REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

ORIGINAL ARTICLE

http://doi.org/10.1590/S1678-9946202062067

Estimation of *Leishmania* spp. infection in asymptomatic people from Muzaffarpur, Bihar, India by antigen-antibody and skin testing

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ABSTRACT

Asymptomatic VL is a concern, considering the risk of transmission in highly endemic areas due to human-to-human transmission. The aim of this study was to report the sero-epidemiological prevalence in Bihar, India, a highly endemic area of VL, using the leishmanin skin test (LST) and the direct agglutination test (DAT). This was a cross-sectional study performed in Muzaffarpur, Bihar, India. Relatives of patients with VL were tested by LST and DAT. Other epidemiological data were evaluated and correlated with tests results. Forty individuals (either previous or current patients), and 109 household contacts were studied. There were 36% of male visceral leishmaniasis family members versus 17.57% of females visceral leishmaniasis family members, thus showing more males with symptomatic disease than females ($p< 0.01$). All visceral leishmaniasis cases had positive DAT tests, but only 37% of past cases were positive on the skin testing. Amongst healthy household contacts, 34% were DAT-positive, whilst 21% were LST-positive. The overall positivity for both assays combined was 44.8% and 23.8% were DAT-positive alone. The finding of high infection prevalence amongst asymptomatic individuals, and the estimation of those at greater risk for overt disease (DAT-positive alone) are important in the development of future disease control policies.

KEYWORDS: Visceral leishmaniasis. Epidemiology. Asymptomatic infection. Survey.

INTRODUCTION

Visceral leishmaniasis (VL), an important vector-borne disease, is largely distributed in 62 countries from Africa, Asia, Europe, and South America, totaling 200 million people at risk, and up to 500,000 new cases per year[1]. The most affected countries are India, Sudan, Nepal, Bangladesh and Brazil, which together account for almost 90% of visceral leishmaniasis cases[2].

Treatment is usually effective, but concerns about toxicity, costs and parasite resistance to commonly used drugs have highlighted the importance of preventing and controlling this medical condition. To implement control strategies, it is paramount to know the parasite and vector biology, the socio-economic conditions found in the transmission area and the population at risk[3]. Unfortunately, in some areas where leishmaniasis is highly endemic, there is a lack of information on this parameters, therefore hindering the effective control planning and implementation[4,5].

Asymptomatic VL is a concern, considering the risk of transmission in highly endemic areas due to human-to-human transmission[6,7]. The seropositivity in blood donors can vary between 0.25to 16%[6,7]. To understand the factors involved in VL transmission and maintenance, particularly related to the prevalence of infected

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Received: 5 June 2020

Accepted: 8 September 2020

asymptomatic individuals, the aim of this study was to report the sero-epidemiological prevalence in Bihar, India, a highly endemic area of visceral leishmaniasis, using the leishmanin skin test (LST) and the direct agglutination test (DAT).

METHODS

Study location and population

This was a cross-sectional study performed in Muzaffarpur, Bihar, India, in July 2005, at the Kala-azar Medical Research Centre. All subjects were current or previous VL patients of the unit and their relatives, who were healthy household contacts. VL contacts from seven villages (Karjachati, Karjadi, Vishunpur, Chainpur, Rajwara West, Rajwara East, and Fanda Goriya) were randomly identified from the Centre records. All the subjects, excepting for pregnant women and children below 12 months old, residing in the same household of at least one VL patient, regardless of whether active or previous disease, were eligible for the study.

The case definition of a VL case was any individual who had been diagnosed and treated for VL (including patients on treatment at the time of the interview). VL was diagnosed either by finding *leishmania* amastigotes in a spleen or bone marrow aspirate, or by clinical features together with positive serology (rK39 test). A household was any kind of structure, made from any kind of material, with the purpose of sheltering the same group of individuals most of the time. A household healthy member was any person, either from the same genetic family or not, who had never had VL, and lived in the same house as the VL contact (shared the same premises, such as kitchen, or bathroom if available, and the same utensils, such as crockery, cutlery, and furniture).

