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Risk Factors for Visceral Leishmaniasis in a New Epidemic Site in Amhara Region, Ethiopia

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Abstract. We conducted a case-control study to evaluate risk factors for visceral leishmaniasis during an epidemic in a previously unaffected district of Ethiopia. We also collected blood and bone marrow specimens from dogs in the outbreak villages. In multivariable analyses of 171 matched case-control pairs, dog ownership, sleeping under an acacia tree during the day, and habitually sleeping outside at night were associated with significantly increased risk. Specimens from 7 (3.8%) dogs were positive by immunofluorescent antibody test (IFAT) and both enzyme-linked immunosorbent assays (ELISAs), whereas *Leishmania* DNA was detected in 5 (2.8%) bone marrow aspirates (from 3 seropositive and 2 seronegative dogs). Insecticide-treated nets may only protect a portion of those at risk. Further research on the vectors, the role of the dog in the transmission cycle, and the effect of candidate interventions are needed to design the best strategy for control.

INTRODUCTION

The Horn of Africa is one of the most important foci of visceral leishmaniasis (VL) in the world, characterized by sustained endemic transmission in several geographic sites, and intermittent epidemics often associated with population displacement and conflict.[1,2] In 2005, a new epidemic of VL was reported in the district of Libo Kemkem in the highlands of northwestern Ethiopia.[3] The outbreak occurred in a region where few cases of the disease had ever been reported before, and was hypothesized to represent introduction of the parasite by migrant agricultural laborers returning to their villages from seasonal work on the border with Sudan. A rapid epidemiologic assessment demonstrated that the outbreak appeared to have begun in Bura *kebele* (subdistrict), Libo Kemkem *wereda* (district) in 2003, and the incidence of cases as well as the number of affected *kebeles* was still rising in October 2005. By December 2007, a cumulative total of 2,450 primary kala-azar patients had been treated at the only VL treatment center in the area (*Médecins sans Frontières*-Greece, unpublished data). In the 2005 research, DNA from several strains of parasites of the *Leishmania donovani* complex was identified in splenic aspirate specimens from kala-azar patients and in the blood of two dogs.[3]

Risk factor data are essential to designing the appropriate public health response to an epidemic. The most valuable contribution of such an analysis is to identify risk factors that can be modified to prevent future cases. Because of time and resource constraints, no risk factor data were collected during the rapid assessment in 2005, but the presence of infected dogs and male predominance among cases led us to hypothesize that men and boys might be at higher risk resulting from the practice of sleeping outside, often in proximity to guard dogs, to protect their cattle from theft. In February 2007, a team of investigators returned to the Amhara Region to conduct a case-control study to evaluate risk factors for outbreak-related VL.

METHODS

Human case-control study. The research was conducted in the *weredas* (districts) of Libo and Fogera in the highlands (average altitude 2,000 meters above sea level) of the Amhara Region of northwestern Ethiopia. Addis Zemen is the capital of Libo district, located between Bahir Dar and Gondar on the major road connecting Addis Ababa to the Red Sea. Cases were selected from the records of *Médecins sans Frontières*-Greece (MSF-G)/Addis Zemen Health Center (AZHC) among patients with treatment dates starting in January 2006. Addis Zemen Health Center is the only health care facility in the area with VL diagnostic capability and antileishmanial drugs. Malaria occurs seasonally in this area. Routine human immunodeficiency virus (HIV) screening had not been instituted at the AZHC at the time of the study, although a small number of HIV-VL co-infections had been recognized. None of the case patients in the current study were known to be HIV co-infected.

Patients were diagnosed by physicians at AZHC using a standard clinical case definition (fever for at least 2 weeks, associated with weight loss and/or splenomegaly), and confirmed by the direct agglutination test (DAT). The DAT was performed using standard methods and leishmanial antigen from the Institute of Tropical Medicine, Antwerp, Belgium. Titers $\geq$ 1: 3200 were considered to be positive.[4] For the DAT using the Antwerp antigen and protocol in an Ethiopian population, the estimated sensitivity and specificity were 94% and 93.6%, respectively.[5] If the DAT results on two separate occasions were inconclusive, splenic aspirate was used to confirm the diagnosis. Most case patients had been treated before the case-control study began, following standard MSF practice, usually 28 days of sodium antimony gluconate. The protocol was reviewed by World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC), and judged to be covered as an outbreak investigation.

