# *Leishmania infantum*: illness, transmission profile and risk factors for asymptomatic infection in an endemic metropolis in Brazil

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(*Received 5 August 2016; revised 10 October 2016; accepted 21 October 2016; first published online 29 November 2016*)

SUMMARY

To evaluate the distribution of asymptomatic infection by *Leishmania infantum* in a metropolis in Brazil with different relative risks (RRs) for disease and risk factors associated with the infection, an ecological study was conducted using a Bayesian approach to estimate the RR of human visceral leishmaniasis (HVL) based on cases between 2008 and 2011. The areas were categorized and selected according to disease incidence: low (area-1), medium (area-2) and high (area-3). Cross-sectional study enrolling 935 children was used to estimate the prevalence of infection by *L. infantum*. Volunteers from these three areas were tested for *L. infantum* infection by ELISA (rK39 and soluble antigens). Infection prevalence rates were estimated and compared with the RR of disease. Multilevel logistic regression model evaluated the relationship between infection and the analysed variables. The RR of HVL was distributed heterogeneously in the municipality. The infection prevalence rates were: 34·9% in area-1; 29·3% in area-2; and 33·6% in area-3, with no significant differences between these areas. The variables 'Presence of backyards in the neighbourhood' and 'Younger children' were associated with *L. infantum* infection. We conclude that infection by *L. infantum* affects a significant proportion of the infant population regardless of the RR of disease.

Key words: visceral leishmaniasis, asymptomatic infection, infection, relative risk, Brazil.

## INTRODUCTION

Human visceral leishmaniasis (HVL) is a serious tropical disease that still neglected accounting for approximately 200 000–400 000 new cases each year worldwide, and a fatality rate of approximately 10% (Alvar *et al*. 2012). In the Americas, the disease is caused by the protozoan parasite *Leishmania* (*L.*) *infantum* (*syn* = *Leishmania* (*L.*) *chagasi*). The parasite is transmitted through the bite of carrier female sand flies of the genus *Lutzomyia*. The sand flies become vectors of the infection when they feed on *L. infantum* reservoirs. In urban areas dogs are considered the major reservoir of this parasite.

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Due to the wide geographic distribution of HVL and the severity of its clinical manifestations, a better understanding of the disease is of utmost relevance. The increased interest in HVL since the 1980s is due to the increased incidence rates and the expansion of the disease into new geographic areas, along with its tendency for urbanization. HVL is also challenging to diagnose and treat and of difficult vector control (Costa *et al*. 1990; Romero and Boelaert, 2010).

In Brazil, an average of approximately 3500 cases of HVL is reported annually, with an incidence rate of 1–3 cases per 100 000 inhabitants and an average fatality rate of 7% (Alvar *et al*. 2012). Between 1994 and 2014, 1656 cases were confirmed in the city of Belo Horizonte, the capital of the Southeastern Minas Gerais state. Belo Horizonte had a mean incidence of 4·1 cases per 100 000 inhabitants between 2008 and 2014 (Secretaria de Saúde de Belo Horizonte, PBH, 2014). The large number of

*Parasitology* (2017), **144**, 546–556. 
doi:10.1017/S0031182016002134

deaths indicates that the city has one of the highest mortality rates in the country (de Araujo *et al*. 2013).

The Visceral Leishmaniasis Control and Surveillance Programme was implemented in Belo Horizonte in the 1990s, and its strategies are focused on: (1) early diagnosis and treatment of infected individuals, (2) sand fly population control, (3) the elimination of reservoirs and (4) health education activities. However, despite the reduction in the number of cases of HVL, the disease control strategies used by the Visceral Leishmaniasis Control and Surveillance Programme have not been effective in preventing the transmission of the parasite, which occurs throughout and heterogeneously across the city (de Araujo *et al*. 2013; PBH, 2014).

*Leishmania infantum* infection in humans does not always result in clinical manifestations. A large number of individuals living in endemic areas are infected with the parasite, but do not develop the typical clinical manifestation of the disease (Badaro *et al*. 1986; Moreno *et al*. 2009; Custodio *et al*. 2012). Understanding the characteristics of the infection and the relevance of these asymptomatic individuals to the epidemiological characterization of HVL by *L. infantum* is essential to identify and monitor endemic areas as well as the relevant factors for the spread of the disease. Such knowledge may contribute to the development of more effective disease control strategies.

In order to expand the current understanding of the HVL transmission profile in Belo Horizonte, we evaluated the occurrence and the factors associated with *L. infantum* asymptomatic infection in three areas of the city with different relative risks (RRs) for the disease.

## METHODS

### Study area

The study area was the Brazilian city of Belo Horizonte, which has a population of 2 375 000 inhabitants living in an urban area of 331·4 km$^2$ giving a population density of 7167·02 inhabitants per km$^2$ (Instituto Brasileiro de Geografia e Estatística, IBGE, 2010). The city is located 852 m above sea level, at latitude 19°49′01″S and longitude 43°57′21″W.

In Belo Horizonte, the regional organization of the municipal health service is divided into nine health districts: North, Northeast, Northwest, South, Southeast, Pampulha, Barreiro, South Central and West. Currently, 147 basic health units are distributed between these regional sectors. Their coverage areas (CAs) are established based on 2563 census sectors, which represent the basic units of each territory and are defined by the Instituto Brasileiro de Geografia e Estatística.

In the first phase of the study, 147 CAs located in the basic health units were considered. In the second phase, the study was conducted in three CAs with low, medium and high risk of developing the clinical form of HVL (Fig. 1).

### Ethical considerations

The project containing the Free and Informed Consent Form was submitted to the Research Ethics Committees of the Universidade Federal de Minas Gerais (UFMG), the City Council of Belo Horizonte, and the Centro de Pesquisas René Rachou/FIOCRUZ and was approved by the three institutions (CAAE–UFMG–03903712·2·3001·5091).