Ethical considerations

The study was approved by both, the London School of Hygiene and Tropical Medicine and the Kala-azar Medical Research Centre Ethics Committees. An information sheet and a consent form translated into Hindi were given to every individual prior to the interview and testing. Consent forms were either signed by the family head, or left thumb printed for those who were illiterate. Only people able to give consent were included in the study.

Laboratory tests

Leishmanin skin test

A leishmanin skin test (LST) was used to assess the delayed-type hypersensitivity. The leishmanin preparation came from the Department of Immunology at the Institute Pasteur of Iran, Islamic Republic of Iran. Each mL of preparation contains 6 x 10^6 *Leishmania* major (MRHO/IR/75/ER strain) promastigotes, 1 mL phosphate buffered saline, and 0.01% thimerosal, at pH 7 to 7.1.

Test performance

After mixing the contents, LST was carried out by intradermal injection of 0.1 mL of leishmanin preparation in the anterior the left forearm skin.

Reading and result interpretation

Results were read 48 hours after injection and the induration diameter was measured by ball-point technique, using a millimetre-graduated ruler[8]. Skin reactions ≥ 5 mm were considered as positive results.

Direct agglutination test

The antigen-antibody test was used to assess humoral response against parasite surface antigens. A liquid antigen eluated on a filter paper test version was used, and the study kit was from the Prince Leopold Institute of Tropical Medicine, Applied Technology and Production Unit, Antwerp, Belgium.

Sample collection

Capillary blood was drawn by finger prick and two drops were placed on the filter paper. After blood collection in the field, filter papers were kept refrigerated at 2 to 8 °C and analyzed at the Kala-azar Medical Research Centre laboratory.

Reading and result interpretation

Titres ≥1:3,200 (without antigen addition) were considered as positive results[9].

Data collection and analysis

Data were presented as means or medians (with interquartile [IQR] ranges 25 and 75%) according to the distribution of variables. Log transformations and continuous variable data analyses were compared by the use of the Student's t test. Categorical data were analyzed by the chi-square or Fisher's exact test, 95% confidence intervals around proportions, and odds ratio calculations were carried out by using the EpiCalc 2000 statistical software (version 1.0, Brixton Health, Brixton, UK). The variable age was distributed according to the ideal categorization in accordance with the distribution data. A p-value ≤ 0.05 was considered a statistically significant difference.

RESULTS

General results

Twenty-six households were surveyed in seven villages in Muzaffarpur District. Two hundred and ten people lived in houses surveyed, median seven residents (IQR25-75% 6-9) per household. Sixty-one residents were either away during the interview and testing day, were pregnant women, were infants less than 12 months old, or refused to participate. One hundred and nine individuals were healthy contacts, and 40 people were either previous or current VL patients and agreed to take part in the study.

Healthy family members

One hundred and nine healthy subjects (48 males 44.03%) were studied. The median age was 22 (IQR25-75% 7-35). None had VL. Table 1 shows DAT and LST results by villages.

DAT results

All available individuals were tested, and there were no missed results: 37 results were positive by DAT (33.95%, 95% CI 25.33% - 43.72%). The overall geometric mean titer for positive cases was 1:32,570 (95% CI 1:23,861 – 1:44,356), which was substantially lower than the geometric mean titer for VL cases (p-value < 0.001). Nineteen out of 48 males (39.58%, 95% CI 26.11% - 54.7%), and 18 out of 61 females (29.5%, 95% CI 18.86% - 42.73%) were positive. DAT results were not associated with gender (p-value 0.368). Fifty-four individuals from 1 to 20 years old were DAT-positive (37.04%, 95% CI 24.62% - 51.3%). Amongst 55 people above 20 years old, 17 (30.9%, 95% CI 19.52% - 44.96%) were positive. Again, age had no significant effect on DAT results (p-value 0.499).

