THE EPIDEMIOLOGY OF VISCERAL LEISHMANIASIS AND ASYMPTOMATIC LEISHMANIAL INFECTION IN A HIGHLY ENDEMIC BANGLADESHI VILLAGE

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Abstract. We examined the epidemiology of kala-azar and asymptomatic leishmanial infection measured by serologic and leishmanin skin test results in a Bangladeshi community. In a subset, we measured serum retinol, zinc and C-reactive protein (CRP). Kala-azar and seroconversion incidence were 15.6 and 63.1 per 1,000 person-years, respectively. Proximity to a previous kala-azar case increased the likelihood of both kala-azar and asymptomatic infection. Bed net use protected against kala-azar (rate ratio = 0.35, P < 0.01), but not subclinical infection (rate ratio = 1.1, P = 0.82). Kala-azar patients were younger (P < 0.001) and reported lower red meat consumption (P < 0.01) than asymptomatic seropositive individuals. Retinol and zinc levels were lower in current kala-azar patients and those who later developed kala-azar compared with uninfected and asymptotically infected subjects. The CRP levels were higher in kala-azar patients compared with the other two groups. Low red meat intake and poor zinc and retinol status may characterize a group at higher risk of symptomatic disease.

INTRODUCTION

The intracellular protozoan parasite Leishmania donovani is the etiologic agent of kala-azar (visceral leishmaniasis), which is characterized by prolonged fever, weight loss, hepatosplenomegaly, and pancytopenia.¹ In southern Asia, infected humans are the sole reservoir.² Kala-azar is a progressive, lethal systemic disease; records of kala-azar outbreaks that occurred before specific drug treatment was available document case-fatality rates > 95%.² Nevertheless, most people infected with leishmanial parasites never develop clinical disease.³,⁴ In asymptomatic individuals, leishmanial infection is usually detected using serologic tests that measure antileishmanial IgG or the leishmanin skin test (LST) for the delayed hypersensitivity response.³,⁵ Positive serologic test results are presumed to reflect a relatively transient increase in serum antibodies caused by recent infection that lasts for months,⁶ whereas a positive LST result is thought to indicate durable cell-mediated immunity after asymptomatic infection or clinical cure of kala-azar. A positive LST result may not appear for months to years after successful treatment of kala-azar, but then lasts for decades after exposure.¹,⁷ The reported ratio of asymptomatic infections to kala-azar cases varies widely from 6:1 in a cohort of children in a community in Brazil endemic for visceral leishmaniasis⁵ to 50:1 in an LST survey in Spain.⁸ This variation is presumed to reflect differences in parasite virulence and host population characteristics, such as nutritional status and immunogenetic factors,⁹,¹⁰ and may also depend on the tests used to define asymptomatic infection.¹¹

Data on risk factors for asymptomatic leishmanial infection are sparse, and its epidemiology is not well understood. Such information is essential for visceral leishmaniasis prevention and control efforts, such as the elimination program currently being mounted in South Asia,¹² because asymptotically infected individuals harbor latent parasite and may become ill if immunosuppression occurs,¹³,¹⁴ and may act as leishmanial infection reservoirs. In one study in India, Leishmania parasites were visualized in peripheral blood smears of persons with asymptomatic infection.¹⁵ In a Brazilian study, 25% of sand flies fed on kala-azar patients became infected, but none of the sand flies fed on asymptotically infected humans were infected.¹⁶ Nonetheless, dogs with asymptomatic or presymptomatic L. infantum infection have been shown to infect sand flies, albeit less frequently than dogs with symptomatic disease.¹⁷–²⁰ In one canine study, the more severe the symptoms, the higher the proportion of fed flies that became infected.²⁰ These findings suggest that humans with asymptomatic leishmanial infection may have the potential to transmit leishmaniasis, but are likely to be significantly less infectious to sand flies than patients with active kala-azar. Nevertheless, in many leishmaniasis-endemic communities, ≥ 30% of the population is infected based on skin testing.³,²¹–²³ Even limited infectiousness of a portion of these people could represent a large reservoir of infection and jeopardize efforts to control or eliminate the disease.

