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ORIGINAL PAPER

Asymptomatic *Leishmania* infection in blood donors from the Southern of Spain

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Abstract

Objectives To investigate the proportion of asymptomatic infection among blood donors in a region endemic for *Leishmania*; and to ascertain epidemiological and genetic factors associated with this condition.

Methods We studied 1260 blood donors in the Province of Granada in the Southern Spain. After obtaining informed consent in each participant, a poll about habits, housing and contact with animals were carried out. Blood samples were obtained for determining antileishmanial antibodies and a PCR assay. HLA typing was performed in a randomly sample among the donors with positive serology.

Results We have found that *L. infantum* antibodies were present in 7.9% of blood donors and DNA in blood was detected in 2.5% of donors. There was no concordance between both determinations, except in one patient. Taking into consideration both techniques, 129 participants were considered to have asymptomatic *Leishmania* infection. No participant in this study developed clinical leishmaniasis during a follow-up period of 2 years. HLA were typed in 51 donors. Asymptomatic *Leishmania* infection might be associated with certain HLA antigens. A multivariate analysis was done with the variables obtained through the participants' interview. The contact with livestock (goats, pigs, and sheep), but not dogs, either at home or in the environment, was significantly and independently associated with asymptomatic leishmania infection.

Conclusions Asymptomatic leishmanial infection among blood donors is frequent in the Granada Province, south of Spain. The presence of livestock in this region is related to this infection, perhaps influencing vector density of this disease. Some HLA genes might be associated with asymptomatic leishmanial state.

Keywords Leishmaniasis · Asymptomatic Leishmania infection · Molecular tests · Serology · HLA

Introduction

The term leishmaniasis (or 'leishmaniosis') [1] refers to a diverse group of syndromes caused by more than 20 different species of intracellular protozoan of the genus *Leishmania*. The parasite infection is widely distributed across the tropical, subtropical, and temperate regions in 98 countries, including the Southern Europe in the Mediterranean basin [1, 2]. In the latter region, leishmaniasis is mainly a zoonotic disease caused by *Leishmania infantum*. Dogs are the main reservoir and the parasites are transmitted to the mammalian host by the bite of female sand flies of the genera *Phlebotomus* [3, 4].

The clinical manifestations of human leishmaniasis depend on the interplay between the virulence factors of the infecting *Leishmania* species and the genetically determined immune responses of their human hosts [3, 4]. Leishmaniasis

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in humans has been divided traditionally into three major clinical syndromes: visceral, cutaneous, and mucocutaneous leishmaniasis [3, 4]. Moreover, in endemic regions, the majority of individuals infected with *Leishmania* spp. never develop the disease; they only will show antileishmanial antibodies, a positive polymerase chain reaction (PCR) in the blood and/or cutaneous delayed-type hypersensitivity to leishmanial antigens [3, 4]. Several studies carried out on blood donors from Southern France [5] and Spain [6–9] have revealed a high proportion of infection in asymptomatic subjects. The precise meaning of this asymptomatic carrier state is not completely understood [10], but a better knowledge of this issue may be crucial for understanding the pathogenesis of human leishmaniasis.

Therefore, the aim of the present study was to investigate the prevalence of asymptomatic *Leishmania* infection in a healthy population of the south of Spain, an endemic region for leishmaniasis; and to ascertain what epidemiological and genetic factors were associated with this condition.

Fig. 1 Location of Granada within Spain and its regions. At the top left-hand corner, map of Spain showing the location of Granada Province (in red). At the bottom right-hand corner, Granada's regions are depicted and the numbers underneath indicate the population of participants in the study for each region