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Case patients were sought in their homes, based on a line listing from AZHC that included name, age, gender, name of the father or husband of the patient, village, and kebele. If a household contained more than one case, only the most recently treated case was included in the study. For each case, one matched control was chosen. The controls were chosen from the nearest house to the case household, identified by leaving the case house and turning right from the doorway. Each control was matched to the respective case by gender and age range (< 5 years, 5–14 years, 15–39 years, and 40 years of age or older). If a case of VL had ever occurred in a member of the candidate control household, or if there was no household member fulfilling the matching criteria, the next nearest household (again, turning right from the doorway) was chosen instead. The risk factor questionnaire collected data concerning domestic animals and where they were kept at night, sleeping location and habits, bed net ownership and use, house construction materials, travel to the Sudan border area, and socioeconomic indicators. For young children, parents provided consent and responded to the questionnaire.

The initial sample size calculations, based on logistic considerations, called for 100 cases and 100 controls that would have enabled the identification of risk factors associated with odds ratios of 2.3–2.5. When it became clear that further recruitment was feasible, the target sample size was increased to 175 case-control pairs, allowing the detection of factors associated with odds ratios of 1.9–2.1. Field work was conducted during two periods of time, February 9–16 and November 8–22, 2007 (before and after the rainy season). The same field workers collected the data during the two field trips, using the same questionnaires. A comparison of the data from the two periods of time demonstrated that the participants were comparable in age and gender distribution, and separate analysis of the two datasets yielded comparable epidemiologic findings. Therefore, only the aggregate data are presented here.

Data were single-entered in a database designed by a CDC data manager with internal quality control checks. All questionnaires were reviewed in the field, and the database and questionnaires were compared by two independent observers. Analysis was conducted in SAS 9.1 (SAS Institute Inc, Cary, NC) and Stata 10 (StataCorp LP, College Station, TX). Case-control data were analyzed using univariate and stepwise multivariable conditional logistic regression with Wald 95% confidence limits, to account for the matched design. The Kruskal-Wallis test was used for comparison of continuous variables. Co-linearity was assessed using Spearman correlation coefficients. Variables with $P < 0.10$ in the univariate analyses were tested in multivariable models; interaction between variables was tested using interaction terms. Case-control pairs with missing data for specific variables were excluded from analyses that included those variables.

Canine infection survey. During the February field work, dogs were sampled in study villages in 3 of the 6 kebeles. The villages were chosen for logistic reasons, because the human case-control study was being conducted in those sites at the time that the veterinary team was in the field. Owners were requested to bring their dogs to a central location in the village; dogs were restrained by the owners while sampling was carried out. Peripheral blood (0.6 mL) was collected in EDTA from the jugular vein, and bone marrow aspiration (0.2 mL) was performed at the junction of the rib and costal cartilage. Blood samples were centrifuged within 6 hours of collection, plasma and cellular portions separated, and stored at 4°C until serologic testing, and polymerase chain reaction (PCR) were carried out in Spain. Bone marrow aspirates were immediately placed in NET 10 buffer (NaCl 10 mM, EDTA 10 mM and Tris–HCl, pH 8.0, 10 mM) in the field; an aliquot of each specimen was inoculated into Novy-MacNeal-Nicolle (NNN) culture medium within 6 hours of collection and maintained at 27°C for 4 weeks. Subcultures were performed weekly.

