Leishmania infantum chagasi in Northeastern Brazil: Asymptomatic Infection at the Urban Perimeter

Iraci D. Lima, Jose W. Queiroz, Henio G. Lacerda, Paula V. S. Queiroz, Nubia N. Pontes, James D. A. Barbosa, Daniella R. Martins, Jason L. Weirather, Richard D. Pearson, Mary E. Wilson, and Selma M. B. Jeronimo*

Health Post-Graduate Program, Department of Infectious Diseases, Health Sciences Center, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil; Department of Biochemistry and Department of Biology and Genetics, Biosciences Center, Universidade Federal do Rio Grande do Norte Natal, RN, Brazil; Fundação Nacional de Saúde, Natal, RN, Brazil; Departments of Medicine and Pathology, University of Virginia, Charlottesville, Virginia; Interdisciplinary Graduate Program in Genetics, Departments of Internal Medicine, Microbiology, and Epidemiology, University of Iowa, Iowa City, Iowa; Veteran's Affairs Medical Center, Iowa City, Iowa; Instituto Nacional de Ciência e Tecnologia de Doenças Tropicais (INCT-DT/CNPQ/MCT), Brazil

Abstract. Visceral leishmaniasis (VL) is endemic in large cities in Brazil, including Natal. We determined the prevalence of asymptomatic human infection with *Leishmania infantum chagasi* and associated environmental risks around Natal. Infection was detected by Leishmania skin test (LST) and anti-leishmanial antibodies in humans and anti-leishmanial antibodies in dogs. Amongst 345 humans, 24.6% were seropositive, and 38.6% were LST-positive. Prevalence of positive serology was similar in both sexes and across all ages. However, positive LST responses increased with age, suggesting that LST is long-lasting and cumulative. Multinomial logistic analysis showed that LST response varied with location ($P = 0.007$) and that males were more frequently LST-positive ($P = 0.027$). Indicators of lower socioeconomic status associated significantly with human infection. Furthermore, there was geographic coincidence of seropositive humans and dogs ($r = 0.7926$, $P = 0.011$). These data suggest that dog and human *L. i. chagasi* infection are intimately interrelated in environmental conditions associated with low income.

INTRODUCTION

Human visceral leishmaniasis (VL) in Brazil, caused by *Leishmania infantum chagasi*, historically occurred in a sporadic manner in rural areas of the northeast region of the country.^{1,2} Over the past 30 years, numerous outbreaks have been reported from Natal and other major cities in Brazil.^{3–7} These outbreaks have coincided with large-scale migration of people, with their dogs and other domestic animals, from rural areas endemic for *L. i. chagasi* to urban areas.⁸ In turn, the city of Natal itself has expanded into previously rural endemic areas.^{4,9} The sand fly vector for visceral leishmaniasis, *Lutzomyia longipalpis*, is present in the northeast and other areas of Brazil and extends south to Argentina.^{10,11} The net result is a coincidence of highly concentrated human populations, reservoir hosts, and infected vectors, creating a situation optimal for disease transmission.¹²

The *Leishmania* species previously referred to separately as *L. chagasi* and *L. infantum* are now thought to be the same species based on the genome sequence and biological characteristics and the fact that clinical manifestations of the diseases are remarkably similar.¹³ For these reasons, we refer to this parasite as *L. infantum chagasi* or *L. i. chagasi* throughout this paper. Domestic dogs are the principle animal reservoir for *L. i. chagasi* in Brazil. Similarly, dogs serve as a reservoir for *L. infantum* in endemic regions of Europe.¹⁴

The clinical manifestations of human *L. i. chagasi* infection vary greatly. The majority of infected persons experience asymptomatic or oligosymptomatic self-resolving infection that can be detected initially with anti-leishmanial antibodies.¹⁵ Serologic responses wane with time, and there is subsequent development of positive delayed-type hypersensitivity (DTH)

reactions to intradermally administered leishmanial antigens and the Montenegro or leishmanial skin test (LST).^{16–18} In those subjects who progress to symptomatic VL, anti-leishmanial antibodies rise to high titers, falling only after successful therapy.^{19,20} The LST is negative during acute VL and becomes positive months after successful chemotherapy.²¹ Thus, a positive LST, whether it occurs after asymptomatic infection or after successful treatment, is an indication that a protective type 1 cellular immune response has developed.²² Serologic responses, in contrast, accompany acute infection whether symptomatic or not. Anti-leishmanial antibodies fall with time after resolution of infection to low or undetectable levels.²³

Whereas there is little doubt that domestic dogs are the primary animal reservoir for *L. i. chagasi* in the region, the relationship between human and canine disease is not straightforward. Measures taken to control *L. i. chagasi* infections in Brazil have included euthanizing dogs with positive anti-leishmanial serology,²⁴ using insecticide-impregnated dog collars,²⁵ and spraying for vector control. However, often, the elimination of infected dogs has not impacted *L. i. chagasi* infection in humans.²⁶ Several possibilities could explain this observation, including a delay between dog euthanasia and the development of VL in humans, the high prevalence of canine leishmaniasis, large numbers of dogs in endemic neighborhoods, potential canine vertical transmission of *L. i. chagasi*, and/or the possibility that other animals, including humans, serve as reservoirs.²⁷ The spread of canine *L. i. chagasi* infection to more populated areas of southern Brazil, the adaptation of *Lu. longipalpis* to the periurban environment, and recent reports of concurrent human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and VL from the northeast of Brazil have raised concern that American VL may come to mimic the pattern observed in southern Europe, where VL emerged as an indicator disease for AIDS.^{28–32}

The goal of the current study was to determine the extent of human *L. i. chagasi* infection among people residing in an endemic area at the perimeter of Natal, Brazil. Specifically,

* Address correspondence to Selma M. B. Jeronimo, Department of Biochemistry and Health Post-Graduate Program, Federal do Rio Grande do Norte, Av. Sen. Salgado Filho, SN Natal, RN, 59078-970, Brazil, CP 1624. E-mail: smbj@cb.ufrn.br

we determined the prevalence of human infection detected by anti-leishmanial antibodies and/or delayed hypersensitivity responses to parasite antigens, the potential role of dogs as a reservoir for human infection, and the presence of a sand fly vector.

MATERIALS AND METHODS

Study area. Parnamirim, a city of 180,000 people, is located on the perimeter of metropolitan Natal in the state of Rio Grande do Norte, Brazil. The locality is home for many people who work in Natal. The study was conducted in neighborhoods accounting for 39.4% (230) of the individuals reported with VL in Parnamirim between 1990 and 2010 (Figure 1). The municipality is composed of urban, periurban, and rural areas that are defined according to the distance between houses and population density. The city is undergoing substantial expansion with resultant urbanization of surrounding rural areas. The demographics of VL in Parnamirim were similar to the demographics in other areas of Rio Grande do Norte and Brazil. The population of the region had increased fourfold over the previous 20 years. Sixty percent of subjects with VL were male, with a mean age of 11.1 years in VL-affected males and a mean age for females of 5.4 years ($P < 0.001$). No cases of cutaneous or mucosal leishmaniasis have been reported in the area. *L. i. chagasi* has been the sole *Leishmania* species isolated from humans and dogs with VL in the region according to isoenzyme analyses kindly performed by Elisa Cupolillo (Fiocruz, Rio de Janeiro, RJ, Brazil) on 25 *Leishmania* isolates (15 isolates from dogs and 10 isolates from humans).

