Asymptomatic Infection with Visceral Leishmaniasis in a Disease-Endemic Area in Bihar, India

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Abstract. A prospective study was carried out in a cohort of 355 persons in a leishmaniasis-endemic village of the Patna District in Bihar, India, to determine the prevalence of asymptomatic persons and rate of progression to symptomatic visceral leishmaniasis (VL) cases. At baseline screening, 50 persons were positive for leishmaniasis by any of the three tests (rK39 strip test, direct agglutination test, and polymerase chain reaction) used. Point prevalence of asymptomatic VL was 110 per 1,000 persons and the rate of progression to symptomatic cases was 17.85 per 1,000 person-months. The incidence rate ratio of progression to symptomatic case was 3.36 (95% confidence interval [CI] = 0.75–15.01, $P = 0.09$) among case-contacts of VL compared with neighbors. High prevalence of asymptomatic persons and clinical VL cases and high density of *Phlebotomus argentipes* sand flies can lead to transmission of VL in VL-endemic areas.

INTRODUCTION

Indian kala-azar or visceral leishmaniasis (VL) is a protozoa disease caused by *Leishmania donovani* and transmitted by the females *Phlebotomus argentipes* sand flies. It is endemic in 62 countries, and 200 million persons are at risk.¹ It is estimated that 500,000 cases of VL occur annually,^{1,2} with a prevalence of 2.5 million.³ More than 90% of VL cases occur in five countries: India, Bangladesh, Nepal, Sudan, and Brazil.^{2,4}

Visceral leishmaniasis has been a major health problem in the State of Bihar, India, and in the adjacent states of West Bengal, Jharkhand, and Uttar Pradesh for the many decades.⁵ Focal and sporadic cases of VL have been observed in many districts of this region since 1977.⁶ Currently, 30 of 38 districts in Bihar have different levels of endemicity, and nearly 67.5 million persons are at risk of acquiring the disease. More than 90% of all the cases in India are reported in Bihar State.⁵ It has been estimated that the total number of cases may be 2–2.5 times higher than the actual incidence and five times higher than official number of reported cases.^{5,7}

Symptomatic cases of VL and cases of post-kala-azar dermal leishmaniasis are considered potential reservoirs of VL, and thus play a major role in transmission of the disease in VL-endemic areas. Most of the infected population in whom VL does not develop is considered asymptomatic; however, these cases can act as potential reservoirs in transmission dynamics of VL.⁸ However, the actual estimate of asymptomatic cases in a VL-endemic area is difficult to assess.

We conducted a study in a region highly endemic for VL to estimate the prevalence of asymptomatic cases on the basis of serologic and molecular diagnosis and to estimate the rate of progression to symptomatic VL cases.

MATERIALS AND METHODS

Study area. The study was carried out at the Masauri Primary Health Center in the village of Bhakhra, Patna District, Bihar, India (Figure 1). Patna is the capital of Bihar, and the

Masauri Primary Health Center is 40 km south of the capital. This region has a high endemicity of VL. The village has a population of 557 persons and 92 houses. The study area was selected on the basis of sporadic occurrence of VL reported from this village in the past few years and on an outbreak of VL during 2005.

Study design. At baseline, a house-to-house survey was carried out in the study area for identification of a cohort of asymptomatic persons. This cohort was followed-up prospectively every month for 12 months for progression of asymptomatic cases of VL to symptomatic cases of VL.

Data collection. A questionnaire was used for obtaining sociodemographic information. The entire village was mapped and households were identified. A team of doctors clinically examined 355 persons (64% of the total population) for VL symptoms such as fever for more than 15 days, an enlarged spleen and liver, and general physical conditions. The remaining 36% of the population were either not available at the time of the survey or refused to participate in the study because of various reasons. Blood samples (3 mL) were obtained from 355 persons and placed in tubes that contained EDTA and tubes that did not contain EDTA (1.5 mL in each type of tube) for an rK39 antigen test, a direct agglutination test (DAT), and a polymerase chain reaction (PCR).

rK39 antigen test. An amastigote-specific recombinant 39-amino acid antigen (rK39) that is encoded by a kinesis-related gene was obtained from *L. chagasi*. It is specific for antibodies in patients with VL caused by the *L. donovani* complex.⁹ The estimated sensitivity of the test is 99% (95% confidence interval [CI] = 95–100%) and its specificity is 89% (95% CI = 86–92%).¹⁰ This test is a reliable tool for use in field-based diagnosis of VL (results remain positive up to three years after treatment), but it cannot differentiate among cases of previous VL, sub-clinical infections, and active disease.^{11,12} The test was obtained from Inbios International Inc. (Seattle, WA).