Methods

Study population

The study was carried out from June 2015 to May 2016, in Granada, a province of the Southern Spain. The population of Granada is 900,000 inhabitants. This Spanish Province has ten geographic regions, namely Huéscar, Baza, Guadix, Alpujarra, Costa, Valle de Lecrín, Alhama, Loja, Los Montes, and Vega de Granada. The sample was collected from each area, with a number of participants proportional to the global population in each region (Fig. 1). As a cross-sectional study, sample size was calculated at a confidence of 95%, an error of 1.5% and an expected prevalence of asymptomatic infection of around 8% based on previous studies [6–9]. Ethics approval was granted by the Human Research Ethics Committee. After informed consent was obtained in each participant, an epidemiological poll was undertaken and blood samples were drawn for leishmanial serology and PCR. For the purpose of this study, a healthy subject was considered to be infected with *L. infantum* if only one test was positive. A total of 1260 blood donors participated in the study. All participants positive for either *Leishmania* antibodies or PCR were followed up for 2 years from the time of blood sample was obtained. At this time, asymptomatic carriers were requested for a clinical evaluation, including complete clinical history and physical examination.

Blood collection

Two peripheral blood samples, one in a tube without anticoagulant (8.5 cubic centimeters of blood) and the other in a tube containing EDTA (3.5 cubic centimeters of blood), were collected from each study participant for serological and molecular studies.

Serological study

We used indirect immunofluorescence assay to detect antileishmanial serum antibodies (Vircell, Granada, Spain), following the manufacturer's instructions. Blood samples were centrifuged at 4 °C and stored at – 80 °C until assayed. A single determination was performed for each serum sample. We considered serum to be positive when a titer of 1:80 or higher was obtained.

DNA extraction

DNA was extracted from 500 µL of blood with 50 µL of elution solution using NucliSense EasyMag (BioMerieux, Marcy l'Etoile, France). We followed the manufacturer's recommendations. The extracted DNA was kept at – 20 °C until its amplification by PCR.

Leishmania PCR

The samples were analyzed with two different PCR techniques that were performed and evaluated by two independent observers: GRANALEISH Multiplex qPCR (University

of Granada, Spain), which is able to differentiate among *L. infantum* from other *Leishmania* species [11] and a PCR–ELISA technique, specific for *L. infantum* [12]. Both techniques were performed according to previous investigations [11, 12]. Human DNA free of *Leishmania* and DNA obtained from 1000 *L. infantum* promastigotes were used as negative and positive controls, respectively.

The quantitative real-time PCR technique (qPCR) was applied to all the samples. Each sample was analyzed adding 11 µL of DNA in a final reaction volume of 25 µL. The parasite load was obtained interpolating the threshold cycle (C_t) values obtained for each biological sample in a previously constructed calibration curve. The final parasite density data were expressed in terms of number of parasites/mL of blood.

The PCR–ELISA technique was applied to confirm the positive and doubtful qPCR results ($C_t \leq 36$). Each sample was analyzed in triplicates adding 5, 7 and 11 µL of DNA in a final reaction volume of 25 µL. The results were read in a spectrophotometer at a λ of 405 nm with an OD threshold of 1.

HLA typing

HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 typing were performed in a subset of 51 seropositive asymptomatic donors selected at random. Typing was performed using LIFECODES SSO Typing Kits (Inmunocor, USA) in low resolution, following the manufacturer's instructions. The allele frequencies and the HLA-DRB1–DQB1 haplotypes for this Caucasian population were compared with 636 healthy controls.

Statistical analysis

Chi-square or Fisher's exact test was used to assess statistical significance for various epidemiological data and to compare HLA typing in asymptomatic *Leishmania* infection donors and matched controls. Bonferroni correction was applied to *P* values for HLA typing. The odds ratio and its confidence intervals were calculated. A backward stepwise logistic regression procedure was used to establish which variables were significantly and independently associated with asymptomatic *Leishmania* infection. The dependent binary variable was asymptomatic leishmanial infection, and the independent variables were the monitored epidemiological factors. All variables with a level of significance at the $P \leq 0.2$ were included in the final multivariate logistic model. All variables were entered in the regression analysis as categorical variables within two categories: present or absent. All tests of significance were two tailed. Statistical calculations were made with IBM SPSS Statistics 20.