Households included in the study were selected through a random point pattern generated for the study region without prior knowledge of the houses in the vicinity. The closest household to each point was selected using a global positioning system (GPS; model 315; Magellan, San Dimas, CA). If the family did not agree to participate or there was not a house at the point, the next closest household was selected. A total of 268 households were studied. The study area with the tessellation considered is shown in Supplemental Figure 1.

Ethical considerations. The protocol for this study was reviewed and approved by the Universidade Federal do Rio Grande do Norte Ethical Committee (CEP-UFRN 94/06).

The certificate of ethical approval is 0086.0.051.000-06 (<http://www.saude.rn.gov.br>). This institutional review board (IRB) committee is registered with the US National Institutes of Health (NIH), and the protocol was approved by the NIH. Approval for collaborators was received from IRBs at the University of Iowa and the University of Virginia.

Human study population. A total of 345 people residing within the 268 houses were enrolled. The sample size was based on prior observations in the Natal area documenting detectable human infection with *L. i. chagasi* in 42% of residents of neighborhoods where human VL was common.^{4,33} Subjects answered a verbal questionnaire indicating their time of residence in the neighborhood and previous medical history. A 10-mL sample of venous blood was collected, and an LST was taken.

Canine study population. All dogs belonging to consenting families in the targeted area were included in the study. Information related to a dog's age, size, type of hair, housing, breed, and health was collected. A subset of the canine population was reevaluated approximately 6 months after the first examination.

Leishmanial infection. Canine and human sera were screened for the presence of anti-leishmanial antibodies with two enzyme-linked immunosorbent assays (ELISAs). The first ELISA tested antibody responses to a soluble lysate of *L. i. chagasi* (SLA) using an isolate from a patient with VL in Natal that was typed in a World Health Organization Reference Laboratory (Elisa Cupolillo, Fiocruz, Rio de Janeiro, Brazil). The second ELISA used recombinant K39 antigen as previously described.²³ Briefly, wells of ELISA plates (Costar) were coated with 200 ng *L. i. chagasi* promastigote antigens (SLA) or 50 ng rK39. The recombinant K39 antigen for ELISA was kindly provided by Steven G. Reed (Infectious Diseases Research Institute, Seattle, WA). Each serum sample was assayed in triplicate. The A_{405} was determined using a Titertek Multiskan ICN plate reader. The cutoff used was the mean plus 3 standard deviations (SDs) absorbance of negative control sera from unexposed individuals. The cutoff values for anti-leishmanial SLA for humans and dogs were 0.119 and 0.124, respectively. For anti-rK39 in dogs, the cutoff value was 0.090.

Although there have been no other *Leishmania* species isolates from infected humans within 400 km of Natal,

FIGURE 1. Incidence of VL between 1990 and 2009 in the city of Parnamirim (bars) and the state of Rio Grande do Norte (lines) in Brazil.

Trypanosoma cruzi infections can give false-positive serological tests for Leishmania. *T. cruzi* infections, however, are uncommon in the region. Only 0.3% of blood bank samples were positive for anti-*T. cruzi* antibodies by ELISA in Natal (<http://saude.rn.go.br>). To further validate the leishmanial serological results, sera from all subjects in this study who were positive for anti-leishmanial antibodies were tested for *T. cruzi* antibodies by ELISA at Hemovida Blood Bank (Natal, RN, Brazil), and three subjects were positive. These positive sera were retested using a second serological assay as well as the radioimmune precipitation assay (RIPA) for confirmation of *T. cruzi* infection,³⁴ which was kindly performed by L. V. Kirchhoff (University of Iowa, Iowa City, IA). All three subjects' retested samples were found to be negative. Although we cannot absolutely exclude cross-reactive antibodies in our subjects, overall, the evidence supported the specificity of the serologic assay for *L. i. chagasi* infection in this study region.

The antigen used for the LST was kindly donated by the Centro de Produção e Pesquisa de Imunobiológico/PR (CPPI, Paraná, Brazil). It was prepared from *L. amazonensis*, and it is the only LST preparation approved for clinical use in Brazil. Thus, all clinical assessments of prior or current infection with any *Leishmania* species in the country use this antigen source. The LST was placed and read in accordance with the ballpoint pen technique.³⁵ Induration greater than 5 mm in diameter was considered positive.³⁶

Quantitative polymerase chain reaction detection and quantification of *L. i. chagasi* in blood from humans or dogs. Serum specimens are frequently obtained and stored from patients being assessed for various illnesses or during serosurveys, whereas buffy coat DNA is usually only collected for research purposes. We hypothesize that a low level of leukocyte lysis during blood draw may result in release of DNA from intracellular *Leishmania* in banked serum specimens from this and other studies. Indeed, DNA has been detected in sera from VL subjects from Bangladesh (Weirather JL and others, unpublished data). To assess the possibility of parasite DNA in sera from subjects in this study, DNA was isolated from the serum specimens drawn from human subjects and dogs using a Qiagen kit (MinuEute PCR Purification Kit). Control DNA was extracted from sera of individuals from a non-endemic region (Iowa). Parasite DNA was amplified by quantitative polymerase chain reaction (qPCR) using primers at 200 nM (forward: 5'-CTTTTCTGGTCCTCCGGGTAGG; reverse: 5'-CCACCCGGCCCTATTTACACCAA) and a Taqman probe (5'-FAM- TTTTCGCAGAACGCCCCCTA CCCGC-TAMRA) directed against the multicopy conserved region of the kDNA mini circles.^{37,38} Duplicate reactions were performed on an ABI 7500 thermal cycler. The number of parasites in each sample was calculated based on a standard curve generated from DNA extracted from a known number of *L. i. chagasi* promastigotes.