LST results

One hundred and five results out of 109 healthy family members were analyzed (4 individuals were not available for the follow-up). The overall number of positive tests was 22 (20.95%, 95% CI 13.86% - 30.21%). Eight out 45 males (17.78%, 95% CI 8.51% - 32.59%), and 14 out 60 females (23.33%, 95% CI 13.78% - 36.63%) were LST-positive. The difference between proportions was not statistically significant (p-value 0.653). Amongst 52 individuals aged 1 to 20 years old, 2 (3.85%, 95% CI 0.67% - 14.34%) were positive, and 20 out of 53 (37.74%, 95% CI 25.12% - 52.13%) individuals above 20 years old tested positive. The difference between age groups was statistically significant (p-value < 0.001).

Combined DAT and LST results

One hundred and five health individuals were tested with both DAT and LST. Of them, 10 members (9.52%) tested positive in both tests. There were 12 (11.43%) individuals who were LST-positive, but DAT-negative, and 25 (23.8%) subjects DAT-positive, but LST-negative. There were 47 (44.76%) out of 105 healthy family members who had either a positive DAT or LST.

Visceral leishmaniasis cases

Forty people were VL patients, 37 previous (the oldest case occurred 25 years ago, but most of them took place within the past year, with a median of 1 year (with IQR25-75% 1-25). Thirty-six VL cases had their diagnosis confirmed by splenic aspiration, and 4 cases by clinical picture and positive rK39 test. Two or more VL cases living in the same household were identified in 10 (38.46%, 95% CI 20.91% – 59.27%) houses (1 house had 5 VL cases). The median age was 13.5 years (IQR25-75% 8-35). The total study population was 149 (40 VL cases and 109

Table 1 - Distribution of DAT and LST results amongst healthy contact and VL cases per village.

Villages	Healthy contacts per village.							VL cases per village.							
	Houses (n)	LST			DAT			VL cases	Active VL	LST			DAT		
		Positive	Tested	(%)	Positive	Tested	%			Positive	Tested	%	Positive	Tested	%
Karjachati	1	1	1	(100)	1	1	(100)	1	1	1	1	(100)	1	1	(100)
Karjadi	2	2	14	(14)	7	14	(50)	5	0	5	1	(20)	5	5	(100)
Vishunpur	3	2	17	(12)	6	17	(35)	3	0	3	0	(0)	3	3	(100)
Chainpur	4	2	5	(33)	4	7	(57)	9	0	4	3	(75)	5	5	(100)
Rajwara West	4	0	18	(0)	7	18	(39)	5	0	5	0	(0)	4	4	(100)
Rajwara East	4	7	19	(37)	8	19	(42)	6	0	5	3	(60)	2	2	(100)
Fanda Goriya	8	8	31	(26)	4	33	(12)	11	2	7	3	(43)	5	5	(100)
Total	26	22	105	(21)	37	109	(34)	40	3	30	11	(37)	25	25	(100)

healthy contacts), and 27/75 (36%) were VL male family members and 13/74 (17.57%) were female family members. Thus, males had more symptomatic disease than females (p-value 0.011).

DAT results

Amongst 40 VL cases, 25 individuals were assessed, and all of them were DAT-positive. The geometric mean titer was 1:82,019 (95% CI 1:59,113 – 1:113,800). Fifteen individuals were not available for sample collection.

LST results

30 out of 40 VL cases were tested. The overall positivity was 36.66% (11/30). Among 30 individuals tested, 27 were previous VL cases, and the LST was positive in 10 members (37.03%). Ten individuals were not available for sample collection. Table 1 summarizes the results.

DISCUSSION

We report a cross-sectional survey of markers of *Leishmania donovani* infection in VL patients and their household healthy contacts in Muzaffarpur District, Bihar, a highly endemic area of VL. Both T-cell and antibody-immune-mediated assays, LST and DAT, respectively, were employed together to assess sub-clinical and asymptomatic infections. A total of 210 individuals lived in 26 households, but 61 were away during the testing period, therefore, being excluded from the statistical analysis.

Our DAT and LST findings suggest a past and steady exposure to the parasite in the study region. The positive skin test builds up continuously throughout the childhood into the adolescence and reaches a plateau in adulthood. Therefore, although all age groups are at risk of infection, as demonstrated by the DAT results distribution, although children are more likely to have overt disease. Several previous cross-sectional and prospective studies have demonstrated a higher number of positive reactors amongst household contacts compared to control groups from sites where VL had been endemic for a long time. In India, one survey showed 17.5% of positive skin reactors amongst household contacts, and another study indicated that amongst the 19% of individuals who had positive skin tests, most of them were close VL contacts and neighbors[10,11].