Whole blood was tested immediately using the rK39 Kala-azar Detect rapid test (InBios International, Seattle, WA) following the manufacturer's specifications. The rapid test used to test dogs was the human format, which employs a non-specific Protein A that also recognizes canine IgG. Plasma specimens were transported to the laboratory of the National Center of Microbiology, Instituto de Salud Carlos III, Madrid, Spain, where they were tested by immunofluorescent antibody test (IFAT), and enzyme-linked immunosorbent assays (ELISAs) using two different antigens. The IFAT followed standard methods,[6] using *Leishmania infantum* MON-1 (reference strain MHOM/FR/78/LEM-75) promastigotes, rabbit anti-dog IgG (H+L) conjugated with fluorescein isothiocyanate (ICN Plaza, Costa Mesa, CA). The positive cut-off was set at 1/80 based on the internationally accepted IFAT cut-off value.[7–9]

For the first ELISA, microtiter plates (Nunc Maxisorp, Thermo Fisher Scientific, Roskilde, Denmark) were coated with 1 μg per well soluble *L. infantum* MON-1 antigen (reference strain MHOM/FR/78/LEM-75).[10] Study sera were diluted 1/100 and tested in duplicate; positive and negative controls were included on each plate. The presence of antibodies was detected by horseradish peroxidase-conjugated dog IgG heavy chain (Bethyl Laboratories, Inc, Montgomery, TX). The positive cut-off was set at optical density (OD) = 0.660 at 405 nm (the mean of the OD values plus 3 standard deviations from 30 healthy control dogs from the study site). The second ELISA used the same technique, but substituted 50 ng of rK39 antigen per well.[11] The positive cut-off was set at OD = 0.280 at 405 nm (the mean of the OD values plus 3 standard deviations from 30 healthy control dogs from the study site shown to be uninfected by the other diagnostic methods used).

For the molecular assays, 100 μL of peripheral blood or bone marrow aspirate was mixed with 300 μL NET 10 and 40 μL 10% sodium dodecyl suphate (SDS), incubated with Proteinase K for 1 hour at 70°C, and subjected to a classic phenol-chloroform extraction and ethanol precipitation. To determine the presence of *Leishmania* DNA, 10 μL of the extracted material was processed following the nested PCR technique developed by Cruz and others.[12] To identify the *Leishmania* species, sequence analysis of the LnPCR products (small subunit ribosomal [SSUrRNA] region) was performed.[12]

RESULTS

A total of 171 case-control pairs were interviewed (Table 1). They came from 3 *kebeles* in Fogera and 3 in Libo Kemkem; their geographic origins reflected the predominant distribution of VL cases in 2006. The mean age of controls was 1.5 years older than cases; other demographic characteristics were comparable among cases and controls. In univariate conditional logistic regression analyses, dog ownership, keeping cattle inside the house at night, report of indoor insecticide spraying, and increasing family size were associated with

TABLE 1

Age, gender, and geographic distribution of 171 case-control pairs, Amhara Region, Ethiopia

Demographics	Cases	Controls	*P* value
Male	127 (74%)	127 (74%)	1.00
Female	44 (26%)	44 (26%)	
Age (years)			
Mean	21.3	22.8	0.010
Median	18	20	
Range	2.5–65	0.5–77	
Among males			
Mean	22.6	24.0	0.041
Median	19	22	
Range	3–65	3–77	
Among females			
Mean	17.6	19.2	0.108
Median	15	16.5	
Range	2.5–55	0.5–60	
Kebele (District) of residence			
Addis Beta Cristian (Fogera)	4 (2%)	4 (2%)	1.00
Dibasifatra (Fogera)	37 (22%)	37 (22%)	
Rib Gebriel (Fogera)	62 (36%)	62 (36%)	
Estifanos (Libo)	1 (1%)	1 (1%)	
Shamo (Libo)	44 (26%)	44 (26%)	
Shina (Libo)	23 (13%)	23 (13%)	

significantly higher risk of VL (Table 2). Habitually sleeping outside at night and daytime naps under an acacia tree were both associated with significantly increased risk of VL. The odds ratio was 2.46 (95% confidence interval [CI], 1.5–4.0) for those having one dog, and 2.88 (95% CI, 1.0–8.2) for those with two or more dogs, compared with the risk for those without dogs; however, only 21 participants had 2 or more dogs. Ownership of a treated bed net appeared to lower risk, but this factor did not reach statistical significance.