### Study design

Two epidemiological studies were conducted: (i) an ecological study and (ii) a cross-sectional study. The ecological study used the frequency of HVL cases between 2008 and 2011 to estimate the RR of illness due *to L. infantum* infection in each of the 147 CAs in Belo Horizonte. The results of this study allowed the identification of three CAs according to RR. These were evaluated in the subsequent phase, a cross-sectional investigation used to estimate the prevalence rate of asymptomatic *L. infantum* infection.

### Ecological study: RR of developing the clinical form of HVL

To estimate the RR of illness due to HVL in the 147 CAs of the basic health units of Belo Horizonte, secondary data on HVL cases reported between 2008 and 2011 were used ($n = 498$). These cases were registered in the Brazilian Notifiable Disease Information System (Sistema de Informação de Agravos de Notificação – SINAN) and were reported by the Management of Epidemiological Data (Gerências de Epidemiologia e Informação–GEEPI) of the Municipal Health Department of Belo Horizonte. The cases were geo-referenced using the MapInfo® software, version 11·0.

The RRs for each area were estimated using a Bayesian approach, considering the spatial dependence between neighbouring areas. The spatial statistical modelling minimizes the instability of the rates resulting from the low frequency of cases in small areas and eliminates much of the randomness not associated with the risk factors. The average RRs of developing clinical HVL in the study areas between 2008 and 2011 were calculated according to Besag's model (Besag *et al*. 1991) as given by:

$$O_i \sim \text{Poisson}\ (\mu_i)$$

$$\xi_i = \frac{\mu_i}{E_i} \rightarrow \ln\ (\mu_i) = \ln\ (E_i) + \ln\ (\xi_i)$$

$$\ln\ (\xi_i) = \alpha_0 + b_i$$

https://doi.org/10.1017/S0031182016002134 Published online by Cambridge University Press

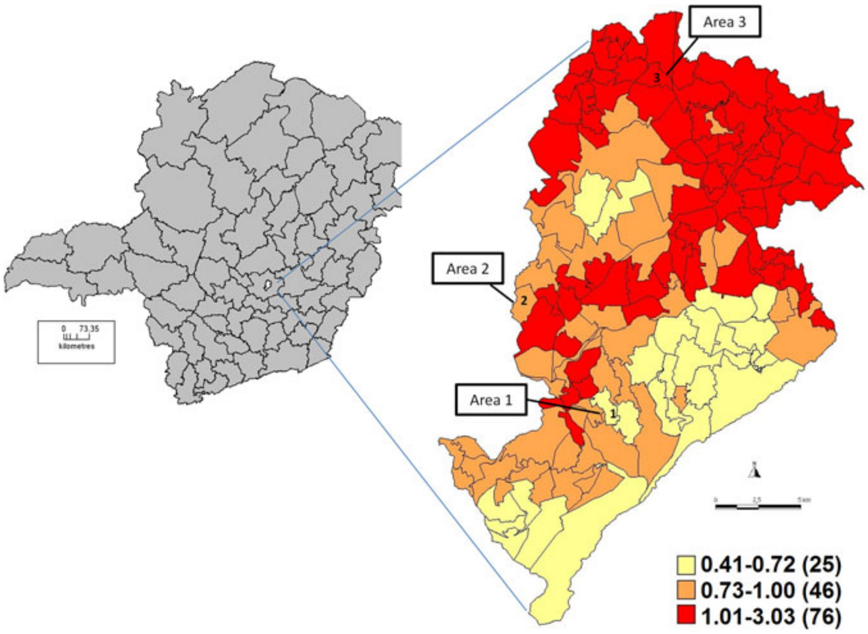


Fig. 1. Relative risk of developing the clinical form of human visceral leishmaniasis (HVL) in Belo Horizonte, Brazil, in 2012. The areas studied were categorized for the risk of developing the disease according to the frequency of HVL cases: area-1: low risk, area-2: medium risk, area-3: high risk.

where $O_i$ is the number of observed cases in area $i$, $\mu_i$ is the average of the cases in area $i$, $E_i$ is the number of expected cases in area $i$ assuming an even distribution of the cases all over the city and $\xi_i$ is the RR of area $i$. This model allows for the calculation of the RR in each area based on the estimated HVL incidence rates smoothed by estimates of incidence rates in neighbouring areas (spatial dependence). The total number of HVL cases during the 4-year period was considered for each area.

The software WinBugs version 1.4, was used to implement Besag's model, taking into account the average RR of developing HVL in each CA, smoothed by the spatial dependence as described above (Supporting Information 1).

From this modelling, a map containing different cut-off points was generated for the RR, allowing for the selection of CAs with distinct RR: low (area 1), medium (area 2) and high (area 3).

### *Cross-sectional study: evaluation of asymptomatic infection*

Three areas were selected according to the risk classification criteria developed during the ecological study and using the operational criteria (the organization of the health services and accessibility to the study areas).

### *Sample size estimation*

The study sample used to estimate the prevalence rate of asymptomatic infection included children aged between three months and 10 years living in the three study areas. The sample size was estimated considering the following parameters: (1) the average prevalence rates of human infection of 15/100 children under 7 years of age obtained from a previous study conducted in Belo Horizonte (dos Santos Marques *et al*. 2012), (2) an error (α) equivalent to 0·05 and (3) design effect constant equating 1·5. The estimated value was 294 children per area, totalling 935 children. The households with children in the assessed age group were randomly selected based on data from the Family Health Program of the Health Centres located in the geo-referenced areas.