Statistical analysis. Statistical analyses were performed using STATISTICA release 6.1 (Stat Soft) and Stata/IC 10.0 (<http://www.stata.com>). Box plot analysis was performed to determine the normality of the data and presence of outliers. One-way analysis of variance (ANOVA) was used to assess differences in anti-leishmanial antibodies between groups. The maximum likelihood (ML) χ^2 test was used to assess the relationship between qualitative variables in contingency tables. Longitudinal canine anti-leishmanial antibody levels were analyzed using McNamer χ^2 test. Categories of pop-

ulation density were defined according to the number and proximity of households. The correlation between human and canine infections was assessed using Pearson's coefficient by two modes: first, considering the response of anti-leishmanial antibodies and LST in each point, and second, considering the mean response by tile at a spatial tessellation built on the study region to reduce the individual variability. A tessellation is a division of space into non-overlapping tiles. In this study, each tile contained at least five georeferenced points corresponding to households. The tiles were chosen based on visual inspection of the major clusters (Supplemental Figure 1). A multinomial logistic model was performed; as a response, it had a four vector representing the joint result of the LST and SLA tests, which yielded four outcomes (LST-SLA-, LST-SLA+, LST+SLA-, and LST+SLA+) with percent distribution named infection profile. The independent variables in the model were household location, sex, age, income, time residing in neighborhood, and number of residents. The model assessed the exposure to *Leishmania*, taking into account variables that could influence the LST and SLA responses.

RESULTS

Factors influencing the prevalence of human *Leishmania* infection. The prevalence of anti-leishmanial antibodies, detected with the anti-SLA ELISA, was 24.6% (85 of 345) in the human subjects tested. Serum samples from 17% (15 of 85) of these subjects were also positive for parasite DNA by qPCR. Although the sensitivity of this test on frozen serum specimens is not known, a validated sequence of species-specific qPCR primers identified all the organisms as *L. i. chagasi* (Weirather JL and others, unpublished data). The estimated numbers of parasites from the above assay varied from 6 to 23 parasites/mL serum according to qPCR using a standard curve generated from promastigote DNA. Seropositive individuals were also tested for anti-*T. cruzi* antibodies, and all were negative. A positive LST was detected in 38.6% (123 of 319) of the individuals available to test. Among these 123 LST-positive individuals, 83% were seronegative for anti-leishmanial antibodies, whereas 17% were seropositive ($P = 0.013$). Overall, there was an inverse correlation between a positive LST and a positive anti-leishmanial antibody response, a result that is consistent with previous studies of symptomatic VL.²¹ The prevalence of a positive LST response increased with age (Figure 2). In contrast, serologic evidence of anti-leishmanial antibodies, which correlates best with recent exposure, did not differ among age groups. However, LST response was greater in males ($P = 0.015$), but no difference in anti-leishmanial antibodies was observed ($P = 0.17683$) when sex was considered, indicating that both sexes were equally infected with *Leishmania*.

No significant differences in the *Leishmania* infection profiles were observed when considering other variables, including income, time of residence in the neighborhood, or number of people residing in the household (Table 1). The time of residence in the area did not correlate with either serologic or LST responses, likely reflecting the fact that the population was reasonably stable and that most subjects had resided in the area for more than 3 years. Although the mean age profiles did not reach statistical significance ($P = 0.052$), the post-hoc Tukey test was indicative of a significant difference in the mean age of LST+SLA- and LST-SLA- ($P = 0.045$).

FIGURE 2. Positive anti-leishmanial antibody and positive LST responses to *Leishmania* antigens according to age and sex. The prevalence of positive LST response increased with age and was greater in both sexes after 35 years of age. In contrast, the prevalence of positive serology for *Leishmania* was similar in all age groups.

There was a significant association between the *Leishmania* infection profile and the location of the household in the three study areas ($P < 0.0001$), which was shown by the different patterns of phenotype prevalence observed in Figure 3 and Table 2. Remarkably, significantly fewer subjects living in periurban areas (8.7%) exhibited the profile of recent infection (LST–SLA+) than those subjects living in urban (25.2%) or rural (20.8%) areas ($P < 0.0001$). Consistently, subjects residing in periurban areas were also less likely than others to have any sign of *Leishmania* infection (i.e., LST–SLA– was 56.5%, 36.0%, and 36.6% in periurban, urban, and rural subjects, respectively). Finally, subjects living in rural areas were the most likely to show signs of prior resolved infection (LST+SLA– = 40.6% in rural versus 25.2% or 30.4% in urban or periurban, respectively).

The presence of fruit trees versus shrubs was also associated with *Leishmania* infection ($P = 0.0088$), although this observation is confounded because of the fact that the type of vegetation is intimately linked to the location of the household in the municipality. Living in a house with walls made of mud as opposed to other materials (e.g., concrete or bricks; $P = 0.001$) was associated with measures of either recent infection (LST–SLA+ in 46.7% or 16.4%, respectively) or resolved infection (LST+SLA– in 46.7% or 31.3%, respectively). Individuals with a septic tank were most likely to have no evidence of infection (LST–SLA–) and least likely to have recent infection (LST–SLA+; $P < 0.0001$), although

this difference was not evident in the remote infection (LST+SLA–) phenotype. Infection was also associated with the presence of animals in the neighborhood ($P = 0.0090$) (Table 2).

Because *Leishmania* infection can be influenced by several cross-correlated variables, a multinomial logistic model was used to assess potential confounding by environmental factors and demographic factors such as age and sex (Table 3). First, a model was tested with variables that included area of location of the household, sex, age, income, time in the area, and number of residents, but because no difference was observed, a simpler model was adjusted by excluding them (Table 3). In this way, the model tested the relative risk of infection based on the LST–SLA– phenotypes. Therefore, the odds ratio of LST–SLA+ over LST–SLA– periurban was 0.24, which was the same ratio risk observed in a rural location ($P = 0.002$). However, when the LST+SLA– response was considered, the odds ratio was 0.42 ($P = 0.007$) (Table 3). The odds ratio of LST+SLA+ over LST–SLA– urban was 6.47 times the same ratio risk observed in a rural location ($P = 0.019$). The overall ratio risk of being LST+SLA–/LST–SLA– for males was 1.87 greater than the same ratio of females ($P = 0.027$) (Table 3). In addition, the odds ratio of LST+SLA– over LST–SLA– for age greater than 35 years was 2.42.

Prevalence of canine *L. i. chagasi* infection. All dogs that were either owned by or resided in households of subjects in the study were examined for serologic evidence of leishmaniasis.

TABLE 1

The size of the LST reaction and the magnitude of the anti-leishmanial antibody responses considering potentially associated variables

Variable	Subjects	LST–SLA– (139 subjects; 43.5%) mean $\pm$ SD (n)	LST–SLA+ (57 subjects; 17.9%) mean $\pm$ SD (n)	LST+SLA– (102 subjects; 32.0%) mean $\pm$ SD (n)	LST+SLA+ (21 subjects; 6.6%) mean $\pm$ SD (n)	P
Age (years)	318	34.1 $\pm$ 19.8 (139)	37.7 $\pm$ 19.0 (57)	41.2 $\pm$ 19.7 (102)	37.3 $\pm$ 19.8 (20)	0.052*
Income (monthly minimal wages)	319	2.0 $\pm$ 1.1 (139)	2.2 $\pm$ 1.1 (57)	2.1 $\pm$ 1.1 (102)	2.0 $\pm$ 1.0 (21)	0.468
Time in neighborhood (years)	305	7.7 $\pm$ 8.1 (133)	7.5 $\pm$ 5.6 (55)	9.8 $\pm$ 11.2 (96)	8.9 $\pm$ 5.7 (21)	0.249
Number of residents in household	306	4.8 $\pm$ 2.0 (136)	5.2 $\pm$ 2.1 (53)	4.9 $\pm$ 2.2 (96)	4.7 $\pm$ 1.7 (21)	0.689

Potentially associated variables were analyzed (results are indicated in the last column) using ANOVA one-way test. Unit measures for income are defined in monthly minimal wage (approximately R\$540 per month).