Direct agglutination test. A 96-hour, stationary-phase culture of promastigotes of a laboratory-adapted reference strain of *L. donovani* (WHOM/IN/80DD8) was grown in monophasic medium containing 20% fetal calf serum and used to prepare DAT antigen.^{13,14} For sub-clinical infections, the DAT has a sensitivity of 91.7% and a specificity of 100%.^{14,15} The cut-off serum dilution for the DAT was $\leq 1:800$.¹⁵

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FIGURE 1. Map of study area in Bihar, India. ▲ = study village of Bhakhra.

Polymerase chain reaction. DNA was extracted from 1.5-mL blood aliquots from 355 persons by using the phenol-chloroform extraction method and ethanol precipitation. Blood samples were treated with erythrocyte lysis buffer (155 mM NH_4Cl , 10 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.4). The test was performed by using described techniques.¹⁶⁻¹⁹

Parasitologic confirmation. Patients who had positive results for any of the three tests and were clinically suspected of having VL were referred to the Rajendra Memorial Research Institute of Medical Sciences (Indian Council of Medical Research) in Patna for parasitologic confirmation of bone marrow or splenic aspirate samples.

Follow-up of patients with asymptomatic cases. Asymptomatic persons who had positive results for any of the three tests were followed-up every month for 12 months. At each follow-up visit, clinicians examined these persons for development of any signs and symptoms. At the end of follow-up, the rK39 antigen test was performed to assess serologic status of persons who did not show any signs and symptoms.

Entomologic survey. An entomologic survey was carried out in 56 houses to observe resurgence of sand flies after spraying with DDT in the study area. Spraying with DDT was not conducted in the study village in past year. A door-to-door survey was conducted to collect sand flies by using a hand aspirator method in each household. Sand flies were collected from cracks and crevices on internal walls, roofs, dark corners, and cattle sheds. Man-hour density (number of sand flies caught per hour per person) was estimated. Temperature and humidity were also recorded.

Statistical analysis. Data collected at the baseline survey and during the follow-up period were entered into computer

by using Epi-Info version 3.2 (Centers for Disease Control and Prevention, Atlanta, GA). Data were analyzed by using SPSS version 15 (SPSS Inc., Chicago, IL).

RESULTS

The study population was composed of 184 (52%) males and 171 (48%) females, which indicated no statistically significant sex-specific difference ($P > 0.05$) among the screened populations. A total of 38% of the population was ≤ 12 years of age and 62% was > 12 years of age. Approximately 93% of the population had a low standard of living index,²⁰ which was based on the type of roof and floor of house, availability of drinking water, electricity, fuel use for cooking, lavatory facilities, and possession of consumer items such as a television, radio, motorbike, bicycle, and sewing machine. Seventy-six percent of the population lived in mud-plastered houses with thatched roofs, and 67% had cattle (cows, buffaloes, and goats) in their house. The average family size was 5.9 persons. Most of the adult populations were agriculture laborers or unskilled workers in the nearby city. Seventy-one (20%) of the screened persons had a history of VL in their family in the past two years.

Age-specific distribution of tests results for the rK39 test, the PCR, and the DAT in 355 persons screened in the study area is shown in (Table 1). Comparative distribution of tests results of for the rK39 test, the PCR, and the DAT in the screened population at the baseline survey is shown in (Table 2). There were different combinations of positive and negative results among the screened population. We assumed that a person would be considered positive for leishmanial infection if he or she had positive tests results for any of the three tests. Of 355 persons

TABLE 1
Age-specific distribution of rK-39, PCR and DAT results, Bihar, India*

Age group, years	rK-39		PCR		DAT		rK-39/PCR/DAT	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
0-4	1 (3.7)	26	3 (11.1)	24	2 (8.1)	25	2 (8.1)	25
5-14	9 (7.7)	108	10 (8.5)	107	9 (7.7)	108	15 (12.8)	102
15-29	7 (8.9)	72	6 (7.6)	73	12 (15.2)	67	14 (17.7)	65
30-44	4 (7.0)	53	6 (10.5)	51	10 (17.5)	47	12 (21)	45
45-59	2 (3.3)	58	3 (5)	57	6 (10)	54	6 (10)	54
≥ 60	1 (6.7)	14	0 (0)	15	0 (0)	15	1 (7.1)	14
Total	24 (6.8)	331	28 (7.9)	327	39 (11)	316	50 (14)	305

* Values are no. (%). PCR = polymerase chain reaction; DAT = direct agglutination test.

screened by the three tests, 50 (14%) were positive at the baseline survey.