### *Data acquisition and blood samples*

Data and blood samples were collected in August and September of 2012 by qualified nurse assistants. After training, these professionals performed visits in the previously mapped and geo-referenced residential properties. Blood samples from the subjects were collected on filter paper, and data were collected using a standardized form to guide the interview. Before data collection, a form for a Free and Informed Consent was presented and signed by each legal guardian.

The following information was collected: general information about the child (age, gender, where the child spends most of his/her time); household characteristics (number of rooms, number of residents, intra-domicile characteristics); peri-domicile characteristics (storage, collection and disposal of domestic waste, waste water management, presence of domestic animals); awareness of HVL and its vector; and neighbourhood characteristics (presence of trees, animals, gardens, vacant land), etc.

Socio-economic variables were evaluated using specific questions related to profession, education and income. The average gross household income was obtained using a specific question and was estimated considering the possession of certain items and the level of education of the family head, according to the scoring system and economic classification established by the Brazilian Economic Classification Criteria (ABEP, 2012).

### *Laboratory tests*

*Serological tests*. Enzyme-linked immunosorbent assays (ELISA) were performed using *L. infantum* soluble antigen (ELISA-SLA) or recombinant antigen K39 (ELISA-rK39) for the detection of IgG antibodies in blood sample eluates.

Soluble antigen was prepared as described by Ho *et al*. (1983) using promastigotes of *L. infantum* (MHOM/BR/2002/LPC-RPV). NUNC Maxisorp polystyrene microplates (Nunc-Immuno Plate Brand Products, Roskilde, Denmark) were coated with 50 $\mu$L well$^{-1}$ of *L. infantum* soluble antigen (3 $\mu$g mL$^{-1}$) diluted in 0·1 M carbonate-bicarbonate buffer (pH 9·6) for approximately 18 h at 4 °C. The microplates were then washed five times with phosphate-buffered saline (PBS) containing 0·05% Tween 20 (PBS-T). Next, 200 $\mu$L of 5% non-fat milk diluted in PBS-T (PBS-T-milk 5%) were added to each well, and the plate was incubated for 2 h at 37 °C. The microplates were again washed five times with PBS-T followed by the addition, in duplicate, of 50 $\mu$L of eluate using a 1:250 ratio, after which the plates were incubated at 37 °C for another hour. After washing again, 50 $\mu$L of peroxidase conjugated anti-human IgG (Sigma Chemical Co., St Louis, MO, USA), diluted in 1% milk in PBS-T using a 1:1000 ratio, were added to each well, and the plate was incubated for 1 h at 37 °C. The plates were washed again, followed by the addition of 50 $\mu$L well$^{-1}$ of substrate solution ($H_2O_2$+TMB – Sigma Chemical Co.). The assays were incubated for 5 min at room temperature in the dark, and the enzymatic reaction was quenched with 50 $\mu$L well$^{-1}$ of 1 N sulphuric acid. The absorbance readings were performed at 450 nm in a microplate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan). Samples that showed differences >20% between the absorbance values of duplicates were tested again. For each sample, we calculated the reactivity index (RI) by dividing the mean absorbance by the cut-off value, determined by the mean absorbance obtained by testing 15 negative samples plus three standard deviations. Samples were considered positive, if RI $\geqslant$ 1·1 and negative if RI < 1·1.

To perform the ELISA-rK39, NUNC Maxisorp (Nunc-Immuno Plate Brand Products, Roskilde, Denmark) plates were coated with 50 $\mu$L well$^{-1}$ of rK39 antigen at a concentration of 1 $\mu$g mL$^{-1}$. A 1:100 dilution of serum and a 1:10 000 dilution ratio of peroxidase-conjugated anti-human IgG (Sigma Chemical Co) in 1% milk in PBS-T were utilized following the same protocol described for the ELISA-SLA.

### *Case follow-up*

A year later, in September 2013, the children who tested positive in any of the tests were invited to attend the health unit of the respective CA of their residence for a clinical evaluation performed by physicians. On that occasion, blood samples were collected by venipuncture to perform complementary laboratory tests. The data obtained during the examination were recorded on the appropriate form.

### *Data analysis*

Interview and blood test data related to asymptomatic infection in the respective study areas were processed using a double-entry method. The files were compared and the differences detected were corrected. EpiData® software, version 3.2, was used for data entry, and STATA® software, version 11·0, was used for data analysis.

### *Estimation of asymptomatic L. infantum infection prevalence*

The analyses were performed on the three CAs with low, medium and high risk of developing the clinical form of HVL as defined above. It included the following steps: (i) estimates of asymptomatic infection prevalence rates in the three study areas, taking into account the percentage of seropositive children in at least one of the tests and the total number of children evaluated, and (ii) comparison between the infection prevalence rates of the designated areas using the chi-squared ($\chi^2$) test assuming a significance level of 5%.

### *Multilevel logistic regression analysis*

A multilevel logistic regression method was used to assess the relationship between asymptomatic infection and the variables analysed using the xtmelogit command of STATA. The effect of the variable 'Household' was considered at one level, while the variable 'Influence of the child's characteristics' was considered at another level. The child's residence was considered to be the first level because more than one child from the same household was sampled during data collection. Therefore, it is likely that these children shared similar attributes considering their common biological and environmental context.

To investigate the association between the variables of different levels, two multilevel models were initially constructed: (i) a model including the

https://doi.org/10.1017/S0031182016002134 Published online by Cambridge University Press

individual variables related to each child and (ii) a model including the variables related to environmental factors in the intra- and peri-domicile, socio-economic and context variables.

The final multilevel model grouped the significant variables of the univariate analyses ($P < 0{\cdot}15$) of the two afore-mentioned models. To define the final model with the best data fit, the likelihood ratio test was used (Hosmer and Lemeshow, 1989).

The strength of the association was assessed using the odds ratio (OR) with a 95% confidence interval (CI).