* A Tukey's post-hoc test shows that there was a significant difference between LST–SLA– (34.1) and LST+SLA– (41.2; $P = 0.045$).

FIGURE 3. Anti-leishmanial antibody and LST profiles by location. The percent of total was based on the percent of individuals falling into the infection profile based on 100% of individuals living in the region. The numbers of subjects were 103 in the urban, 115 in the periurban, and 101 in the rural regions. Based on the ML χ^2 test, the profiles of infection were significantly different among the three areas.

A total of 32.5% (101 of 346) dogs were seropositive when tested with SLA, whereas 2.3% (7 of 311 tested) were anti-rK39 positive. A subset of dogs (47 of 101) had parasitemia ranging from 5 to 282 parasites/mL that was quantified by qPCR. The seven dogs that were anti-rK39 positive were euthanized, and all necropsies were consistent with active VL. Necropsy signs of VL included enlargement of the spleen and liver, muscle wasting suggestive of weight loss, and amastigotes visualized in histologic sections of liver and spleen. A total of 177 dogs were located and reexamined 6 months after the initial enrollment. Of those dogs that were seropositive, 72.2% (39 of 54) had converted to negative, 30.1% (37 of 123) that were seronegative had become seropositive, 60.9% (86 of 123) remained seronegative, and 27.7% (15 of 54) remained seropositive. The net effect after conversions in both directions was a relatively constant percentage of dogs with positive anti-leishmanial antibodies over time ($P = 0.755$).

Spatial aggregation of human and canine *L. i. chagasi* infections. Using anti-leishmanial seropositivity as a marker

of canine infection and analyzing pairs (x, y) of human and canine responses within each household, no significant correlation was observed. However, when pairs of the mean response by tile in the tessellation tiles (Supplemental Figure 1) were compared, there was a significant correlation between the serologic responses of humans and dogs in tessellation tiles ($r = 0.7926$, $P = 0.011$) (Figure 4A). It is logical to examine correlations in these larger regions as opposed to houses, because dogs are not confined to households but rather, wander through neighborhoods close to their homes. In contrast to this measure of recent infection, there was no significant spatial correlation between a positive or negative human LST response, a measure of resolved infection, and positive canine anti-leishmanial serology when analyzed either by household or in tessellation tiles ($r = 0.4138$, $P = 0.206$), which was as shown in Figure 4B.

DISCUSSION

Similar to other Brazilian states, much of the population of Rio Grande do Norte has migrated from rural to urban areas over the past 30 years, resulting in a population residing predominantly in urban or periurban areas. The state capital city of Natal has been the site of epidemic VL along its expanding urban borders for several decades, similar to other cities in Brazil.^{3-7,39,40} Furthermore and as described previously in India and elsewhere in Brazil, the regional incidence of VL has varied over time, with peaks in 1992 and again in 2001.⁴¹⁻⁴³ This variability is reflected by disease incidence both in Parnamirim and the overall state of Rio Grande do Norte. Hypotheses to explain the cyclical pattern of VL epidemics have included herd immunity and fluctuations in the sand fly population density, but a definitive explanation is lacking. Beyond these hypotheses, it has been speculated that macroenvironmental phenomena, such as El Niño, contribute to outbreaks of VL and other vector-borne diseases by affecting rainfall in semi-arid endemic regions.^{43,44} Indeed, an increase in the incidence of VL has been observed in post-El Niño years in Brazil.⁴³

TABLE 2
Association between SLA response and/or positive LST with environmental parameters

Variable and category	n	LST-SLA-		LST-SLA+		LST+SLA-		LST+SLA+		P (χ^2 test)
		n	Percent	n	Percent	n	Percent	n	Percent	
Location of the household										< 0.0001
Urban	103	37	36.0	26	25.2	26	25.2	14	13.6	
Periurban	115	65	56.5	10	8.7	35	30.4	5	4.4	
Rural	101	37	36.6	21	20.8	41	40.6	2	2.0	0.0088
Type of vegetation in the yard										
Fruit	208	81	38.9	43	20.7	68	32.7	16	7.7	
Shrub	89	46	51.7	4	15.7	24	27.0	5	5.6	0.0010
Mixed	22	12	54.5	—	—	10	45.5	—	—	
Type of wall in the house										< 0.0001
Brick/roughcast	304	138	45.4	50	16.4	95	31.3	21	6.9	
Mud	15	1	6.6	7	46.7	7	46.7	—	—	
Sanitation										0.0090
Partial sanitation	86	24	27.9	25	29.1	29	33.7	8	9.3	
Septic tank	181	97	53.6	20	11.1	56	30.9	8	4.4	
No sanitation	47	15	31.9	12	25.5	15	31.9	5	10.7	< 0.0001
Presence of any animals in the neighborhood										
No	121	39	50.5	29	14.1	43	29.8	10	5.6	
Yes	198	100	32.2	28	24.0	59	35.5	11	8.3	

Data indicate the numbers of subjects in each subcategory and whether the subcategories are associated with distribution into the four phenotypes listed in the columns. Statistical analysis was performed by χ^2 test.

TABLE 3
Multinomial logistic model to investigate the association between the SLA and LST status and demographic factors

Response	n (%)	Independent variables	Odds ratio	95% confidence interval		P
				Lower bound	Upper bound	
LST-SLA+	57 (17.87)	Urban/rural	1.14	0.54	2.40	0.729
LST-SLA+	57 (17.87)	Periurban/rural	0.25	0.11	0.60	0.002
LST-SLA+	57 (17.87)	Male	0.77	0.37	1.62	0.496
LST-SLA+	57 (17.87)	Age greater than 35 years	1.74	0.91	3.33	0.091
LST+SLA-	102 (31.97)	Urban/rural	0.56	0.28	1.12	0.102
LST+SLA-	102 (31.97)	Periurban/rural	0.42	0.22	0.79	0.007
LST+SLA-	102 (31.97)	Male	1.87	1.07	3.25	0.027
LST+SLA-	102 (31.97)	Age greater than 35 years	2.42	1.41	4.16	0.001
LST+SLA+	21 (6.58)	Urban/rural	6.47	1.36	30.70	0.019
LST+SLA+	21 (6.58)	Periurban/rural	1.31	0.24	7.13	0.757
LST+SLA+	21 (6.58)	Male	1.29	0.47	3.54	0.616
LST+SLA+	21 (6.58)	Age greater than 35 years	1.71	0.66	4.45	0.269
LST-SLA-	139 (43.57)	Base group	—	—	—	—

Category of response LST $\times$ SLA using the LST-SLA- as the base group and considering the variables income, time in neighborhood, and number of residents, which were shown not to influence the results.

