral leishmaniasis (VL, kala azar) is a systemic infection of the reticuloendothelial system. VL patients present with fever, weight loss, weakness and loss of appetite, and enlargement of liver and spleen. Such progressive infection is associated with poor delayed-type hypersensitivity and high antibody production (11-12). While the gold standard for diagnosis is still demonstration of parasites in splenic or bone marrow smears, serological tests such as the direct agglutination test (DAT) and rK39 based tests (i.e. immunochromatic strip tests, ELISA) are increasingly used for diagnosis (3). It is well known that anti-leishmanial antibodies persist after clinical cure (4, 6, 13, 17), however, it is unknown for how long they persist in VL patients in the Indian subcontinent. In the present study we used the DAT and the rK39 ELISA to assess the persistence of antibodies against *Leishmania donovani,* the VL agent in the Indian subcontinent, in past VL cases in Muzaffarpur district, India.

Three house to house surveys were conducted in 16 VL endemic villages in Muzaffarpur district in November 2006, 2007 and 2008. Details on the selection of the study villages and their demographic characteristics are provided elsewhere (14). Age, gender and past history of VL were gathered from participants using semi-structured questionnaires. Individuals who reported a past episode of VL were further interviewed (parents/guardians in case of minors) to collect data on date of onset of symptoms, date and place of treatment, and type of drug used. Medical records, if available, were checked. The case ascertainment was made by a physician. A blood sample was collected from all consenting individuals over 2 years old by finger prick onto Whatman® (Maidstone, UK) filter paper #3.

Self-reported VL cases treated with anti-leishmanial drugs before November 1st of 2006 who provided a blood sample were included in this study. The time between VL treatment and blood sampling was calculated in months. The date of treatment was used instead of the onset of symptoms to minimise the recall bias. For those who reported several episodes of VL (relapse or recurrence), the most recent episode was taken into consideration.

DAT and rK39 ELISA were performed to detect *L. donovani* antibodies on blood samples as detailed elsewhere (7-9). For DAT, sera were tested at titres 1:400 up to 1:25600. Sera that did not agglutinate were assigned a titre of 1:200 and those with an end-titre above 1:25600 were coded as 1:51200. Titre 1:3200 was used as a cut off (5). Results from rK39 ELISA were expressed as Percent Positivity (PP) (8). A cut off of 22 PP for ELISA was determined from 37 non-endemic controls samples as detailed elsewhere (8).

Spearman's correlation coefficient was used to evaluate the agreement between DAT and rK39 ELISA (8). The geometric means (GM) (and 95% confidence interval) for DAT titres and rK39 PP were calculated and plotted for the following time periods: 0-6, 7-12, 13-24, 25-36, 37-48 months, 5-6, 7-8, 9-11, 12-17, 18-24 and over 24 years. The GM were calculated on the basis of the number of past VL cases in each category using Stata v10.

The study protocol was approved by Institutional Review Boards of the Banaras Hindu University and University of Antwerp. Written informed consent was obtained from all participants or guardians before enrolling the study.

Out of 13343 subjects, 845 had been treated for VL before the 1st November 2006. DAT and rK39 ELISA results were available for 780 (92.3%) past VL cases. Most of the

participants were male (500/780) and reported a single VL episode (96.3%; 751/780). The median age when they suffered VL was 17 (range < 1 to 87) years old. Blood samples were taken at time points ranging from one month to 53 years post completion of therapy (median 3 years).

The majority of VL cases were treated in private institutions or Non Governmental Organisations, especially in the last 15 years (Table 1). The fact that incentives are now provided to patients attending public facilities (10) should increase the number of people treated in governmental facilities and reduce the underreporting (15). A significant number of patients were still treated with sodium stibogluconate (SSG) (Table 1) even if SSG was progressively replaced by Amphotericin B since mid 1990s (16). Miltefosine, the first line treatment for VL in India since 2005 (1), was administered to only 30 (4%) patients, all of them after 2002.