## RESULTS

### *Ecological Study: RR of developing the clinical form of HVL*

According to secondary data provided by SMSA-BH, a total of 498 cases of clinical HVL were accounted for between 2009 and 2011. These were distributed as follows: 155 cases in 2008; 133 cases in 2009; 123 cases in 2010; 87 cases in 2011.

For incorporation of the spatial dependence in Besag's model, two areas were considered neighbouring if they shared frontiers. After the model coefficients were estimated, an RR map for Belo Horizonte was generated (Fig. 1).

Of the 147 CAs in Belo Horizonte, 76 (51·7%) showed RR values ⩾1 (1·01–3·03), 46 CAs (31·3%) had RR values between 0·73 and 1·00, and 25 CAs (17·0%) had low RR values, ranging between 0·41 and 0·72.

Considering this heterogeneity, it was possible to select CAs with different risks of illness due to HVL in order to perform the cross-sectional study. The areas selected were: 'Professor Amilcar Viana', with low risk (Area-1), located in the West health district; 'Pindorama', with medium risk (area-2), located in the Northwest health district; and 'Minas Caixa', with high risk (area-3), located in the Venda Nova health district (Fig. 1).

### *Cross-sectional study: evaluation of asymptomatic infections*

The study included 935 children living in 679 households located in the three areas selected and distributed as shown in Table 1.

The participating children's ages ranged from 3 to 120 months and the mean ± standard deviation (S.D.) and median (25th and 75th percentiles) children's age (in months) by study area were as follows:

| | Mean ± S.D. | Median |
|---|---|---|
| Area 1 | 47·73 ± 26·9 | 48 months (27·0 and 70·5) |
| Area 2 | 64·67 (±27·82) | 48 months (46·5 and 86·0) |
| Area 3 | 47·53 (±25·02) | 67·5 months (24·0 and 72·0) |

Table 1 shows the distribution of children according to their area of residence as well as individual and familial characteristics. There was no gender predominance (48·3% male and 51·7% female). Regarding individual habits, most children remained indoors in the evening (83·5%).

The results related to the socio-economic variables indicated that most of the participating families residing in areas 1 and 2 had an income of 2–3 monthly minimum wages. A higher number of families with an income of 1–2 minimum wages (one minimum wage equivalent to $311 [USD] per month in 2012, $1.00 [USD] = R$ 2·00) lived in area 3.

Data on the peri-domiciliar environment, including the presence of backyards, backyard flooring material and the presence of fruit trees and other vegetation, organic matter, garbage, debris, etc. were recorded for each area. Dogs were present in 45·7% of the households evaluated, and in 73·0% of the cases, they did not have access to the outside (Table 2).

In order to assess the participants' awareness of the HVL vector, each participant was shown three flasks containing different insect species: (i) *Musca domestica*, (ii) *Aedes aegypti* and (iii) *Lutzomyia longipalpis*. Sand flies were correctly identified by 28·7% of the respondents (Table 2), 11·7% of which had spotted the insect in their homes.

Data on the peri-domiciliar environment was processed following the guidelines for environmental observation. Data on the presence of dogs, backyards and nearby vacant plots of land were also collected. Backyards were accounted for depending on lateral, frontal and back neighbouring properties. A total of 95·5% of the residents had a neighbour who owned a dog, and 59·7% of the households were surrounded by neighbours who had backyards (Table 2).

### *Estimation and comparison of infection prevalence rates in Belo Horizonte*

Considering the children with positive results in at least one serological test (ELISA-SLA or ELISA-rK39), the infection prevalence rates in the three designated areas of Belo Horizonte were: 34·9% (95% CI, 29·7–40·4) in area-1, 29·3% (95% CI, 24·5–34·5) in area-2 and 33·5% (95% CI, 28·5–38·9) in area-3 (Table 3).

Statistical analysis ($\chi^2$ test) of infection prevalence rates amongst children revealed no statistically significant difference between the three areas of Belo Horizonte ($P = 0{\cdot}29$) (Table 3). Similarly, the multilevel analysis indicated no significant differences in the probability of infection in these areas (Table 4). Therefore, it is plausible to assume that the areas were evenly affected by asymptomatic *L. infantum* infection.

https://doi.org/10.1017/S0031182016002134 Published online by Cambridge University Press

Table 1. Characteristics of the study participants living in three areas of different risk of developing human visceral leishmaniasis. Belo Horizonte, Brazil, in 2012