Anti-leishmanial antibodies develop during acute VL and fall to the negative range after symptomatic resolution. There is simultaneous development of a positive delayed-type hypersensitivity skin test response to *Leishmania* antigens (LST) that signifies recovery from disease. As such, positive serology in humans seems to be the best measure of recent or acute infection with the parasite. In contrast, a positive LST is a measure of a degree of cellular immunity associated with control of symptomatic disease.^{22,45} Males were shown to have a more robust LST response than females, although both sexes were infected as shown by positive anti-leishmanial antibodies. Previous studies conducted in other area of Brazil showed similar results.⁴⁶ Whether this finding reflects capacity of males to mount a strong DTH response or whether it reflects differences in parasite burden is not known. In the current study, the frequency of positive serology remained similar in all age groups, whereas the prevalence of a positive LST increased with age. This finding is consistent with observations of *L. donovani* infection in the Sudan.⁴⁷ It is also consistent with the disappearance of detectable anti-leishmanial antibodies after spontaneous resolution of asymptomatic infec-

tion or successful chemotherapy of VL.^{20,46,48-53} In contrast to the transient serologic status, a positive LST may be long lasting in most individuals,^{47,54} although it can become negative in some over time (Lacerda H and others, unpublished data).

The ratio of symptomatic to asymptomatic infection is highly variable in different geographic regions.^{47,55,56} Earlier studies in northeast Brazil reported a symptomatic to asymptomatic infection ratio in seropositive individuals of approximately 1:6 in children less than 10 years of age and 1:18 in adults ages 18 years or over.^{17,18} Using these parameters, we would have predicted approximately four cases of VL among our seropositive subpopulation. Although the absence of symptomatic VL cases in our seropositive subjects could represent sampling variability, it is also possible that the ratio of symptomatic to asymptomatic infection has changed with economic development and improved nutrition over the years since the earlier studies. Relevant to this hypothesis, it is noteworthy that there has been a substantial decrease in overall childhood mortality in Rio Grande do Norte in recent decades.⁵⁷ Although difficult to compare because of the likelihood of different *L. i. chagasi* zymodemes and different genetic and nutritional characteristics

FIGURE 4. Correlation between *Leishmania* infection in humans and dogs. A total of 272 residents were grouped in a tessellation with 11 polygonal tiles that considered the respective mean of human LST response, human anti-leishmanial antibodies, and canine anti-leishmanial antibodies in each tile. (A) Correlation of human anti-leishmanial antibodies and canine anti-leishmanial antibodies. (B) Correlation of human LST response and canine anti-leishmanial antibodies.

of the populations, the ratio of symptomatic to asymptomatic *L. infantum* infection in Europe may be as low as 1:50.⁵¹

Environmental factors that positively correlated with anti-leishmanial antibodies in humans included residence in a house with a mud floor and/or mud walls, lack of garbage collection, vegetation type, and dogs, birds, or donkeys/horses in the neighborhood. These factors could reflect the coincidence of poorer housing with other unmeasured effects of poverty and/or an environment that was conducive to sand fly breeding, which is similar to observations made in India.^{41,58,59} Domestic animals other than dogs are thought to produce an environment conducive to sand fly reproduction, although many may not serve as reservoirs for the parasite in Latin America. Although these observations suggest that the environment surrounding houses of poorer families may be conducive to the presence of sand flies and thus, disease transmission, these same factors could also correlate with host factors influencing disease susceptibility, such as inadequate sanitation or nutrition. Furthermore, a role of host genetics in disease development cannot be ignored.⁶⁰ Our data merely document the coincidence of environmental factors with infection/disease, and they do not indicate underlying causality.

Lutzomyia longipalpis, *Lu. lenti*, and *Lu. evandroi* were the major species identified in the study area; 49.3% of the sand flies collected were identified as *Lu. longipalpis*, the only vector known to be competent to transmit *L. i. chagasi* infection to humans in the region (data not shown) as documented previously.^{9,61,62} However, the circle was not completed by documenting that captured sand flies carried *L. i. chagasi*. Furthermore, sand flies are the major vector for transmission, but alternate routes for dog infection have been suggested. Indeed, non-arthropod transmission has been observed during a large outbreak of *L. infantum* infection among foxhounds in the United States, and it may be because of vertical and/or horizontal transmission.⁶³ Although difficult to document in our neighborhoods, such alternate routes of transmission are of substantial concern, because they may contribute to the maintenance of the parasite in dogs during periods when the numbers of sand flies are low.

Dogs are considered the major peridomestic reservoir for *L. i. chagasi* infections in Brazil.⁶⁴ However, in the current study, the presence of infected dogs in the household was not associated with human *L. i. chagasi* infection. The lack of household association between human and dog infections could be because of the fact that dogs are often kept outside the house and/or that they are frequently free to wander in the neighborhood, suggesting a model in which dog presence should be measured on the scale of a whole neighborhood rather than individual household. This concept is supported by the significant correlation between dog infection and acute human infection observed when the same parameters were assessed in tiles of the tessellation model. Serial measures of dog serology revealed fairly equal rates of conversion between positive and negative states, resulting in a state of equilibrium with a relatively constant proportion of infected dogs in the region (data not shown). Notably, subsets of both seropositive humans and dogs had evidence of *L. i. chagasi* DNA in their blood detected by qPCR. Although parasitemia seemed to be greater in dogs, the presence of *L. i. chagasi* DNA in humans suggests that they may potentially serve as a reservoir. Additional studies are needed to determine whether uninfected sand flies acquire *L. i. chagasi* when they feed on seropositive and/or PCR-

positive dogs or humans. An understanding of when canine infection transmission is most likely to occur and whether humans are a reservoir is obviously important to the formulation of strategies for control of *L. i. chagasi*.⁶⁴⁻⁶⁶

A number of cases of concurrent VL and HIV/AIDS have been identified in Natal and the state of Rio Grande Do Norte.³⁰ The presence of high rates of asymptomatic human and canine *L. i. chagasi* infections in an area where HIV/AIDS infection is emerging may lead to an increase in VL cases. In addition, there is concern over potential transmission through organ transplants and blood transfusions from reports of VL associated with these procedures.^{29,67,68} Considering the changing epidemiologic patterns of *L. i. chagasi* infection and expanding numbers of immunocompromised persons, the above data suggest that VL may emerge as an increasingly important health problem in Brazil.

Received September 6, 2010. Accepted for publication July 14, 2011.

Note: Supplemental figure is available at www.ajtmh.org.