Distribution of test-positive screened persons with or without symptoms of VL on the basis of clinical examination is shown in (Table 3). Among 24 persons with positive results for the rK39 test, 8 (33%) had clinical manifestation of the disease at the baseline screening. Six of these persons had a confirmed diagnosis of VL on the basis of parasite-positive results for bone marrow or splenic aspirates, and two had a history of VL of less than six months.

Among 28 persons with positive results for the PCR, 8 (29%) had clinical manifestation of disease at baseline screening. Five of these persons had a confirmed diagnosis of VL on the basis of parasite-positive results for bone marrow or splenic aspirates, two had started treatment at a private clinic, and one had a history of VL of less than six months.

Among 39 persons with positive results for the DAT, 9 (23%) had clinical manifestation of the disease at baseline screening. Five of these persons had a confirmed diagnosis of VL on the basis of parasite-positive results for bone marrow or splenic aspirates, two had started treatment at a primary health center, and two had a history of VL of less than six months. Seventeen persons had positive results for all three tests, of whom seven had clinical symptoms at baseline and three had a history of VL.

Of 50 persons who had positive results for any of the three tests, 10 (20%) had clinical symptoms such as fever, and enlargement of the spleen, liver, or both organs at baseline screening. Among 40 asymptomatic persons with positive test results, two had a history of VL of less than six months. These persons were excluded from the cohort of asymptomatic persons. Only 38 persons with no history of VL or any clinical symptoms subjected to were follow-up.

Clinical symptoms such as enlargement of the spleen, liver, or both organs, and fever developed in 7 of 38 asymptomatic

persons. These persons had clinical manifestations of VL and a confirmed diagnosis of VL on the basis of parasite-positive results for bone marrow or splenic aspirates. Visceral leishmaniasis developed in five persons within three months and in two persons in less than six months from the baseline screening. At the end of follow-up (12 months), 8 (21%) persons remained asymptomatic and 23 (60%) showed self-healing of the infection.

The point prevalence of asymptomatic cases was 110 per 1,000 persons at baseline. The rate of progression was 17.85 per 1,000 persons-months. The overall rate of progression to symptomatic cases was 18.42% in less than six months. However, the incidence rate ratio of progression to symptomatic disease among case-contacts of patients with VL in comparison to neighbors of these patients was 3.36 (95% CI = 0.75-15.01), which was not statistically significant ($P = 0.09$). The point prevalence of VL was 11.33 per 1,000 persons, and incidence of disease was 20 per 1,000 persons, which indicated a high level of endemicity of VL in the study area. The period prevalence of VL from the baseline survey until the end of follow-up was 31 per 1,000 persons.

Man-hour density of *P. argentipes* was 21-24 during summer and rainy season and 4 during the winter. This density during the summer and rainy season was 3-4 times higher than the critical density of *P. argentipes*.²¹

DISCUSSION

Asymptomatic cases of VL have been reported from disease-endemic areas of Bihar. A study conducted with asymptomatic contacts of VL cases showed that 36.67% were seropositive by the rK-39 test; VL developed in 49% within three months and in 69% within a year.²² Another study showed subclinical infections in 12-39% of healthy persons in a VL-endemic region in India.¹⁰ A third study showed that approximately 13% of neighbors of persons with VL were seropositive by the rK39 test at baseline and that 100% of case-contacts were seropositive; VL developed in 25% of these persons

TABLE 2
Comparative results of rK-39, PCR, and DAT among screened population at baseline survey, Bihar, India*

DAT	rK-39	PCR		Total
		No. positive	No. negative	
Positive	Positive	17	4	21
	Negative	3	15	18
	Total	20	19	39
Negative	Positive	0	3	3
	Negative	8	305	313
	Total	8	308	316

* PCR = polymerase chain reaction; DAT = direct agglutination test.

TABLE 3
Distribution of leishmaniasis-positive persons with or without symptoms, Bihar, India*

Serologic test	Symptomatic persons, no. (%)	Asymptomatic persons, no. (%)	Total
rK39	8 (33)	16 (67)	24
PCR	8 (29)	19 (71)	28
DAT	9 (23)	30 (77)	39
Any test	10 (20)	40 (80)	50

* PCR = polymerase chain reaction; DAT = direct agglutination test.

within 3 months.⁸ In our study, the point prevalence of asymptomatic infection was lower than in previous studies. We also observed a lower rate of progression from asymptomatic to symptomatic VL in less than six months.