| Variables | Area 1 *N* | Area 1 % | Area 2 *N* | Area 2 % | Area 3 *N* | Area 3 % | Total *N* | Total % | *P*-value |
|---|---|---|---|---|---|---|---|---|---|
| Number of children | 304 | 32·5 | 318 | 34·0 | 313 | 33·5 | 935 | 100 | – |
| Number of households | 216 | 31·8 | 251 | 37·0 | 212 | 31·2 | 679 | 100 | – |
| Gender | | | | | | | | | |
| Male | 150 | 48·3 | 154 | 48·4 | 148 | 47·3 | 452 | 48·3 | 0·87 |
| Female | 154 | 51·7 | 164 | 51·6 | 165 | 52·7 | 483 | 51·7 | |
| Where the child spends time between 6 and 10 pm | | | | | | | | | |
| Inside the residence | 273 | 90·1 | 243 | 76·4 | 265 | 84·7 | 781 | 83·5 | 0·01 |
| In the peri-domicile | 22 | 7·3 | 52 | 16·4 | 36 | 11·5 | 110 | 11·8 | |
| Other places in the same neighbourhood | 7 | 2·3 | 19 | 6·0 | 9 | 2·9 | 35 | 3·7 | |
| Other neighbourhoods | 1 | 0·3 | 4 | 1·3 | 3 | 1·0 | 8 | 0·9 | |
| Education level of the family head | | | | | | | | | |
| Higher education | 36 | 11·9 | 27 | 8·5 | 6 | 1·9 | 69 | 7·4 | 0 |
| High school education | 115 | 38·0 | 140 | 44·2 | 119 | 38·1 | 374 | 40·0 | |
| Elementary education | 75 | 24·8 | 69 | 21·8 | 81 | 26·0 | 225 | 24·1 | |
| Primary education | 59 | 19·5 | 51 | 16·1 | 68 | 21·8 | 178 | 19·0 | |
| Illiterate | 18 | 5·9 | 30 | 9·5 | 38 | 12·2 | 86 | 9·2 | |
| Family income (BMW) | | | | | | | | | |
| Up to 1 | 63 | 20·7 | 64 | 20·1 | 76 | 24·3 | 203 | 21·7 | 0·01 |
| 1–2 | 86 | 28·3 | 104 | 32·7 | 126 | 40·3 | 316 | 33·8 | |
| 3–4 | 34·0 | 11·2 | 32 | 10·1 | 20 | 6·4 | 86 | 9·2 | |
| 4–7 | 16 | 5·3 | 10 | 3·1 | 9 | 2·9 | 35 | 3·7 | |
| 7–10 | 8 | 2·6 | 6 | 1·9 | 0 | 0 | 14 | 1·5 | |
| 10–20 | 4 | 1·3 | 2 | 0·6 | 2 | 0·6 | 8 | 0·9 | |
| Does not know | 27 | 8·9 | 38 | 12·0 | 42·0 | 13·4 | 107 | 11·4 | |

BMW, Brazilian minimum wage.
The areas studied were categorized for the risk of developing the disease according to the frequency of visceral leishmaniasis cases: area-1: low risk, area-2: medium risk, area-3: high risk.

### *Assessment of the risk factors contributing to L. infantum infection*

Multilevel modelling was used to explain the asymptomatic infection in children. The residence was considered a random effect variable, and the presence or absence of infection according to the serological tests was considered the dependent variable.

The following variables presented $P < 0·15$ in the univariate analysis and were entered into the multivariate analysis: area, level of education, having a washing machine (proxy variable of income), gender, having a refrigerator, type of roof and walls on the property, place where the dog spends the night, places where the child stays between 6 and 10 p.m., having neighbours with backyards, and the age of the participating children (Table 4).

After multivariate analysis of the aforementioned variables, the following variables remained in the final model with house effect: 'Presence of backyards in the neighbourhood' (OR 1·14; 95% CI, 1·01–1·29; $P = 0·037$) and 'Age of the children' as continuous variables (OR 0·91; 95% CI, 0·84–0·98; $P = 0·008$). 'Living in distinct areas' was not a significant variable in the final model, but the variable 'Area' remained in the model as an adjustment variable (Table 5).

### *Case follow-up*

In 2013, we attempted to contact the guardians of the 304 children with positive results in any of the serological tests (ELISA-SLA or ELISA-rK39) during the cross-sectional study. A total of 65 children were found in area 1, 70 in area 2 and 50 in area 3, totalling 185 children (60·8%).

The blood test results and clinical data were analysed by pediatricians and indicated no changes suggestive of the development of the clinical form of VL in the respective children. Most of the few changes observed in the blood tests were associated with mild anaemia due to iron deficiency or mild leucocytosis, and these cases were forwarded to paediatric care at the health units.

## DISCUSSION

The HVL RR distribution map in the 147 CAs generated by the ecological study revealed a heterogeneous risk spread across the study areas, allowing the stratification of disease risk into three levels: low, medium and high.

The use of spatial statistical modelling allowed the estimation of incidence rates, smoothed by the spatial dependence between neighbouring areas. The

Table 2. Characteristics of the households from the studied areas. Belo Horizonte, Brazil, 2012