We recently characterized the epidemiology of kala-azar in a highly endemic Bangladeshi village.²⁴ We found that the strongest determinant of an individual’s risk of kala-azar was living with or near a person with recent kala-azar. Living in the same house with a kala-azar patient was associated with a 26-fold increased risk, and living within 50 meters increased risk by 3-fold compared with those living ≥ 50 meters away. Consistent use of locally available untreated bed nets in the summer months and increased density of cattle around the individual’s house were associated with significant protection from kala-azar. In the course of our investigation, we also surveyed the population with the recombinant rK39 enzyme-linked immunosorbent assay (ELISA) and LST and measured serum retinol and zinc levels in a subset of participants. In this analysis, our objectives are to describe the incidence and determinants of asymptomatic leishmanial infection compared with those of kala-azar, and to assess the correlation between micronutrient status and the clinical outcome of leishmanial infection.
METHODS

The study site was a village in Fulbaria subdistrict, Mymensingh district, Bangladesh chosen based on a high incidence of kala-azar reported in government surveillance data in the two years prior to initiation of the study.\textsuperscript{24,25} From January to April of 2002, 2003, and 2004, we performed house-to-house surveys to enumerate all residents who had spent at least six cumulative months in the village in the three years prior to data collection. The data collected included demographic information, ascertainment of past and current kala-azar cases, risk factor data, and in participants ≥ 3 years of age collection of a capillary blood specimen and intradermal application of the LST. The protocol was reviewed and approved by the International Centre for Diarrhoeal Disease Research, Bangladesh, Research and Ethical Review Committees and the Institutional Review Board of the Centers for Disease Control and Prevention (CDC). Informed consent was obtained from all adult participants and the parent/guardian of all participating children. Assent was also obtained from children ≥ 7 years of age.

The skin test antigen consisted of a suspension of 5 × 10\textsuperscript{7} L. infantum (World Health Organization reference strain MHOM/TN/80/IPT1) promastigotes/mL and was provided by the Istituto Superiore di Sanità (Rome, Italy). The test was applied and read following standard methods: 0.1 mL of antigen was injected intradermally; 48–72 hours later, the induration was measured in two perpendicular directions using the ball-point pen method.\textsuperscript{26} The LST result was considered positive if the mean of the two measurements was ≥ 5 mm. Because of substantial loss of leishmanial antigen quality in 2003 and 2004, the current analysis includes only the LST results from 2002.\textsuperscript{7}

Serum specimens were tested by ELISA using recombinant K39 antigen (Corixa Corporation, Seattle WA) and a modified protocol that included a standard curve based on a pool of known positive sera on each plate.\textsuperscript{6,27} The positive ELISA cut-off was placed at 20 standard curve concentration units (the 99th percentile of specimen readings from 38 people living in a region of Bangladesh not endemic for visceral leishmaniasis), while a cut-off of 61 concentration units demonstrated the best performance for diagnosis of kala-azar.\textsuperscript{27}

We defined a past case of kala-azar as an illness with two or more weeks of fever plus at least one of the following: weight loss, abdominal fullness or skin darkening, with clinical improvement after 20 days of intramuscular injections (corresponding to the sodium stibogluconate regimen prescribed by the Bangladesh national kala-azar treatment guidelines at the time). We defined a current case of kala-azar as illness meeting the above definition plus physical examination consistent with kala-azar (splenomegaly and/or hepatomegaly with or without measured fever, evidence of weight loss, skin darkening, or jaundice), and a positive rK39 ELISA result and/or rK39 immunochromatographic strip test (Kalazar Detect; Inbios International, Seattle, WA) result. Among participants with no symptoms suggestive of past or current kala-azar, remote asymptomatic infection was defined by a positive LST result, and recent asymptomatic infection by a positive ELISA result and negative LST result. Participants with a negative ELISA result, a negative LST result, and no history of kala-azar were considered uninfected.