Acknowledgments: The authors thank the Health Agents from the Municipality of Parnamirim for their assistance during the field work and Mr. Manoel Fernandes (Fundação Nacional de Saúde) for field assistance. We also thank Dr. Maria Gorete L. de Queiroz Laboratório Central de Saúde Pública (LACEN/RN) for making available sera from subjects with Chagas disease, Dr. Louis V. Kirchhoff (University of Iowa) for performing the confirmatory testing for Chagas disease in our samples, and Dr. Elisa Cupulillo (Fiocruz, Rio de Janeiro, RJ, Brazil) for identification of *Leishmania* species.

Financial support: This work was supported in part by funds from National Institutes of Health Grant NHP50 AI-030639 and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Studies were also supported by grants from the Department of Veterans' Affairs (to J.L.W. and M.E.W.) and National Institutes of Health Grants R01 AI045540 (to M.E.W.), R01 AI067874 (to M.E.W.), and R01 AI076233 (to M.E.W.).

Authors' addresses: Iraci D. Lima, Health Post-Graduate Program, Universidade Federal do Rio Grande do Norte and Fundação Nacional de Saúde, Natal, RN, Brazil, E-mail: iraci.duarte@ig.com.br. Jose W. Queiroz, Paula V. S. Queiroz, Nubia N. Pontes, and James D. A. Barbosa, Health Post-Graduate Program, Federal do Rio Grande do Norte, Natal, RN, Brazil, E-mails: jwq@supercabo.com.br, paul.avivianne@hotmail.com, nnatalir@yahoo.com.br, and jamesdary@oi.com.br. Henio G. Lacerda, Health Post-Graduate Program and Department of Infectious Diseases, Federal do Rio Grande do Norte, Natal, RN, Brazil, E-mail: heniolacerda@ufrnet.br. Daniella R. Martins, Department of Biology and Genetics, Biosciences Center, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil, E-mail: daniel.lamartins@cb.ufrn.br. Jason L. Weirather, Interdisciplinary Graduate Program in Genetics, University of Iowa, Iowa City, IA, E-mail: jason-weirather@uiowa.edu. Richard D. Pearson, Division of Infectious Diseases and International Health, Departments of Medicine and Pathology, Center for Global Health, University of Virginia School of Medicine, Charlottesville, VA, E-mail: rdp9g@virginia.edu. Mary E. Wilson, Departments of Internal Medicine, Microbiology, and Epidemiology, University of Iowa and the Veteran's Affairs Medical Center, Iowa City, IA, E-mail: mary-wilson@uiowa.edu. Selma M. B. Jeronimo, Department of Biochemistry and Health Post-Graduate Program, Federal do Rio Grande do Norte, Natal, RN, Brazil, E-mail: smbj@cb.ufrn.br.

REFERENCES

1. Lainson R, Shaw JJ, Lins ZC, 1969. Leishmaniasis in Brazil. IV. The fox, *Cerdocyon thous* (L) as a reservoir of *Leishmania donovani* in Para state, Brazil. *Trans R Soc Trop Med Hyg* 63: 741-745.
2. Lainson R, Ward RD, Shaw JJ, 1977. Experimental transmission of *Leishmania chagasi*, causative agent of neotropical visceral