We found a modest correlation between the DAT and rK39 ELISA results (Spearman's rho=0.57 p-value<0.001). The agreement between tests was better than the one observed in asymptomatic individuals in Nepal (8). The kinetics of *L. donovani* antibodies over time detected by both tests were similar. Titres (DAT) and PP values (rK39 ELISA) were high for recent cases, decreased rapidly in the first 12 months and then declined more slowly (Figure 1). There were however some differences between rK39 ELISA and DAT e.g. the early decline was steeper for ELISA than agglutination antibodies (Figure 1). This difference could be related to differences in ELISA and DAT antibodies half-life times (6). Nevertheless, antibody titres remained high for an extended period of time e.g. a significant number of individuals who suffered VL 15 or more years before the blood

sample was taken tested positive to DAT (53%) and rK39 ELISA (39%) (Table 1). In endemic areas, anti-leishmanial antibodies remain detectable many years after the episode of VL. This may be due to repeated exposure to *L. donovani*, or incomplete elimination of the parasite as suggested in a study in Nepal where 26.1% (6/23) of past VL cases were PCR positive (2). The study samples could not be tested by PCR as this method has not been validated for filter paper samples (2).

Previous cohort studies on past VL showed that rK39 ELISA detected anti-*Leishmania* antibodies up to 4 years in India (13) and remained positive up to 12 years in Brazil (4) and 24 months in Sudan (17). Similarly 89% of past VL were DAT-positive 56-90 months post-treatment in Ethiopia (6). In contrast to our retrospective study, cohort designs have less problems of recall and misclassification bias but are limited on the number of patients that can be followed for a long time. The results of this study highlight the need to carefully elicit the history of patients with VL symptoms as serological tests used for diagnosis may remain positive for a long time in past VL patients.

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Captions:

Table 1: Number of past Visceral Leishmaniasis (VL) cases per time period and information on (1) place (Government vs Private/Non Governmental Organisations) where they were treated and (2) anti-leishmania drug (sodium stibogluconate vs Amphotericine B) received. Percentage and number of past VL cases that would test positive to Direct Agglutination Test (DAT) and rK39 ELISA per period using the titre 1:3200 and 22 Percent Positivity (PP) as cut off values respectively.

Figure 1: Kinetics of *Leishmania donovani* antibodies on past Visceral Leishmaniasis (VL) cases. Geometric mean and 95% confidence interval for Direct Agglutination Test (DAT) titres (red dashed line) and rK39 ELISA percentage positivity (PP) (blue solid line) per time periods between (reported) date of VL treatment and date of blood sampling. Vertical lines indicate the time periods considered to calculate the geometric means: 0-6, 7-12, 13-24, 25-36, 37-48 months, 5-6, 7-8, 9-11, 12-17, 18-24 and over 24 years.

134 Table 1:

Time VL treatment – blood sampling (Nov 2006) (N=subjects)	Place treatment[4] - % (n)		VL drug used[5] - % (n)		DAT positive % (n)	rK39 ELISA positive % (n)
	Government[1]	Private/NGO[2]	SSG[3]	Amphotericine B	1:3200 cut off	22 PP[6] cut off
<1 year (N=124)	19,4 (24)	80,6 (100)	31,5 (39)	61,3 (76)	93,5 (116)	86,3 (107)
1 to <5 years (N=447)	16,1 (72)	83,7 (374)	38,0 (170)	55,9 (250)	89,3 (399)	68,2 (305)
5 to < 10 years (N=123)	13,0 (16)	87,0 (107)	53,7 (66)	45,5 (56)	80,5 (99)	57,7 (71)
10 to < 15 years (N=35)	25,7 (9)	74,3 (26)	68,6 (24)	31,4 (11)	68,6 (24)	42,9 (15)
≥15 years (N= 51)	29,4 (15)	62,7 (32)	84,3 (43)	7,8 (4)	52,9 (27)	39,2 (20)

135 [1]District Hospital, Medical College and Community Health Centres.

136 [2]Private hospitals/health centres and Non Governmental Organisations - NGO (i.e. Kala azar Medical Research Centre in Muzaffapur)

137 [3]sodium stibogluconate

138 [4]Other sites and "unknown" not shown

139 [5]Other drugs (i.e. Miltefosine) and "unknown" not shown

140 [6]PP=Percent Positivity

References:

1. **Bhattacharya, S. K., D. Sur, P. K. Sinha, and J. Karbwang.** 2006. Elimination of leishmaniasis (kala-azar) from the Indian subcontinent is technically feasible & operationally achievable. Indian J Med Res **123:**195-6.
2. **Bhattarai, N. R., G. Van der Auwera, B. Khanal, S. De Doncker, S. Rijal, M. L. Das, S. Uranw, B. Ostyn, N. Praet, N. Speybroeck, A. Picado, C. Davies, M. Boelaert, and J. C. Dujardin.** 2009. PCR and direct agglutination as Leishmania infection markers among healthy Nepalese subjects living in areas endemic for Kala-Azar. Trop Med Int Health **14:**404-11.
3. **Boelaert, M., S. El-Safi, A. Hailu, M. Mukhtar, S. Rijal, S. Sundar, M. Wasunna, A. Aseffa, J. Mbui, J. Menten, P. Desjeux, and R. W. Peeling.** 2008. Diagnostic tests for kala-azar: a multi-centre study of the freeze-dried DAT, rK39 strip test and KAtex in East Africa and the Indian subcontinent. Trans R Soc Trop Med Hyg **102:**32-40.
4. **De Almeida Silva, L., H. D. Romero, A. Prata, R. T. Costa, E. Nascimento, S. F. Carvalho, and V. Rodrigues.** 2006. Immunologic tests in patients after clinical cure of visceral leishmaniasis. Am J Trop Med Hyg **75:**739-43.
5. **el Harith, A., A. H. Kolk, J. Leeuwenburg, R. Muigai, E. Huigen, T. Jelsma, and P. A. Kager.** 1988. Improvement of a direct agglutination test for field studies of visceral leishmaniasis. J Clin Microbiol **26:**1321-5.
6. **Hailu, A.** 1990. Pre- and post-treatment antibody levels in visceral leishmaniasis. Trans R Soc Trop Med Hyg **84:**673-5.

7. **Jacquet, D., M. Boelaert, J. Seaman, S. Rijal, S. Sundar, J. Menten, and E. Magnus.** 2006. Comparative evaluation of freeze-dried and liquid antigens in the direct agglutination test for serodiagnosis of visceral leishmaniasis (ITMA-DAT/VL). Trop Med Int Health **11:**1777-84.

8. **Khanal, B., S. Rijal, B. Ostyn, A. Picado, K. Gidwani, J. Menten, D. Jacquet, V. Lejon, F. Chappuis, and M. Boelaert.** 2010. Serological markers for Leishmania donovani infection in Nepal: agreement between Direct Agglutination Test and rK39 ELISA. Trop Med Int Health **15**: 1390-4

9. **Kurkjian, K. M., L. E. Vaz, R. Haque, C. Cetre-Sossah, S. Akhter, S. Roy, F. Steurer, J. Amann, M. Ali, R. Chowdhury, Y. Wagatsuma, J. Williamson, S. Crawford, R. F. Breiman, J. H. Maguire, C. Bern, and W. E. Secor.** 2005. Application of an improved method for the recombinant k 39 enzyme-linked immunosorbent assay to detect visceral leishmaniasis disease and infection in Bangladesh. Clin Diagn Lab Immunol **12:**1410-5.

10. **NVBDCP.** Template for developing District Action Plan (Kala-azar). National Vector Borne Disease Control Programme - Kala-azar.

11. **Reiner, S. L., and R. M. Locksley.** 1995. The regulation of immunity to Leishmania major. Annu Rev Immunol **13:**151-77.

12. **Sharma, U., and S. Singh.** 2009. Immunobiology of leishmaniasis. Indian J Exp Biol **47:**412-23.

13. **Singh, S., V. Kumari, and N. Singh.** 2002. Predicting kala-azar disease manifestations in asymptomatic patients with latent Leishmania donovani infection

by detection of antibody against recombinant K39 antigen. Clin Diagn Lab Immunol **9:**568-72.

14. **Singh, S. P., A. Picado, M. Boelaert, K. Gidwani, E. W. Andersen, B. Ostyn, F. Meheus, M. Rai, F. Chappuis, C. Davies, and S. Sundar.** 2010. The epidemiology of Leishmania donovani infection in high transmission foci in India. Trop Med Int Health **15 Suppl 2:**12-20.

15. **Singh, S. P., D. C. Reddy, M. Rai, and S. Sundar.** 2006. Serious underreporting of visceral leishmaniasis through passive case reporting in Bihar, India. Trop Med Int Health **11:**899-905.

16. **Thakur, C. P., A. Kumar, G. Mitra, S. Thakur, P. K. Sinha, P. Das, S. K. Bhattacharya, and A. Sinha.** 2008. Impact of amphotericin-B in the treatment of kala-azar on the incidence of PKDL in Bihar, India. Indian J Med Res **128:**38-44.

17. **Zijlstra, E. E., N. S. Daifalla, P. A. Kager, E. A. Khalil, A. M. El-Hassan, S. G. Reed, and H. W. Ghalib.** 1998. rK39 enzyme-linked immunosorbent assay for diagnosis of Leishmania donovani infection. Clin Diagn Lab Immunol **5:**717-20.