Venous blood specimens for micronutrient assays were collected in 2002 from currently ill kala-azar patients, participants found to be positive by the rK39 ELISA on capillary specimens, and a sample of seronegative participants. Participants were classified by their findings throughout the study period up to the end of follow-up in 2004: those with negative ELISA results and LST results were considered uninfected, and participants with positive ELISA results and/or LST results without clinical findings or history of kala-azar were considered to have an asymptomatic infection. Participants who developed kala-azar ≥ 1 month after specimen collection were considered to have subsequent kala-azar. For the micronutrient analysis, the groups of participants without infection and with asymptomatic infection were frequency-matched by age group to current and subsequent kala-azar patients. The blood specimens for zinc assay were collected in trace element-free tubes from a lot shown to be zinc-free by the CDC National Center for Environmental Health laboratory prior to use. Venous blood specimens were placed immediately after collection in a cold box, protected from light exposure, and transported on ice packs to Dhaka. The serum was separated within six hours of collection and stored at −20°C until processing. Serum retinol levels were measured by high-performance liquid chromatography (Millipore Corp., Bedford, MA).\textsuperscript{28} The C-reactive protein levels were determined by the immune turbidimetry method,\textsuperscript{29} and were used to assess the presence of inflammation caused by kala-azar, or to other intercurrent infections, which are common in this community, and may affect levels of micronutrients. Serum zinc levels were measured by atomic absorption spectrophotometry (Shimadzu AA 6501S; Shimadzu, Nakagyouku, Japan).\textsuperscript{30} The generally accepted threshold for defining zinc deficiency based on serum measurements is 60 μg/dL (9.2 μmol/L).\textsuperscript{31} Retinol levels less than 30 mg/dL (105 μmol/L) are considered low or possibly responsive to greater intake, and those less than 20 mg/dL (70 μmol/L) are considered as vitamin A deficiency.\textsuperscript{32}

Data were analyzed using SAS version 9.0 (SAS Institute Inc., Cary, NC). We examined logistic regression models of risk factors for asymptomatic leishmanial infection in the 2002 survey. The eligible population consisted of those study participants with defined leishmanial status in 2002 (kala-azar or no kala-azar and results available for both ELISA and LST). All study households were mapped by global positioning system and data were uploaded into ArcView Geographic Information System (GIS) version 3.3 (Environmental Systems Research Institute Inc., Redlands, CA). Using GIS data, we calculated the distance from each household to the closest kala-azar case in any of the preceding years. To evaluate the impact of cattle (cows, oxen, or calves) on kala-azar risk for nearby residents, kernel density estimation was used to estimate cattle per 1,000 meters\textsuperscript{2}.\textsuperscript{24} The current analysis focused particularly on the risk factors identified in the published analyses of kala-azar cases with onset from 2000 to 2003: proximity to previous kala-azar cases, use of untreated bed nets in the summer months, and the kernel density of cattle around the subject’s house.\textsuperscript{24} We tested the following additional variables: household income, materials used in the walls, roof and floor of the house, animal ownership, and dietary intake of fish, goat, beef, and chicken. For comparisons within the current analysis, we also modeled risk factors...
Multivariable logistic regression models for determinants of kala-azar, positive rK39 ELISA results, and positive leishmanin skin test result in 2002*