Results

A total of 1260 serological studies were carried out. 654 donors were female (51.9%) and the average age was 41 years (range 18–65 years). In the total of participants, serology was positive in 100 (7.9%, 95% confidence interval [CI] 6.5–9.6). Ninety-three had a titer of 1:80 (7.4%, 95% CI 6.0–9.0); five a titer of 1:160 (0.4%, 95% CI 0.1 to 0.9); and two a titer of 1:320 (0.1%, 95% CI 0.03–0.6). Fifty-three of 606 males had a positive serology (8.7%, 95% CI 6.7–11.2) and 47 of 654 females were positive for serology (7.1%, 95% CI 5.4–9.3). The seroprevalence for the different regions of Granada was as follows: Alhama, 6.9%; Alpujarra, 6.8%; Baza, 12.3%; Costa, 9.5%; Guadix, 9.0%; Huéscar, 12.9%; Loja, 8.4%; Los Montes, 10.0%; Valle de Lecrín, 8.3%; and Vega de Granada, 5.1%. When we compare the percentage of seroprevalence attending to sex and different regions of Granada, there was statistical difference neither for sex nor for regions.

In these participants, a total of 1189 molecular studies were carried out. PCR study was positive in 30 donors (2.5%, 95% CI 1.7–3.5). Among the participants on which both studies had been performed, 95 subjects had a positive serology (7.9%, 95% CI 6.0–9.6). As is shown in Table 1, only one participant was positive for both immunofluorescence and PCR; the remaining 29 participants with a positive PCR had a negative serology assay. Among the participants with a positive PCR result, the parasite burden was low; in 15 donors ≤ 1 parasite/mL and averaging 3.67 parasite/mL in the remaining (95% CI 1.88–5.47). Three donors presented a higher parasite burden (6.37; 9.47; and 11.75 parasite/mL, respectively). The participant positive for both determinations showed a serological titer of 1:80 and 0.61 parasite/mL in the PCR.

Frequencies of HLA-B*08 and HLA-B*15 were higher in the donors than in matched controls (9.8% vs. 5%, $P = 0.03$; and 9.8% vs. 5.2%, $P = 0.04$, respectively). The haplotypes HLA-DRB1*15–DQB1*06 also had a higher frequency among asymptomatic donors for *Leishmania* than in controls (33.3% vs. 19.5%, $P = 0.02$). When *P* values were adjusted with the Bonferroni correction, there was no statistical significance for these findings.

Table 1 Results of PCR and immunofluorescence assay of 1189 blood donors

	Immunofluorescence assay		
qPCR	Positive	Negative	Total
Positive	1	29	30
Negative	94	1065	1159
Total	95	1094	1189

Blood donors participating in the study were self-questioned about demographic, habits, housing condition, and contact with animals. An univariate and multivariate statistical analyses were carried out to ascertain which variables were significantly and independently associated with asymptomatic leishmanial infection. The statistical results are shown in Table 2. We have found that the presence of certain particular animals (cats, goats, pigs, sheep), either at home or in the house environment, and frequently using a second residency as recreational house were statistically associated with presenting asymptomatic leishmanial infection in blood donors.

All donors enrolled in the study were followed up for 2 years. No participant was microbiologically diagnosed of leishmaniasis during this period of time, according to the registry for infectious diseases of the Province of Granada. In addition, the 129 donors with leishmanial asymptomatic infection were invited for a clinical visit. Lack of contact or unavailability for the appointment, however, greatly reduced the attendance to 75 subjects. Many of them went on to being regular donors during this time, and any of them neither referred symptoms consistent with clinical leishmaniasis nor showed any compatible sign in the physical examination.

Discussion

In the present study, we have found that *L. infantum* antibodies were present at low titers in 7.9% of a large cohort of blood donors and DNA in blood was detected in 2.5% of them. These results indicate that the prevalence of asymptomatic *Leishmania* infection among blood donors from the Province of Granada in the Southern Spain is high, which concurs with previous investigations carried out on blood donors from the Mediterranean area [5–9] and Brazil [13]. We performed serology and PCR in 1189 blood donors. In only one participant, both determinations were positives, showing the lack of concordance between serological techniques and PCR, as has been stated in several investigations [5–9, 13–16].