The variation in proportion of asymptomatic persons in VL-endemic regions can be explained. The rate progression of symptomatic cases among case-contacts of persons with VL was approximately three times higher than in neighbors in VL-endemic regions. This estimate is similar to the probability of acquiring sub-clinical infections among persons in the same households with VL cases than to the probability of acquiring sub-clinical infections among neighbors of persons with VL.^{8,9}

Because we did not perform the PCR at the end of follow-up period, it was difficult to predict the PCR status of these subjects. However, on the basis of PCR status, a high (80%) proportion of persons were asymptomatic up until six months of follow-up. These asymptomatic persons likely harbor *Leishmania* parasites in their peripheral blood, and thus can act as reservoirs of parasites in the transmission of disease by bites of sand flies.¹⁹

We observed a high man-hour density of *P. argentipes* during the summer and rainy season in the study area. One reason for the high (80%) rate of PCR positivity and high vector density in the study area might be the fact that sampling was performed during the high-transmission period. However, the presence of asymptomatic persons, high vector density, and high prevalence of persons with VL could lead to rapid transmission of disease in the study area.

The presence of other factors in the study area, such as a low-income population, mud-plastered houses, and VL contacts, were also conducive for high transmission of VL. *Phlebotomus argentipes* is commonly found in cracks and crevices of mud walls, mud-plastered walls, or unplastered brick walls in rural areas of Bihar. These factors have been identified as potential risk factors for VL.²⁰ Most of the asymptomatic persons were children (5–14 years of age) and adults (15–44 years of age).

Self-cure has been reported in a case report of VL.²³ In a follow-up study, approximately 65% of seropositive persons cleared their infections within six months.¹² In a longitudinal study of 55 asymptomatic subjects, approximately 31% showed self-cure at the end of one year of follow-up.¹⁷ In a retrospective study conducted in VL-endemic areas of Brazil, self-healing of *L. chagasi* infection occurred after one or two years.²⁴ It has been reported that some persons with evidence of sub-clinical infection (i.e., serologic reactivity to *Leishmania* antigen) were capable of clearing infections before parasites induced any symptomatic disease, a finding probably caused by strong cellular immunity.²⁵

In our study, symptomatic VL developed in approximately 18.42% of the asymptomatic persons within 3–6 months. Similar observations have been reported.¹⁷ Also, in VL-endemic areas in Brazil, VL developed in approximately 20–45% of persons infected with *L. chagasi*.^{26,27} In our study, no new cases of VL were detected after six months of follow-up. Similar observations have been reported from VL-endemic areas of Bihar.¹² It appears that most *L. donovani* infections that will develop into symptomatic VL take no longer than six months to do so.

We identified a shorter incubation and latent period for *L. donovani* infection. Our study may also provide information to predict development of disease in persons living in

areas endemic for VL where a significant proportion of persons are infected with *L. donovani*. Serologic tests, such as the rK39 test, combined with the PCR could be the most sensitive and specific tools for diagnosing asymptomatic infections. Our study suggests implementation of this strategy in regions of high endemicity for VL to detect early cases of VL. Early case detection and treatment would then provide an impetus for on-going strategies to eliminate VL in India by 2012.

Our study had some limitations. The study area was selected on the basis of an outbreak of VL reported in a daily newspaper. Nearly 35% of persons in the study area could not be enrolled for baseline screening because of various reasons. Some adult men, who were daily wage laborers in the nearby city, were not present in the study area during baseline screening. Some persons, especially women and children, refused to participate in the study. These factors could lead to selection bias if unscreened persons were more susceptible to VL than persons who were screened at baseline.

In conclusion, asymptotically infected persons in VL-endemic areas may alter transmission dynamics and lead to a high incidence of disease. Thus, assessment of the magnitude of asymptotically infected persons by using serologic screening and follow-up for at least six months to detect seroconversion can help in early detection of VL cases in areas endemic for this disease. Early treatment of persons with VL can help decrease transmission and lead to decreases in disease morbidity and mortality.

Received June 20, 2009. Accepted for publication April 2, 2010.

Acknowledgments: We thank R. N. Sah and N. K. Sinha for technical support during the study and Ranjeet Kumar and Anup Kumar for assistance in preparing the manuscript.

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