- leishmaniasis, by the sandfly *Lutzomyia longipalpis*. *Nature* 266: 628–630.
3. Costa CH, Pereira HF, Araujo MV, 1990. Visceral leishmaniasis epidemic in the State of Piauí, Brazil, 1980–1986. *Rev Saude Publica* 24: 361–372.
 4. Jeronimo SM, Oliveira RM, Mackay S, Costa RM, Sweet J, Nascimento ET, Luz KG, Fernandes MZ, Jernigan J, Pearson RD, 1994. An urban outbreak of visceral leishmaniasis in Natal, Brazil. *Trans R Soc Trop Med Hyg* 88: 386–388.
 5. Silva ES, Gontijo CM, Pacheco RS, Fiuza VO, Brazil RP, 2001. Visceral leishmaniasis in the Metropolitan Region of Belo Horizonte, State of Minas Gerais, Brazil. *Mem Inst Oswaldo Cruz* 96: 285–291.
 6. Marzochi MC, Fagundes A, Andrade MV, Souza MB, Madeira MF, Mouta-Confort E, Schubach Ade O, Marzochi KB, 2009. Visceral leishmaniasis in Rio de Janeiro, Brazil: eco-epidemiological aspects and control. *Rev Soc Bras Med Trop* 42: 570–580.
 7. Albuquerque PL, Silva Junior GB, Freire CC, Oliveira SB, Almeida DM, Silva HF, Cavalcante Mdo S, Sousa Ade Q, 2009. Urbanization of visceral leishmaniasis (kala-azar) in Fortaleza, Ceara, Brazil. *Rev Panam Salud Publica* 26: 330–333.
 8. Mott KE, Desjeux P, Moncayo A, Ranque P, de Raadt P, 1990. Parasitic diseases and urban development. *Bull World Health Organ* 68: 691–698.
 9. Ximenes MF, Souza MF, Castellon EG, 1999. Density of sand flies (Diptera: psychodidae) in domestic and wild animal shelters in an area of visceral leishmaniasis in the state of Rio Grande do Norte, Brazil. *Mem Inst Oswaldo Cruz* 94: 427–432.
 10. Salomon O, Sinagra A, Nevot M, Barberian G, Paulin P, Estevez J, Riarte A, Estevez J, 2008. First visceral leishmaniasis focus in Argentina. *Mem Inst Oswaldo Cruz* 103: 109–111.
 11. Salomon OD, Quintana MG, Bruno MR, Quiriconi RV, Cabral V, 2009. Visceral leishmaniasis in border areas: clustered distribution of phlebotomine sand flies in Clorinda, Argentina. *Mem Inst Oswaldo Cruz* 104: 801–804.
 12. Mott KE, Nuttall I, Desjeux P, Cattand P, 1995. New geographical approaches to control of some parasitic zoonoses. *Bull World Health Organ* 73: 247–257.
 13. Mauricio IL, Howard MK, Stothard JR, Miles MA, 1999. Genomic diversity in the *Leishmania donovani* complex. *Parasitology* 119: 237–246.
 14. Bray RS, 1982. The zoonotic potential of reservoirs of leishmaniasis in the Old World. *Ecol Dis* 1: 257–267.
 15. Evans TG, Vasconcelos IA, Lima JW, Teixeira JM, McAulliffe IT, Lopes UG, Pearson RD, Vasconcelos AW, 1990. Canine visceral leishmaniasis in northeast Brazil: assessment of serodiagnostic methods. *Am J Trop Med Hyg* 42: 118–123.
 16. Evans TG, Teixeira MJ, McAulliffe IT, Vasconcelos I, Vasconcelos AW, Sousa AA, Sousa Ade A, Lima JW, Pearson RD, 1992. Epidemiology of visceral leishmaniasis in northeast Brazil. *J Infect Dis* 166: 1124–1132.
 17. Badaro R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, Teixeira R, Johnson WD Jr, 1986. New perspectives on a subclinical form of visceral leishmaniasis. *J Infect Dis* 154: 1003–1011.
 18. Badaró R, Jones TC, Lorenço R, Cerf BJ, Sampaio D, Carvalho EM, Rocha H, Teixeira R, Johnson WD Jr, 1986. A prospective study of visceral leishmaniasis in an endemic area of Brazil. *J Infect Dis* 154: 639–649.
 19. Evans TG, Teixeira MJ, Sousa AQ, Pearson RD, 1995. Short report: extended follow-up of the natural history of persons infected with *Leishmania chagasi*. *Am J Trop Med Hyg* 53: 360–361.
 20. Jeronimo SM, Teixeira MJ, Sousa A, Thielking P, Pearson RD, Evans TG, 2000. Natural history of *Leishmania (Leishmania) chagasi* infection in northeastern Brazil: long-term follow-up. *Clin Infect Dis* 30: 608–609.
 21. Miles SA, Conrad SM, Alves RG, Jeronimo SM, Mosser DM, 2005. A role for IgG immune complexes during infection with the intracellular pathogen *Leishmania*. *J Exp Med* 201: 747–754.
 22. Carvalho EM, Badaro R, Reed SG, Jones TC, Johnson WD Jr, 1985. Absence of gamma interferon and interleukin 2 production during active visceral leishmaniasis. *J Clin Invest* 76: 2066–2069.
 23. Braz RF, Nascimento ET, Martins DR, Wilson ME, Pearson RD, Reed SG, Jeronimo SM, 2002. The sensitivity and specificity of *Leishmania chagasi* recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. *Am J Trop Med Hyg* 67: 344–348.
 24. Quinnell RJ, Courtenay O, 2009. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. *Parasitology* 136: 1915–1934.
 25. David JR, Stamm LM, Bezerra HS, Souza RN, Killick-Kendrick R, Lima JW, 2001. Deltamethrin-impregnated dog collars have a potent anti-feeding and insecticidal effect on *Lutzomyia longipalpis* and *Lutzomyia migonei*. *Mem Inst Oswaldo Cruz* 96: 839–847.
 26. Dietze R, Barros GB, Teixeira L, Harris J, Michelson K, Falqueto A, Corey A, 1997. Effect of eliminating seropositive canines on the transmission of visceral leishmaniasis in Brazil. *Clin Infect Dis* 25: 1240–1242.
 27. Costa CH, Gomes RB, Silva MR, Garcez LM, Bozza M, Satskar A, Dissanayake S, Santos RS, Silva MR, Shaw JJ, David JR, Maguire JH, 2000. Competence of the human host as a reservoir for *Leishmania chagasi*. *J Infect Dis* 182: 997–1000.
 28. Daher EF, Fonseca PP, Gerhard ES, Leitao TM, Silva Junior GB, 2009. Clinical and epidemiological features of visceral leishmaniasis and HIV co-infection in fifteen patients from Brazil. *J Parasitol* 95: 652–655.
 29. Oliveira RA, Silva LS, Carvalho VP, Coutinho AF, Pinheiro FG, Lima CG, Leandro JE Jr, Silva GB Jr, Daher EF, 2008. Visceral leishmaniasis after renal transplantation: report of 4 cases in northeastern Brazil. *Transpl Infect Dis* 10: 364–368.
 30. Nascimento ET, Moura MLN, Queiroz JW, Barroso AW, Araujo AF, Rego EF, Wilson ME, Pearson RD, Jeronimo SM, 2011. The emergence of concurrent HIV-1/AIDS and visceral leishmaniasis in northeast Brazil. *Trans R Soc Trop Med Hyg* 105: 298–300.
 31. Fernandez-Guerrero ML, Aguado JM, Buzon L, Barros C, Montalban C, Martin T, Bouza E, 1987. Visceral leishmaniasis in immunocompromised hosts. *Am J Med* 83: 1098–1102.
 32. Montalban C, Calleja JL, Erice A, Laguna F, Clotet B, Podzamczar D, Podzamczar D, Cobo J, Mallolas J, Yebra M, Gallego A, 1990. Visceral leishmaniasis in patients infected with human immunodeficiency virus. Co-operative Group for the Study of Leishmaniasis in AIDS. *J Infect* 21: 261–270.
 33. Jeronimo SM, Duggal P, Braz RF, Cheng C, Monteiro GR, Nascimento ET, Martins DR, Karplus TM, Ximenes MF, Oliveira CC, Pinheiro VG, Pereira W, Peralta JM, Sousa J, Medeiros IM, Pearson RD, Burns TL, Pugh EW, Wilson ME, 2004. An emerging peri-urban pattern of infection with *Leishmania chagasi*, the protozoan causing visceral leishmaniasis in northeast Brazil. *Scand J Infect Dis* 36: 443–449.
 34. Kirchhoff LV, Paredes P, Lomeli-Guerrero A, Paredes-Espinoza M, Ron-Guerrero CS, Delgado-Mejia M, Peña-Muñoz JG, 2006. Transfusion-associated Chagas disease (American trypanosomiasis) in Mexico: implications for transfusion medicine in the United States. *Transfusion* 46: 298–304.
 35. Sokal JE, 1975. Editorial: measurement of delayed skin-test responses. *N Engl J Med* 293: 501–502.
 36. Melo MN, Mayrink W, da Costa CA, Magalhaes PA, Dias M, Williams P, Araujo FG, Coelho MV, Batista SM, 1977. Standardization of the Montenegro antigen. *Rev Inst Med Trop Sao Paulo* 19: 161–164.
 37. Gibson LJ, McCann SH, Brewster S, Barker DC, 2002. A kinetoplast DNA minicircle probe (B18) specific for the *Leishmania* subgenus *Viannia*. *Trans R Soc Trop Med Hyg* 96 (Suppl 1): S71–S73.
 38. Mary C, Faraut F, Lascombe L, Dumon H, 2004. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. *J Clin Microbiol* 42: 5249–5255.
 39. Tolezano JE, Uliana SR, Taniguchi HH, Araujo MF, Barbosa JA, Barbosa JE, Barbosa JE, Floeter-Winter LM, Shaw JJ, 2007. The first records of *Leishmania (Leishmania) amazonensis* in dogs (*Canis familiaris*) diagnosed clinically as having canine visceral leishmaniasis from Aracatuba County, Sao Paulo State, Brazil. *Vet Parasitol* 149: 280–284.
 40. Iversson LB, Camargo ME, da Rocha e Silva EO, Chieffi PP, de Barros JA, 1979. Epidemiological survey of a case of visceral leishmaniasis autochthonous in greater Sao Paulo, Brazil. *Rev Saude Publica* 13: 159–167.