In the absence of a gold standard to diagnose asymptomatic leishmanial infection, the positive PCR result for *Leishmania* might raise questions about possible false-positive results. Because we confirmed qPCR findings with PCR–ELISA, this assertion seems unlikely. On the other hand, it has been observed an intermittent positivity of qPCR in asymptomatic subjects [9] what may underestimate the prevalence of asymptomatic leishmanial infection in cross-sectional studies. Moreover, we detected a low parasite load by means of qPCR, usually less than 1 parasite per mL of blood, which is in contrast with that observed in patients with active visceral leishmaniasis, suggesting that asymptomatic *Leishmania* infection is generally accompanied by very low parasitemia, at least in immunocompetent subjects [9, 17]. The level of parasitemia in blood from immunocompetent or immunocompromised patients with active visceral leishmaniasis ranges from 32 to 188,700 parasite/mL [17]. In contrast, in asymptomatic carriers very low numbers of *L. infantum* parasites are detected, averaging from 0 to 1 parasite equivalent/mL of blood by quantitative PCR [9, 17]. The results on serological titers in asymptomatic carriers from the medical literature are scarce. Some of these studies have used western blot as serological procedure [7, 9] or the titer of indirect immunofluorescence assay is not given [13]. However, when antibodies to *Leishmania* have been screened by ELISA, antibody levels only slightly exceeded the cut-off of the procedure in 656 blood donors [6], which is in concordance with the low titers found in this study.

No participant in our study developed clinical leishmaniasis during a follow-up period of 2 years. Jiménez-Marco et al. [9] followed up 20 asymptomatic donors for 3 years and França et al. [13], in Brazil, studied 75 asymptomatic donors for 4 years and none of these donors developed clinical leishmaniasis during the follow-up period. Therefore, it seems that immunocompetent individuals may maintain an asymptomatic state for long time. Interestingly, Maritati and colleagues have recently found that patients treated with disease-modifying antirheumatic drugs have a higher prevalence of subclinical leishmanial infection, determined by a positive PCR in blood, than in healthy subjects in Italy [18]. In these patients, subclinical infection may be an issue of concern. Asymptomatic carrier state can evolve to clinical visceral leishmaniasis in immunocompromised people, either HIV-positive individuals or patients under immunosuppressive therapy [17]. In light of the risk presented in these subjects, some authors recommend screening asymptomatic infection prior to immunosuppressive therapy [15, 17].

The risk of transfusion-transmitted leishmaniasis from blood of asymptomatic infected donors is a controversial issue. Transmission of *Leishmania* through blood transfusion cannot be ruled out in endemic regions. However, in a thorough literature review between 1948 and 2011, only 14 case reports were found of suspected transfusion transmission of *Leishmania* [19]. In these cases, it was very difficult to demonstrate transfusion transmission, because the donor was only identified in four cases and the infection status of the patient before transfusion was unknown. In any way, cases of leishmaniasis due to blood transfusion have so far not been reported in Spain [19]. The use of leukocyte removal by filtration of the blood at the time of collection is common practice in Spanish blood banks. It has been proved that depletion of leukocytes from blood components by filtration greatly reduces the risk of *Leishmania*-parasite transmission by blood [7]. At present, no routine screening test to detect *L. infantum* infection in blood donors is in place in our region.