Moreover, people aged more than 20 years had a positive skin test more often than younger individuals. Another study from an endemic region of Bihar found around 31% of positive DAT results evenly distributed amongst asymptomatic individuals from both genders and age groups, confirming the findings of the present study. The clustering of positive individuals for either serology or skin test around a VL case has also been studied outside India, and results seem to be comparable[12]. In Sudan, one study demonstrated that 16.5% of household VL contacts were DAT-positive, in comparison with 8.9% in non-contacts[13].

In the Mediterranean region, one study carried out in Italy found that 40.3% and 64% of individuals who were positive for LST, from two different sites, were either close contacts or neighbours of VL cases, respectively[14]. Likewise, in Brazil, one study found 42% of positive skin reactors among contacts, compared to 14% in non-contacts, and another studyrevealed that 38% of individuals who lived with at least one VL patient were positive in skin tests[15,16]. People who live in the nearby area seem also to be at a risk in comparison with those who live with a VL case, as shown in a study carried out in Bahia, Brazil, where the proportion of infection was similar between cases and their neighbours who lived up to 100 meters from one index house[17].

Data from the present study and previous surveys suggest the possibility of micro-foci in the transmission of *Leishmania donovani*[13]. More healthy contacts had a positive DAT alone (23.8%), compared to a positive LST alone (11.4%), but as shown by the overlapping confidence intervals, the difference might have occurred only due to sampling variability. Children represented the greatest portion of subjects who were DAT-positive but not for LST. Individuals who were positive for both tests represented only 9.5% of the total studied population of healthy contacts. In Sudan, one study who recruited 622 individuals showed similar results, with very few individuals (1.3%) that were positive by both, DAT and LST, and children responded more often to the serological test than to the skin assay[13].

The overall asymptomatic infection prevalence (sum of both positive tests results) was 44.8%, and the proportion of VL cases: asymptomatic infection was 1:1.18, which reflects a condition associated with high endemicity areas and probably an area in which a vast number of both, symptomatic and asymptomatic infected individuals are found. The overall prevalence of infection amongst asymptomatic contacts of VL cases in our study, although lower than in few reported endemic areas of the world, for instance more than 64% in Italy, 80.5% in Southern Ethiopia, 59% and 63.4% in Eastern Venezuela, and 70% in North-Eastern Brazil[14,18-21]. A three-year cohort in Nepal and India showed higher levels of seroconversion (1:8.9), probably due to the longitudinal follow-up, a limitation of our study[22]. Other limitations of our study include the lack of comparison with a non-exposed population in the same region, the cross-sectional evaluation (a non-prospective cohort), the intrinsic limitations of the LTS (sensitivity and operational technique).

Moreover, asymptomatic infected patients who live with VL, even LST-positive ones, may theoretically act as reservoirs, as shown by a study carried out in an endemic area of Brazil that detected *Leishmania chagasi* kinetoplast DNA in blood samples of suspected cases, using a PCR-based assay[21]. Even if this is true, the lifelong infection in these asymptomatic cases can only represent a hypothetical reservoir for the reemergence of VL.

CONCLUSION

Proper follow-up is essential to disclose any status conversion from asymptomatic to overt disease from the very beginning, decreasing the burden of this life-threatening condition. In addition, , future field studies, especially prospective ones, will collaborate to a better understanding of the local risk factors associated with disease progression amongst infected asymptomatic individuals and their role as reservoir, guiding control measures specifically designed for the Muzaffarpur population. A future study is important to assess household contacts of new cases with follow-up of contacts, and the dynamic of LST and DAT over time.

ACKNOWLEDGMENTS

The authors would like to thank the Kala-azar Medical Research Centre staff for the laboratory support and assistance with the local population, and the University of London for the grant for the travel costs.

FUNDIND

FFT received research grants from TEVA, Pfizer and MSD, and is a CNPq researcher.

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