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Kala-azar (onset 2001 or 2002)</th>
<th>rK39 ELISA positive in 2002</th>
<th>Leishmanin skin test positive in 2001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)†</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Distance from closest previous kala-azar case</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Same household</td>
<td>6.37 (3.30–12.28)</td>
<td>1.85 (1.09–3.16)</td>
<td>2.86 (1.98–4.14)</td>
</tr>
<tr>
<td>Outside household but within 50 meters</td>
<td>1.85 (0.95–3.60)</td>
<td>1.37 (0.88–2.11)</td>
<td>1.72 (1.24–2.39)</td>
</tr>
<tr>
<td>&gt; 50 meters away</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Use of bed net in summer months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Always</td>
<td>0.48 (0.31–0.74)</td>
<td>0.82 (0.51–1.33)</td>
<td>0.98 (0.70–1.38)</td>
</tr>
<tr>
<td>Sometimes or never</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cattle density (additional cow per 1,000 meters)‡</td>
<td>0.75 (0.58–0.96)</td>
<td>0.97 (0.81–1.16)</td>
<td>1.17 (1.00–1.36)</td>
</tr>
<tr>
<td>Each 10-year increase in age</td>
<td>0.83 (0.74–0.93)</td>
<td>1.12 (1.01–1.23)</td>
<td>1.48 (1.38–1.59)</td>
</tr>
</tbody>
</table>

* ELISA = enzyme-linked immunosorbent assay.
† Odds ratio (95% confidence interval) adjusted for intra-household correlation by generalized estimating equations.
‡ Past and current kala-azar cases excluded from analysis.
§ Leishmanin skin test-positive subjects excluded from analysis.

We attempted to isolate the factors associated with kala-azar and asymptomatic infection by constructing separate logistic regression models using the 2002 survey data (Table 1). As shown in our previous analyses,24 living in proximity to a recent case of kala-azar carried an increased risk of kala-azar in 2001–2002, and sleeping under a bed net in the summer months, higher density of cattle near the house, and increasing age were protective (Table 1, model 1). When those with past or current kala-azar were excluded from analysis, the likelihood of asymptomatic infection as measured by either ELISA or LST increased with proximity to a previous kala-azar patient and with increasing age (Table 1, models 2 and 3). In contrast to the model for kala-azar, higher cattle density was associated with an increased likelihood of a positive LST result, but had no significant effect on the likelihood of a positive ELISA result. A model that compared kala-azar cases to recent asymptomatic infection demonstrated that living in the same house as a previous kala-azar patient was associated with significantly increased risk, and increasing age and higher consumption of beef or goat meat with protection from symptomatic disease (Table 2). None of the other examined variables (listed in the methods) demonstrated any significant association with risk of asymptomatic infection as measured by ELISA or LST.

In 2003, 396 people with negative ELISA results, LST results, and kala-azar history entered the study, yielding 1,184

### RESULTS

At the time of the 2002 survey, the study population comprised 1,379 people with no history of kala-azar, 151 treated for kala-azar before 2002, and 16 people with current untreated kala-azar. A total of 579 people listed in the 2002 household census data were excluded from this analysis: 229 were < 3 years of age, 114 were unavailable for blood and skin testing, 135 had moved out of the village, 12 had died, 53 had a kala-azar status that could not be determined, and 36 refused to participate. Of those with no history of kala-azar, 788 (57%) were negative by the rK39 ELISA and LST, 396 (29%) positive by the LST only, 138 (10%) positive by the ELISA only, and 57 (4%) positive by the ELISA and LST in 2002.
individuals who met the inclusion criteria for the Poisson regression analysis of follow-up data through mid-2004. The incidence density of kala-azar and seroconversion were 15.6 and 63.1 per 1,000 person-years, respectively. In Poisson regression models adjusted for age, the consistent use of a bed net in the summer months was protective against incident kala-azar (rate ratio [RR] = 0.35, 95% confidence interval [CI] = 0.16–0.73, \( P < 0.01 \)), but not against new subclinical infection (RR = 1.1, 95% CI = 0.58–2.0, \( P = 0.82 \)).