| | Area 1 | | Area 2 | | Area 3 | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| Variables | *N* | % | *N* | % | *N* | % | *N* | % | *P*-value |
| Presence of backyard | | | | | | | | | |
| No | 39 | 18·1 | 26 | 10·4 | 38 | 17·9 | 103 | 15·2 | 0·03 |
| Yes | 176·0 | 81·9 | 225 | 89·6 | 174 | 82·1 | 575 | 84·8 | |
| Type of floor in the backyard | | | | | | | | | |
| Cement or coated | 105 | 59·7 | 123 | 54·7 | 88 | 50·6 | 316 | 55·0 | 0·06 |
| Soil | 7 | 4·0 | 22 | 9·8 | 22 | 12·6 | 51 | 8·9 | |
| Mixed (soil and cement) | 64 | 36·4 | 80 | 35·6 | 64 | 36·8 | 208 | 36·2 | |
| Presence of river banks | | | | | | | | | |
| No | 107 | 61·5 | 135 | 61·1 | 97 | 55·8 | 339 | 59·6 | 0·08 |
| Yes | 67 | 38·5 | 86 | 38·9 | 77 | 44·3 | 230 | 20·4 | |
| Presence of fruit trees | | | | | | | | | |
| No | 103 | 58·5 | 130 | 58·0 | 113 | 64·9 | 346 | 60·3 | 0·32 |
| Yes | 73 | 41·5 | 94 | 42·0 | 61 | 35·1 | 228 | 39·7 | |
| Presence of plant pots or gardens | | | | | | | | | |
| No | 47 | 26·7 | 50 | 22·2 | 56 | 32·4 | 153 | 26·7 | 0·08 |
| Yes | 129 | 73·3 | 175 | 77·8 | 117 | 67·6 | 421 | 73·3 | |
| Presence of organic matter | | | | | | | | | |
| No | 130 | 73·9 | 170·0 | 75·6 | 133 | 76·4 | 433 | 75·3 | 0·85 |
| Yes | 46 | 26·1 | 55 | 24·4 | 41 | 23·6 | 142 | 24·7 | |
| Presence of rubble | | | | | | | | | |
| No | 107 | 60·8 | 125 | 56·3 | 103 | 59·2 | 335·0 | 58·6 | 0·65 |
| Yes | 69 | 39·2 | 97 | 43·7 | 71 | 40·8 | 237 | 41·4 | |
| Presence of garbage | | | | | | | | | |
| No | 166 | 94·9 | 187 | 83·9 | 147 | 85·0 | 500 | 87·6 | 0 |
| Yes | 9 | 5·1 | 36 | 16·1 | 26 | 15·0 | 71 | 12·4 | |
| Presence of pens | | | | | | | | | |
| No | 156 | 88·1 | 195 | 86·7 | 145·0 | 83·8 | 496 | 86·3 | 0·49 |
| Yes | 21 | 11·9 | 30 | 13·3 | 28 | 16·2 | 79 | 13·7 | |
| Presence of kennels | | | | | | | | | |
| No | 151 | 85·8 | 188 | 83·6 | 149 | 86·1 | 488 | 85·0 | 0·73 |
| Yes | 25 | 14·2 | 37 | 13·9 | 24 | 13·9 | 86 | 15·0 | |
| Presence of dogs | | | | | | | | | |
| Yes | 99 | 45·8 | 122 | 48·6 | 89 | 42·0 | 310 | 45·7 | 0·36 |
| No | 117 | 54·2 | 129 | 51·4 | 123 | 58·0 | 369 | 54·3 | |
| Dogs have access to the street | | | | | | | | | |
| Yes | 26 | 26·3 | 34·0 | 28·3 | 23 | 26·1 | 83 | 27·0 | 0·68 |
| No | 73 | 73·7 | 86 | 71·7 | 65 | 73·9 | 224·0 | 73·0 | |
| Level of awareness of the disease vector[a] | | | | | | | | | |
| Certain | 58 | 27·5 | 71 | 28·4 | 64 | 30·2 | 193·0 | 28·7 | 0·19 |
| Doubtful | 7 | 3·3 | 24 | 9·6 | 12 | 5·7 | 43 | 6·4 | |
| Wrong | 108 | 51·2 | 115 | 46·0 | 98 | 46·2 | 321 | 47·7 | |
| Does not know (declined to respond) | 38 | 18·0 | 40 | 16·0 | 38 | 17·9 | 116 | 17·2 | |
| Presence of dogs in residences in the neighbourhood | | | | | | | | | |
| No | 13·0 | 6·2 | 12 | 4·9 | 5 | 2·4 | 30 | 4·5 | 0·17 |
| Yes | 197 | 93·8 | 234 | 95·1 | 202 | 95·6 | 633 | 95·5 | |
| Presence of backyards in the neighbourhood | | | | | | | | | |
| No | 13·0 | 6·0 | 5 | 2·0 | 2 | 0·9 | 20 | 3·0 | 0·00 |
| 1 backyard | 20 | 9·3 | 9 | 3·6 | 10 | 4·7 | 39 | 5·7 | |
| 2 backyards | 26 | 12·0 | 22 | 8·8 | 10 | 4·7 | 58 | 8·5 | |
| 3 backyards | 53 | 24·5 | 73 | 29·1 | 31 | 14·6 | 157 | 23·1 | |
| 4 backyards | 104 | 59·7 | 142 | 56·6 | 159 | 75·0 | 405 | 59·7 | |

[a] Identification of the vector after the display of three insect species. The areas studied were categorized for the risk of developing the disease according to the frequency of human visceral leishmaniasis cases: area-1: low risk, area-2: medium risk, area-3: high risk.

Bayesian approach minimizes instability of the rates resulting from the low frequency of cases in small areas, eliminates much of the randomness not associated with risk factors, and ignores the political boundaries between areas (Besag *et al*. 1991; Assuncao *et al*. 1998; de Almeida *et al*. 2011; de Araujo *et al*. 2013).

Therefore, the calculated RR values refer to the ratio of the probability of individuals from one area to develop the clinical form of HVL and this same probability if all cases were distributed homogeneously in Belo Horizonte. Using this approach, it is possible to calculate the approximate number of

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Table 3. ELISA results for each studied area, considering seropositivity in at least one test. Belo Horizonte, Brazil, 2012

| | Area 1 | | Area 2 | | Area 3 | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| Test | *N* | % | *N* | % | *N* | % | *N* | % | *P*-value |
| ELISA-SLA and/or ELISA-rK39 | | | | | | | | | |
| Negative | 198 | 65·1 | 225 | 70·8 | 208 | 66·5 | 631 | 67·5 | 0·29 |
| Positive | 106 | 34·9 | 93 | 29·3 | 105 | 33·5 | 304 | 32·5 | |
| Total | 304 | 100 | 318 | 100 | 313 | 100 | 935 | 100 | |

Chi-square. The areas studied were categorized for the risk of developing the disease according to the frequency of human visceral leishmaniasis cases: area-1: low risk, area-2: medium risk, area-3: high risk.