Table 2 Results of the univariate and multivariate analyses. The variables studied were different for each category due to the participants did not answer all questions

Variables	Individuals with IFAT <80 and PCR– ($N=1071$)	Individuals with IFAT ≥ 80 or PCR+ ($N=129$)	Univariate			Multi-variate		
			Odds Ratio	95% CI	*P* value	Odds Ratio	95% CI	*P* value
Gender								
Male (Ref)	507	61						
Female	564	68	1.00	0.69–1.44	0.942			
Age [18–65 years old]								
≤ 41 (ref)	461	64						
> 41	606	64	0.76	0.53–1.10	0.144			
Country of bird								
Spain (Ref)	1045	127						
Other	26	2	0.54	0.15–2.70	0.536			
Inhabitants in the house	1–10 Average: 3.37	1–6 Average: 3.51	1.10	0.95–1.28	0.198			
Contact with animals								
Never, almost never, sometimes (ref)	526	55						
Always or almost always	521	70	1.29	0.89–1.87	0.188			
Outdoor stays								
Never, almost never, sometimes (ref)	343	31						
Always or almost always	548	79	1.60	1.03–2.47	0.036			
House situation					0.066			
Center (Ref)	643	75						
Periphery	377	48	1.09	0.74–1.60	0.655			
Outskirt	12	5	3.57	1.23–10.4	0.020			
Type of housing					0.813			
Block of flats (Ref)	376	39						
House with garden	200	25	1.21	0.71–2.05	0.491			
House without garden	127	18	1.37	0.76–2.47	0.303			
House with courtyard	336	42	1.21	0.76–1.91	0.427			
Animals at home								
No (Ref)	492	53						
Yes	538	75	1.29	0.89–1.88	0.175			
Dogs at home								
No (Ref)	647	78						
Yes	384	50	1.08	0.74–1.57	0.266			
Cats at home								
No (Ref)	868	114						
Yes	163	14	0.65	0.37–1.17	0.151	0.55	0.30-1.00	0.051
Horses at home								
No (Ref)	1020	128						
Yes	11	0	0.00	0.00	0.999			
Birds at home								
No (Ref)	854	101						
Yes	177	27	1.29	0.82–2.03	0.272			
Goats, pigs or sheep at home								
No (Ref)	1022	123						

Table 2 (continued)

Variables	Individuals with IFAT <80 and PCR– ($N=1071$)	Individuals with IFAT ≥ 80 or PCR+ ($N=129$)	Univariate			Multi-variate		
			Odds Ratio	95% CI	*P* value	Odds Ratio	95% CI	*P* value
Yes	9	5	4.62	1.52-14.0	0.007	4.53	1.32–15.5	0.016
Animals in the house environment								
No (Ref)	246	25						
Yes	656	96	1.44	0.90–2.29	0.125			
Dogs in the house environment								
No (Ref)	309	34						
Yes	594	87	1.33	0.88–2.03	0.182			
Cats in the house environment								
No (Ref)	693	113						
Yes	18	8	1.29	0.84–1.97	0.241			
Birds in the house environment								
No (Ref)	817	103						
Yes	86	18	1.66	0.96–2.87	0.070			
Goats, pigs or sheep in the house environment								
No (Ref)	885	113						
Yes	18	8	3.48	1.48–8.19	0.004	3.64	1.42–9.33	0.007
Vegetation in the house								
No (Ref)	118	14						
Yes	754	107	1.20	0.66–2.16	0.552			
Pots inside the house								
No (Ref)	246	55						
Yes	446	66	1.15	0.78–1.68	0.484			
Pots outside the house								
No (Ref)	210	26						
Yes	662	95	1.16	0.73–1.84	0.530			
Garden at home								
No (Ref)	706	104						
Yes	166	17	0.70	0.41–1.19	0.187			
Vegetation in the house environment								
No (Ref)	198	29						
Yes	673	94	0.95	0.61–1.49	0.835			
Garden in the house environment								
No (Ref)	700	102						
Yes	171	21	0.84	0.51–1.39	0.501			
Field in the house environment								
No (Ref)	454	61						
Yes	417	62	1.11	0.76–1.61	0.599			
Vacant lot in the house environment								
No (Ref)	850	121						
Yes	21	2	0.67	0.16–2.89	0.590			

Table 2 (continued)