Among 432 participants with positive ELISA result in 2002 or 2003, 299 converted to seronegative in a subsequent survey. The incidence of negative seroconversion was 502.1 per 1,000 person-years of follow-up. The magnitude of the positive ELISA result affected the incidence of conversion to negative serologic results: the incidence was 706.0 per 1,000 person-years if the reading was 20–60 CU compared with 104.1 per 1,000 person-years for \( \geq 61 \) CU. In a Poisson regression analysis, those with a history of kala-azar and higher ELISA results were significantly less likely to convert to negative serologic results (RR = 0.94 for each increase of 10 ELISA concentration units, 95% CI = 0.89–0.99, \( P < 0.05 \); RR = 0.40 for history of kala-azar, 95% CI = 0.26–0.61, \( P < 0.001 \)).

Micronutrient data were available for 16 patients with active kala-azar, 13 individuals who developed kala-azar a median of 5.3 months (range = 1–22 months) after the specimens were collected, 120 participants with positive ELISA and/or LST results (asymptomatic infection) by the end of follow-up in 2004, and 68 participants with no evidence of leishmanial infection throughout the study. Currently ill kala-azar patients had lower zinc and retinol levels and higher C-reactive protein levels than those without infection or with asymptomatic infection (Table 3). There was also a trend for lower zinc and retinol levels among the 13 participants who subsequently developed kala-azar, but these differences did not reach statistical significance. The prevalence of low zinc and retinol values was high among all groups (Table 4).

### DISCUSSION

Visceral leishmaniasis has a predominantly bimodal pattern of clinical manifestations. Although mild, self-resolving disease has been reported in cohort studies in Brazil, these cases appear to be rare. Most overtly symptomatic visceral leishmaniasis patients have progressive disease that eventually results in death without treatment, and those with infection detected by serologic analysis or LST usually report no symptoms. Our data document a ratio of four cases of kala-azar, but also of asymptomatic infection. The lack of a protective association between bed net use and asymptomatic infection is somewhat surprising in light of our earlier finding that bed net use was strongly protective against kala-azar, but permitting asymptomatic infection. Having cattle around the household may function in a similar manner to limit but not eliminate exposure, leading to the positive association between cattle density and risk of kala-azar.

In our analyses, not surprisingly, proximity to a previous case of kala-azar increased the likelihood not only of kala-azar, but also of asymptomatic infection. The lack of a protective association between bed net use and asymptomatic infection is somewhat surprising in light of our earlier finding that bed net use was strongly protective against kala-azar. Possibly these locally available, untreated bed nets are only partially protective against sand fly bites, decreasing the average parasite inoculum and therefore the risk of kala-azar, but permitting asymptomatic infection. Having cattle around the household may function in a similar manner to limit but not eliminate exposure, leading to the positive association between cattle density and risk of kala-azar.

Our analyses were limited in two major ways. The progressive loss of leishmanin skin test antigen quality in 2003 and 2004 prevented the inclusion of LST conversion without de-

### Table 3

Mean serum zinc, retinol, and C-reactive protein (CRP) levels among subjects without leishmanial infection, subclinical infection, current kala-azar, and kala-azar with onset one or more months after specimen collection

<table>
<thead>
<tr>
<th>Serum micronutrient</th>
<th>Group 1, no infection (n = 68)</th>
<th>Group 2, asymptomatic infection (n = 120)</th>
<th>Group 3, active kala-azar (n = 16)</th>
<th>P* for comparison to Group 1</th>
<th>Group 4, subsequent kala-azar (n = 13)</th>
<th>P for comparison to Group 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc (µg/dL)</td>
<td>59.5</td>
<td>59.6</td>
<td>56.8</td>
<td>0.08</td>
<td>0.06</td>
<td>57.0</td>
</tr>
<tr>
<td>Retinol (mg/dL)</td>
<td>31.8</td>
<td>34.7</td>
<td>22.9†</td>
<td>0.009</td>
<td>0.002</td>
<td>29.1</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>4.5</td>
<td>3.2</td>
<td>12.2†</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* By Kruskal-Wallis test.
† Retinol and CRP data missing for one subject.