In multivariable conditional logistic regression models, dog ownership, sleeping under an acacia tree during the day, and habitually sleeping outside at night were associated with significantly increased risk (Model 1, Table 3). When the variable "number of family members" was included as a predictor in the multivariable model, the variable "habitually sleeps outside" no longer demonstrated a significant association with risk (Model 2, Table 3). Further analysis revealed a possible interaction between these two variables, although the interaction term failed to reach statistical significance ($P = 0.19$). However, the 33 case-control pairs missing family size data showed much stronger association between sleeping outside and VL risk than pairs with family size data.

The canine serosurvey included 186 dogs from seven of the same villages in which case-control participants lived. All sampled dogs were adults, with reported ages between 10 months and 12 years; 104 (55.9%) were male, and 44 (23.7%) came from houses with at least one reported human case of visceral leishmaniasis. All dogs underwent physical examination; none had signs of VL. All dogs had peripheral blood collected; bone marrow aspirates were obtained from 178 (95.7%) dogs. Specimens from 7 (3.8%) dogs were positive by IFAT and both ELISA assays; 5 of these were positive by rK39 rapid test in the field. An additional 13 (7.0%) specimens were positive by IFAT at the cut-off value of 1/80, but negative or borderline by the ELISA assays. None of the cultures yielded parasite; 33 (23%) had bacterial contamination. None of the peripheral blood specimens was positive by PCR, but 5 (2.8%) bone marrow aspirates yielded DNA; sequence analysis of the SSU rRNA region demonstrated that all belonged to the *Leishmania donovani* complex. Of the 5 dogs with PCR-positive bone marrow aspirates, 3 were seropositive and 2 seronegative.

TABLE 2

Risk factors for visceral leishmaniasis in Amhara, Ethiopia, based on univariate conditional logistic regression models of data from 171 case-control pairs*

Factor	Cases	Cases	Controls	Controls	Matched odds ratio	95% confidence interval	*P* value
Household level variables	n	%	*N*	%			
Owns dog	101	59.1	64	37.4	2.61	1.6–4.2	< 0.0001
Has dog that sleeps in or near house	46	27.2	25	14.6	2.24	1.3–4.0	0.006
Owns cattle (cow, bull, calf)	153	90.0	150	87.7	1.31	0.6–2.7	0.47
Cattle stay in house at night (dry season)	9	5.3	3	1.8	4.00	0.9–18.8	0.08
Cattle stay in house at night (wet season)	90	52.9	75	44.4	1.88	1.1–3.4	0.04
Owns donkey	55	32.4	44	25.9	1.37	0.9–2.2	0.19
Owns goats	60	35.5	59	35.3	1.03	0.7–1.6	0.91
Owns radio	28	16.4	22	13.0	1.32	0.7–2.4	0.37
Has latrine	26	15.2	31	18.1	0.71	0.3–1.5	0.36
Had bed net before case kala-azar onset	37	22.7	46	28.2	0.47	0.2–1.1	0.08
House reported to have been sprayed	60	35.3	50	29.4	6.0	0.7–50	0.10
Earthen walls	148	86.6	154	90.6	0.53	0.2–1.3	0.15
Thatch roof	118	69.4	112	66.7	1.12	0.7–2.0	0.52
Earthen floor	170	100.0	169	99.4	undefined		0.99
Owns ≥ 4 hectares of land	65	38.0	56	32.8	1.29	0.8–1.1	0.28
Monthly expenditure per person < 40 birr†	67	51.9	65	49.6	0.92	0.5–1.6	0.77
Household head can write his name	91	53.2	83	48.8	1.17	0.8–1.8	0.46
Family ≥ 6 members‡	72	52.2	39	28.3	3.54	1.9–6.6	0.0001
Individual behaviors							
Ever sleeps outside the house	114	67.5	99	58.6	1.88	1.1–3.4	0.04
Usually sleeps outside house	83	48.5	58	33.9	2.67	1.5–4.8	0.002
Usually sleeps on ground near cattle	55	33.7	34	20.9	2.31	1.3–4.2	0.005
Ever sleeps under acacia at night	25	14.8	12	7.1	3.17	1.3–7.9	0.02
Ever sleeps under acacia during day	99	59.3	81	48.5	2.64	1.3–5.3	0.006
In Metema/Humera before illness onset	16	9.4	11	6.4	1.56	0.7–3.6	0.30