Table 4. Multilevel univariate analysis of the risk factors for *L. infantum* infection in Belo Horizonte, Brazil, 2012

| | Odds ratio | 95% CI | *P*-value |
|---|---|---|---|
| Residence in area 1 | 1 | | |
| Residence in area 2 | 0·73 | 0·49–1·09 | 0·13 |
| Residence in area 3 | 0·89 | 0·60–1·33 | 0·57 |
| Child's age (years) | 0·90 | 0·85–0·96 | 0·003 |
| Male | 1 | | |
| Female | 0·79 | 0·58–1·08 | 0·15 |
| Performs activities indoors at night | 1 | | |
| Performs activities in the backyard or street | 0·60 | 0·36–1·02 | 0·06 |
| Performs activities in other regions in the neighbourhood | 0·91 | 0·39–2·07 | 0·81 |
| Performs activities in other neighbourhoods | 1·31 | 0·25–6·74 | 0·75 |
| Education | | | |
| Higher education | 1 | | |
| 12 years of education | 1·82 | 0·94–3·56 | 0·08 |
| 8 years of education | 1·46 | 0·73–2·91 | 0·29 |
| 4 years of education | 1·19 | 0·58–2·45 | 0·62 |
| Illiterate | 1·47 | 0·66–3·29 | 0·35 |
| Does not own a washing machine | 1 | | |
| Owns a washing machine | 1·18 | 0·99–1·41 | 0·06 |
| Absence of yards in the neighbourhood | 1 | | |
| Presence of backyards in the neighbourhood | 1·13 | 0·87–2·88 | 0·05 |
| Property with concrete ceiling slab and tiled roof | 1 | | |
| Property only with concrete ceiling slab | 0·94 | 0·67–1·33 | 0·77 |
| Property without concrete ceiling slab | 0·6 | 0·37–0·99 | 0·04 |
| Brick walls with plastering | 1 | | |
| Brick walls without plastering | 1·85 | 0·82–4·15 | 0·14 |
| Mixes walls | 0·92 | 0·57–1·48 | 0·73 |
| Wooded walls | 2·43 | 0·11–54·24 | 0·56 |
| Dog does not spend the night in a kennel | 1 | | |
| Dog spends the night in a kennel | 1·6 | 0·89–2·89 | 0·12 |

CI, confidence interval.
Multilevel regression, considering the household as a random effect. The areas studied were categorized for the risk of developing the disease according to the frequency of human visceral leishmaniasis cases: area-1: low risk, area-2: medium risk, area-3: high risk.

cases of HVL in relation to what would be expected if all cases were distributed homogeneously within the city. This approach proved to be useful and appropriate for the identification of CAs with different risks of illness due to HVL, allowing the calculation of asymptomatic infection prevalence (Besag *et al*. 1991; Assuncao *et al*. 1998, 2001; de Araujo *et al*. 2013)

The observed heterogeneous distribution of disease risk in Belo Horizonte has also been verified in recent studies. These suggest that some risk factors may have an effect on the prevalence rates of clinical HVL. These include: altitude (Margonari *et al*. 2006; Saraiva *et al*. 2011), greater number of seropositive dogs (Saraiva *et al*. 2011; de Araujo *et al*. 2013), presence of domestic animals (Gavgani *et al*. 2002; Oliveira *et al*. 2006); socio-economic status and education (Elnaiem *et al*. 2003; de Araujo *et al*. 2013).

The cross-sectional study compared the prevalence rates of asymptomatic infection in these areas

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Table 5. Final multilevel model of the risk factors for *L. infantum* infection. Belo Horizonte, Brazil, 2012

| | Odds ratio | *P*-value | 95% CI |
|---|---|---|---|
| Area of residence | | | |
| Low risk of illness | 1 | – | – |
| Intermediate risk of illness | 0·81 | 0·32 | 0·54–1·22 |
| High risk of illness | 0·84 | 0·41 | 0·56–1·26 |
| Child's age (years) | 0·91 | 0·01 | 0·84–0·98 |
| Presence of backyards in the neighbourhood | | | |
| No | 1 | – | – |
| Yes | 1·14 | 0·04 | 1·01–1·29 |

CI, confidence interval.
Multilevel regression, considering the household as a random effect. The areas studied were categorized for the risk of developing the disease according to the frequency of human visceral leishmaniasis cases: area-1: low risk, area-2: medium risk, area-3: high risk.

and found similar rates between them: 34·9% in area-1, 29·3% in area-2 and 33·5% in area-3.

The results of the present study indicate no statistically significant differences in the prevalence rates of infection between areas with distinct RRs of developing HVL, suggesting no correlation between disease risk and transmission levels in these areas. Considering that the characteristics of these areas were predominantly homogeneous, the tendency is to assign individual factors, including genetic and nutritional factors as primarily responsible for illness or worsening of the clinical conditions among the infected individuals (Karplus *et al*. 2002; Alonso *et al*. 2007; Maciel *et al*. 2008; de Almeida *et al*. 2011).

The selection of children to participate in the infection prevalence study is justified by the fact that younger individuals are a better indicator of recent infections and transmission in the areas under investigation. Moreover, children generally remain in neighbourhoods for longer periods of time, which reduces the chance of having been infected in other geographic areas. In addition, nearly 38% of cases of HVL reported in BH between 2002 and 2009 occurred in children under 10 years of age (de Araujo *et al*. 2012). In this ecologic study, the proportion of children under 10 years was 24% among those who became ill in the period studied, showing the high percentage of affected individuals in this age group.

The occurrence of asymptomatic individuals is often reported in studies conducted in endemic areas of Brazil. These cases are often more numerous than the number of clinical cases (Badaro *et al*. 1986; Costa *et al*. 2002; Moreno *et al*. 2006; Souza *et al*. 2008; Maia *et al*. 2016). Thus, identifying and understanding the relevance of asymptomatic infection in the transmission cycle of the parasite is essential for the control of HVL in endemic areas.

Considering the positive test results for one or more tests, the overall prevalence rate of infection with *L. infantum* was 32·5% (Table 3). This high prevalence can be explained by the fact that combining diagnostic methods increases the detection sensitivity. Consequently, the probability of achieving a greater coverage in the identification of asymptomatic individuals increases in the region where transmission occurs (Souza *et al*. 2008; Moreno *et al*. 2009).