41. Dye C, Wolpert DM, 1988. Earthquakes, influenza and cycles of Indian kala-azar. *Trans R Soc Trop Med Hyg* 82: 843–850.
42. Le PF, Mollinedo S, Mouchet J, Desjeux P, 1989. Leishmaniasis in Bolivia. IV. The dog in the cycles of leishmaniasis in Bolivia. *Mem Inst Oswaldo Cruz* 84: 417–421.
43. Franke CR, Ziller M, Staubach C, Latif M, 2002. Impact of the El Nino/Southern Oscillation on visceral leishmaniasis, Brazil. *Emerg Infect Dis* 8: 914–917.
44. Rebelo JM, 2009. El Nino episodes and temporal distribution of kala azar on Sao Luis island, Maranhao State, Brazil. *Cad Saude Publica* 24: 1713–1714.
45. Carvalho EM, Teixeira RS, Johnson WD Jr, 1981. Cell-mediated immunity in American visceral leishmaniasis: reversible immunosuppression during acute infection. *Infect Immun* 33: 498–500.
46. Gouvea MV, Werneck GL, Costa CH, de Amorim Carvalho FA, 2007. Factors associated to Montenegro skin test positivity in Teresina, Brazil. *Acta Trop* 104: 99–107.
47. Ali A, Ashford RW, 1993. Visceral leishmaniasis in Ethiopia. II. Annual leishmanin transformation in a population. Is positive leishmanin reaction a life-long phenomenon? *Ann Trop Med Parasitol* 87: 163–167.
48. Bacellar O, D'Oliveira A Jr, Jeronimo S, Carvalho EM, 2000. IL-10 and IL-12 are the main regulatory cytokines in visceral leishmaniasis. *Cytokine* 12: 1228–1231.
49. Abramson MA, Dietze R, Frucht DM, Schwantz R, Kenney RT, 1995. Comparison of New and Old World leishmanins in an endemic region of Brazil. *Clin Infect Dis* 20: 1292–1297.
50. Akuffo H, Darce M, Maasho K, Berhan TY, 1995. *In vivo* evaluation of immune responses in leishmaniasis: the use of cross-species leishmanin preparations for skin testing. *Am J Trop Med Hyg* 53: 16–22.
51. Moral L, Rubio EM, Moya M, 2002. A leishmanin skin test survey in the human population of l'Alacanti region (Spain): implications for the epidemiology of *Leishmania infantum* infection in southern Europe. *Trans R Soc Trop Med Hyg* 96: 129–132.
52. Saran R, Gupta AK, Sharma MC, 1991. Leishmanin skin test in clinical and subclinical kala-azar cases. *J Commun Dis* 23: 135–137.
53. Zijlstra EE, El-Hassan AM, 1993. Leishmanin and tuberculin sensitivity in leishmaniasis in the Sudan, with special reference to kala-azar. *Trans R Soc Trop Med Hyg* 87: 425–427.
54. Ali A, Ashford RW, 1994. Visceral leishmaniasis in Ethiopia. IV. Prevalence, incidence and relation of infection to disease in an endemic area. *Ann Trop Med Parasitol* 88: 289–293.
55. Riera C, Fisa R, Lopez-Chejade P, Serra T, Girona E, Jimenez M, Muncunill J, Sedeño M, Mascaró M, Udina M, Gállego M, Carrió J, Forteza A, Portús M, 2008. Asymptomatic infection by *Leishmania infantum* in blood donors from the Balearic Islands (Spain). *Transfusion* 48: 1383–1389.
56. White AC Jr, Castes M, Garcia L, Trujillo D, Zambrano L, 1992. *Leishmania chagasi* antigens recognized in cured visceral leishmaniasis and asymptomatic infection. *Am J Trop Med Hyg* 46: 123–131.
57. Andrade IG, Queiroz JW, Cabral AP, Lieberman JA, Jeronimo SM, 2009. Improved sanitation and income are associated with decreased rates of hospitalization for diarrhoea in Brazilian infants. *Trans R Soc Trop Med Hyg* 103: 506–511.
58. Sundar S, Mondal D, Rijal S, Bhattacharya S, Ghalib H, Kroeger A, Varghese B, Thakur CP, Huda MM, Mondal D, 2008. Implementation research to support the initiative on the elimination of kala azar from Bangladesh, India and Nepal—the challenges for diagnosis and treatment. *Trop Med Int Health* 13: 2–5.
59. Singh R, Lal S, Saxena VK, 2008. Breeding ecology of visceral leishmaniasis vector sandfly in Bihar state of India. *Acta Trop* 107: 117–120.
60. Jeronimo SM, Duggal P, Ettinger NA, Nascimento ET, Monteiro GR, Cabral AP, Pontes NN, Lacerda HG, Queiroz PV, Gomes CE, Pearson RD, Blackwell JM, Beaty TH, Wilson ME, 2007. Genetic predisposition to self-curing infection with the protozoan *Leishmania chagasi*: a genomewide scan. *J Infect Dis* 196: 1261–1269.
61. Ximenes Mde F, Silva VP, Queiroz PV, Rego MM, Cortez AM, Batista LM, Medeiros AS, Jeronimo SM, 2007. Phlebotomine (Diptera: Psychodidae) and leishmaniasis in Rio Grande do Norte State Brazil: anthropic environment responses. *Neotrop Entomol* 36: 128–137.
62. Queiroz PV, Monteiro GR, Macedo VP, Rocha MA, Batista LM, Queiroz JW, Jeronimo SM, Ximenes MF, 2009. Canine visceral leishmaniasis in urban and rural areas of northeast Brazil. *Res Vet Sci* 86: 267–273.
63. Duprey ZH, Steurer FJ, Rooney JA, Kirchhoff LV, Jackson JE, Rowton ED, Schantz PM, 2006. Canine visceral leishmaniasis, United States and Canada, 2000–2003. *Emerg Infect Dis* 12: 440–446.
64. Evans TG, Vasconcelos IA, Lima JW, Teixeira JM, McAulliffe IT, Lopes UG, Pearson RD, Vasconcelos AW, 1990. Canine visceral leishmaniasis in northeast Brazil: assessment of serodiagnostic methods. *Am J Trop Med Hyg* 42: 118–123.
65. Schantz PM, Steurer FJ, Duprey ZH, Kurpel KP, Barr SC, Jackson JE, Breitschwerdt EB, Levy MG, Fox JC, 2005. Autochthonous visceral leishmaniasis in dogs in North America. *J Am Vet Med Assoc* 226: 1316–1322.
66. Cummins D, Amin S, Halil O, Chiodini PL, Hewitt PE, Radley-Smith R, 1995. Visceral leishmaniasis after cardiac surgery. *Arch Dis Child* 72: 235–236.
67. Singh S, Chaudhry VP, Wali JP, 1996. Transfusion-transmitted kala-azar in India. *Transfusion* 36: 848–849.
68. le Fichoux Y, Quaranta JF, Aufeuvre JP, Lelievre A, Marty P, Suffia I, Rousseau D, Kubar J, 1999. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. *J Clin Microbiol* 37: 1953–1957.