* Number and percentage for each risk factor provided for reference only.
† Data missing for 41 pairs.
‡ Data missing for 33 pairs.

TABLE 3
Multivariable conditional logistic regression models of risk factors for visceral leishmaniasis in Amhara, Ethiopia

Factor	Odds ratio	95% confidence intervals	*P* value
Model 1			
Owns a dog	2.28	1.4–3.8	0.002
Sleeps under an acacia tree during the day	2.24	1.2–4.3	0.03
Usually sleeps outside at night	2.27	1.1–4.7	0.01
Model 2			
Owns a dog	2.76	1.5–5.1	0.001
Sleeps under an acacia tree during the day	2.65	1.2–6.1	0.02
Family size (each additional member)*	1.27	1.1–1.5	0.006

* Data missing for 33 case-control pairs.

DISCUSSION

The epidemiology of VL in the Horn of Africa is complex, and a number of key questions remain unanswered. At least two distinctive ecologic settings have been described, the endemic region spanning eastern Sudan and northwestern Ethiopia, in which the vector *Phlebotomus orientalis* is found in association with black cotton-clay soils and acacia forests,[13] and foci in southern Ethiopia, Kenya, and Uganda where *Phlebotomus martini* and *Phlebotomus celiae* transmit the disease, and termite mounds are thought to provide resting and possibly breeding sites for sandflies.[14,15] Transmission appears to include both anthroponotic and zoonotic components; the relative importance of animal versus human infection reservoir remains undetermined, and probably varies among settings.[16–18]

The area most affected by this epidemic differs from both of the classic ecologic settings. Libo and Fogera districts are located at an altitude of 2,000 meters, are substantially cooler, and have different vegetation from the lowland Sudan border focus. The likely vector is still *P. orientalis*, though possibly a distinct higher altitude sub-population.[19] The affected villages are settled highland agricultural communities, but the residents live in precarious economic conditions, prompting many adult men to seek seasonal employment on the border with Sudan, where sesame and sorghum fields absorb large numbers of migrant workers. In our 2005 rapid assessment data, 10% of all adult men reported having worked in the border areas, the same areas where intense VL transmission is known to occur. Moreover, young men who had worked in Humera and Metema were more likely to be leishmanin skin test (LST) positive (73%) than the ones who had never traveled to those endemic areas (48%), although this difference did not reach statistical significance.[3] The initiating event behind the highland epidemic is thought to be introduction of the parasite by returning migrant workers from these areas.

Our risk factor analysis supports a complex view of the transmission dynamics. We identified infected dogs during both the 2005 and 2007 investigations, and the strong association of dog ownership, with a suggestion of a dose-response relationship, supports a role for dogs in the transmission cycle. However, the prevalence of infection among surveyed dogs in the outbreak villages was only 10.8% by IFAT, 3.8% by ELISA, and 2.8% by PCR. The finding that some PCR-positive dogs were seronegative and *vice versa* is consistent with earlier observations.[20,21] Nevertheless, the low canine infection prevalence came as a surprise. In established zoonotic cycles, such as those in Brazil and southern Europe, reported canine infection rates are as high as 35–80%.[22–24] In an endemic village in eastern Sudan, 43–74% of dogs were seropositive by IFAT.[18] The low prevalence seen in our data may reflect high mortality among dogs infected early in the epidemic; anecdotally, several families reported that canine deaths from illness preceded human VL cases in their households. In Brazil, high canine seroprevalence preceded the human epidemic by several years.[25] Our case-control study was conducted after the peak of the epidemic and may simply have been too late to pick up many canine infections. Alternatively, it may indicate that humans are the main infection reservoir in this site. An important role for anthroponotic transmission has been proposed in several studies from sites classically considered zoonotic.[23,26]