The high rates observed suggest that the presence of asymptomatic individuals infected with *L. infantum* in endemic areas could be an indicator of the presence and extent of transmission of the parasite, of which dogs are the main reservoirs in urban areas (Caldas *et al*. 2002; Costa *et al*. 2002; Romero *et al*. 2009).

Some studies have shown that ELISA is not an accurate diagnostic tool for asymptomatic HVL infection. False-negative test results may underestimate the rates and is more suitable for the identification of individuals with the clinical form of the disease (de Gouvea Viana *et al*. 2008; Moreno *et al*. 2009). Nevertheless, this technique was selected as a screening method because it could be performed on numerous samples coming from field studies. To minimize the effects of this limitation we employed two different antigens. Another limitation is the use of a cross-sectional study, which precludes the temporal assessment of the factors investigated and the risk of *L. infantum* infection.

Multilevel logistic regression was used to evaluate the possible association between the presence of infection and the assessed variables, considering the influence of the household at one level and the children's biological characteristics at another level. The model allowed the degree of dependence between variables from different hierarchical levels and their interactions to be controlled. This modelling simultaneously includes multiple levels of aggregation, making the standard errors, CIs and hypothesis tests more stable (Goldstein, 1995).

The age-related variable remained significant in the final multilevel model, regardless of the area of residence, suggesting that younger children would have an increased likelihood of being infected. In Brazil, age-related differences are associated with

https://doi.org/10.1017/S0031182016002134 Published online by Cambridge University Press

clinical cases of HVL, indicating a significant increase in the risk of illness among children younger than 10 years (Costa *et al*. 1990; Marzochi *et al*. 1994).

The variable 'presence of backyards in the neighbourhood' was maintained in the multilevel logistic model (Table 5). The results indicate that having backyards in the neighbourhood increases the likelihood of having an infected child in that residence by approximately 14%. This data corroborate the findings reported by other authors studying increased availability of shelter and favourable environments for the reproduction of phlebotomines in households with backyards (Oliveira *et al*. 2006; Gouvea *et al*. 2007).

One year after the collection of biological samples, further tests were conducted on seropositive children. During the follow-up, none of the children evaluated developed any clinical signs or symptoms of the classical form of HVL. These results agree with those of other studies, which clinically monitored asymptomatic individuals with positive results for ELISA-rK39 in Brazil. The authors also observed that none of the monitored individuals developed the disease (Nascimento Mdo *et al*. 2005; Moreno *et al*. 2006, 2009; de Gouvea Viana *et al*. 2008; Carneiro *et al*. 2011; Silva *et al*. 2011). In India, a different profile was observed during the follow-up of asymptomatic individuals infected with *L. donovani*. Singh *et al*. (2002) showed that 69% of the seropositive patients for ELISA-rK39 developed the clinical form of HVL over a 12-month period. The absence of clinical signs and symptoms of HVL has been observed in individuals infected with *L. infantum* in studies involving a long follow-up period. In such studies, molecular tests were used to confirm the presence of infection (Costa *et al*. 2002; Singh *et al*. 2002; de Gouvea Viana *et al*. 2008; Carneiro *et al*. 2011).

Among the limitations of this study, we highlight the possible occurrence of selection bias, considering that the sampling process took into account only the families registered by the Family Health professionals working for the Brazilian Unified Health System (Sistema Único de Saude – SUS). Moreover, it is possible that not all families living in the assessed areas enrolled in the study or attended the basic health units. However, all areas evaluated presented high coverage by the Family Health Programme, which minimizes this risk and ensures the representativeness of the population of children in each CA and the external validity of the study.

Data related to income and socio-economic status had the highest loss rates, considering that some interviewees did not respond to the direct questions involving monetary values. To minimize data loss, we used the classification indicator CCEB 2012 (ABEP, 2012), which evaluates income indirectly through the identification of property owned by the family, education of the household head, and presence of a maid, allowing the generation of a score and the classification of the population into income classes (ABEP, 2012).

The study loss during follow-up was 39·2%. It is possible that the children not found during follow-up did not develop the clinical form of HVL, considering the absence of clinical cases from these areas notified to the SINAN.

### *Concluding remarks*

The prevalence rates of infection and the distribution of asymptomatic children in the assessed areas indicate that, regardless of the heterogeneous risk of disease, *L. infantum* infection affects a significant percentage of the children population in Belo Horizonte. Because of the homogeneous distribution of favourable conditions to infection transmission, it is plausible to conclude that the factors associated with illness are distinct and likely related to individual characteristics. The study showed that despite the uneven distribution of disease risk across Belo Horizonte, transmission is present in the municipality homogeneously. These are important findings that should be brought to the attention of the Visceral Leishmaniasis Control and Surveillance Programme as they may be relevant in the development of effective strategies to mitigate transmission of this disease in this highly affected city in Brazil.

## SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182016002134.

## ACKNOWLEDGEMENTS

The authors would like to thank the Health Service Department of Belo Horizonte for helping with the municipal census data and the logistics necessary to undertake this work. This manuscript was reviewed by a professional science editor and by a native English-speaking copy editor to improve readability.

## FINANCIAL SUPPORT

L.H.S.M. is grateful to CNPq-Brazil for a doctoral Research Fellowship. M.C. is grateful to CNPq-Brazil for the research fellowships and FAPEMIG/PPM. The authors are also grateful to the Pró-Reitoria de Pesquisa of Universidade Federal de Minas Gerais for supporting this publication. This study was supported by the following grants: CNPQ/Universal 478528/2012-4, CNPQ/Universal 448449/2014-5, Ministry of Health/Brazilian National Leishmaniasis Control Program (no. 25000·204799/2013-65).

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