### Table 4

Prevalence of low and deficient zinc and retinol levels among subject without leishmanial infection, asymptomatic infection, current kala-azar, and kala-azar with onset one or more months after specimen collection

<table>
<thead>
<tr>
<th>Serum micronutrient level</th>
<th>Group 1, no infection (n = 68) n (%)</th>
<th>Group 2, asymptomatic infection (n = 120) n (%)</th>
<th>Group 3, active kala-azar (n = 16) n (%)</th>
<th>Group 4, subsequent kala-azar (n = 13) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc &lt; 60 µg/dL</td>
<td>35 (52)</td>
<td>58 (48)</td>
<td>12 (75)</td>
<td>9 (69)</td>
</tr>
<tr>
<td>Retinol &lt; 30 mg/dL</td>
<td>34 (50)</td>
<td>50 (42)</td>
<td>12 (80)*</td>
<td>6 (46)</td>
</tr>
<tr>
<td>Retinol &lt; 20 mg/dL</td>
<td>12 (18)</td>
<td>16 (13)</td>
<td>9 (60)*</td>
<td>4 (31)</td>
</tr>
<tr>
<td>C-reactive protein &gt; 5 mg/L</td>
<td>9 (13)</td>
<td>4 (3)</td>
<td>9 (56)*</td>
<td>4 (31)</td>
</tr>
</tbody>
</table>

* Retinol and C-reactive protein data missing for one subject.
tected seroconversion as a criterion for asymptomatic infection in the longitudinal analysis. Our findings also suggest that except for those with kala-azar and/or very high ELISA results, reversion to negative serologic results usually occurs within months after infection. Because our serosurveys were performed at yearly intervals, we may have missed some instances of seroconversion; our estimate of asymptomatic infection incidence is thus a lower bound, rather than a comprehensive assessment. In addition, the relatively small number of kala-azar cases that occurred among those for whom we had zinc and retinol data decreased the statistical power of that analysis.

Nevertheless, our findings add to the published evidence that diet and micronutrient status play a critical role in the susceptibility of *Leishmania*-infected individuals to progress to kala-azar. The characteristics that help determine whether an infection leads to overt disease appear to include age and dietary factors such as intake of iron- and zinc-rich red meat. Our data are consistent with earlier epidemiologic studies that demonstrated a higher risk of kala-azar in younger individuals and in those with protein-energy malnutrition. Recent experimental data in protein energy-, zinc-, and iron-deficient mice suggest that this effect may be mediated primarily through the functional failure of the lymph node barrier and increased early visceralization of the parasite. Immuno genetic factors have been clearly implicated in the risk of symptomatic disease in *L. infantum/chagasi* infection. These factors are also likely to be important in *L. donovani* infection. Nonetheless, modifiable factors such as dietary micronutrient intake may be more immediately relevant from the standpoint of public health.

We found that patients with active kala-azar had significantly lower retinol and zinc levels, and much higher levels of the acute-phase reactant C-reactive protein. The well-known association of many infections with lower retinol levels is usually attributed to reduced retinol transport and synthesis as a direct result of the inflammatory process, but increased excretion may also be a contributor. The trend for lower retinol and zinc levels in those who later developed kala-azar likely reflects in part an early disease stage in a proportion of these individuals, as shown by elevated C-reactive protein levels. However, it is also possible that lower meat intake and poor zinc and retinol status may characterize a group with an increased risk of disease progression. Moreover, uninfected community members had poor micronutrient status: more than 50% had zinc levels below the commonly accepted threshold for deficiency. Differences in micronutrient levels between clinical groups may thus be obscured by the poor status of the population as a whole, a concept expressed by Rose 20 years ago as the sick population. Improved micronutrient status in the population through supplementation and improved dietary intake may have the potential to decrease the proportion of infected individuals who progress to kala-azar and to decrease the burden of visceral leishmaniasis in highly affected areas.

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