Sleeping outside may place people at risk of sand fly exposure, and in proximity to infected dogs acting as guards on cattle herds. However, the risk associated with keeping cattle indoors and the trend toward protection for net ownership suggests that some transmission may also occur inside the house. Sand flies disturbed in daytime resting sites, when people nap under acacia trees, may also transmit the disease. A larger family size may appear as a risk factor based on attraction of sand flies by greater biomass[27]; however, the data for this variable were problematic: the data were missing for nearly 20% of our case-control pairs, and there appeared to be an interaction with other key variables. We have no evidence of a systematic error in the collection of data for this variable, and believe that this effect was a result of chance. In any case, data for factors more easily modified in the short term are more useful for the design of preventive interventions.

Published VL risk factor data for the Horn of Africa are sparse.[16,28,29] In an investigation of an outbreak in a community in eastern Sudan, a large proportion of villagers appeared to have been infected over a 4-year period.[16] Risk factors for VL included ownership of dogs and cattle, younger age, and male gender, whereas the presence of a neem tree was protective. Ethnicity appeared to be another important predictor of risk.[16] In a case-control study conducted in southern Ethiopia, having unplastered walls, keeping animals inside the human dwelling, proximity to termite hills, and poor antecedent nutritional status increased risk of VL.[30,31] A case-control study conducted in a region on the border of northwestern Kenya and eastern Uganda demonstrated a significant increase in VL risk associated with lower socioeconomic status and insecticide application to cattle, and a protective effect associated with sleeping close to animals, ownership of a bed net, and knowledge of VL symptoms.[28] The authors hypothesized that cattle may provide zooprophylaxis, and that application of insecticide to cattle could cause sand flies to increase their feeding on humans. A study in Kenya analyzed factors associated with leishmanial infection as measured by serology; this analysis demonstrated significant spatial clustering, but failed to show consistent risk factors for infection.[29] Only the study in Sudan was conducted in an area where the vector is *P. orientalis*; in southern Ethiopia, Kenya, and Uganda, *P. martini* and *P. celiae* are the presumed vectors. Other studies from Sudan demonstrate an association between the presence of acacia trees and *P. orientalis*,[13] consistent with our finding of risk associated with daytime naps under acacia trees. We could not address clustering of disease or infection in the case-control analysis, but our earlier LST

survey demonstrated strong clustering at the village level.[3] Our current data suggest that sleeping in proximity to cattle increased rather than decreased risk, but we were unable to examine spatial proximity and these results may simply reflect the effect of sleeping outside.

The risk factor study had a number of limitations because of logistical constraints. For example, the retrospective design was incapable of examining factors, such as nutritional status, which affect risk of progression from infection to kala-azar. Controls were not tested for asymptomatic leishmanial infection. However, the presence of undetected infection in a proportion of controls would have the effect of biasing toward the null hypothesis, and making associations appear weaker; for this reason, we do not believe this limitation invalidates our findings.

Our data have several implications for the design of a control program. On the basis of the risk factor analysis, the dog appears to play an important role in the transmission cycle, but the canine survey data decrease the certainty of this assumption. In any case, canine leishmaniasis control is widely recognized as challenging in Brazil, a setting with substantially more infrastructure and resources than highland villages in Ethiopia.[22,23] Novel approaches, such as topical insecticide application or impregnated dog collars, have been suggested as potentially more effective methods.[32] Our data suggest that insecticide-treated nets could have promise as a preventive intervention, but would likely protect only a portion of those at risk. If the use of nets is not feasible in the conditions under which men and boys sleep outside, innovative solutions must be sought for these villagers. Further research on the vectors, the circulating parasite strains, the role of the dog in the transmission cycle, and the effect of candidate interventions will be essential before drawing final conclusions as to the best combination of control measures.

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