Variables	Individuals with IFAT <80 and PCR– (*N* = 1071)	Individuals with IFAT ≥ 80 or PCR+ (*N* = 129)	Univariate			Multi-variate		
			Odds Ratio	95% CI	*P* value	Odds Ratio	95% CI	*P* value
Use of insecticides								
Always or almost always (ref)	231	29						
Sometimes	267	39	1.16	0.69–1.94	0.562			
Never or almost never	376	55	1.17	0.72–1.88	0.531			
Mosquito nets and curtains in windows								
No (Ref)	509	80						
Yes	370	41	0.71	0.47–1.05	0.086			
Curtains on doors								
No (Ref)	530	68						
Yes	349	53	1.18	0.81–1.74	0.389			
Use of air conditioning at night								
Never or almost never	802	112						
Sometimes	28	3	0.77	0.23–2.57	0.667			
Always or almost always (Ref)	48	6	0.90	0.37–2.14	0.803			
Open windows at night								
Never or almost never	114	14						
Sometimes	73	7	0.78	0.30–2.03	0.611			
Always or almost always (Ref)	684	98	1.17	0.64–2.11	0.611			
Use of fan at night								
Never or almost never	723	92						
Always or almost always (Ref)	153	29	1.49	0.95–2.34	0.084			
Night outings								
Never or almost never	78	10						
Sometimes	87	8	0.72	0.27–1.91	0.596			
Always or almost always (Ref)	713	103	1.13	0.57–2.25	0.735			
Second residence								
No	1066	126						
Yes	5	3	5.08	1.20–21,5	0.027	4.51	1.06–19.2	0.041

IFAT immunofluorescence assay test

Recently, it has been shown that certain common polymorphisms in the HLA region are genetic risk and protective factors for visceral leishmaniasis [20, 21]. In the present study, we investigated a potential association between asymptomatic *Leishmania* infection and class I and class II HLA loci. As far as we know, this is the first time that HLA association with asymptomatic infection is studied in a population from the South of Europe. The case–control study did not show any statistically significant association between HLA antigens and asymptomatic infection after correcting for multiple comparisons. However, our preliminary results might suggest a role for some class I and class II HLA genes in determining the asymptomatic leishmanial state.

Several epidemiological studies have been carried out trying to identify epidemiological risk factors associated with leishmaniasis. These investigations have shown conflicting results with respect to contact with animals in regions endemic for *Leishmania donovani* [22–24]. Cattle may affect risk in complex ways, through their effect on sand fly abundance, infection rates, and feeding frequency on humans. In a recent paper studying risk factors associated with skin disease by *L. tropica* in Morocco, the presence of livestock was not associated with skin disease

[25]. In this paper, only the density of the transmitting vector, *Phlebotomus sergenti*, was found to be a risk factor for cutaneous disease. Obviously, the epidemiology of *L. tropica* is different from that of *L. infantum*, and the epidemiological findings on that parasite may not be interchangeable to the transmission cycle of *L. infantum* [1, 2, 26].

In our research, we have found a significant and independent epidemiological relationship between the presence of goats, pigs and sheep, either at home or in the house environment, and asymptomatic infection. Perhaps, this association translates the influence of livestock on vector density. The lack of relationship in our study between dogs at home or in the environment and *Leishmania* infection may be related to the fact that infected dogs would equally pose a risk for owners and neighbors, because it is common that dogs live outside in rural areas in Spain [8]; and our investigation was mostly conducted in rural zones.

Our study has several limitations. First of all, in the absence of a gold standard to measure asymptomatic infection, it is hard to know whether these seropositive individuals who remained healthy were truly infected with *Leishmania* or whether the serology results were simply false-positive results [10]. Second, the fluctuating nature of PCR positivity may underestimate the prevalence of asymptomatic subjects in cross-sectional studies. Third, in epidemiological studies it is difficult to simultaneously account for all of the factors intervening in the transmission of the parasite. Some of the conflicting results of these observational studies may be related to this fact.

In conclusion, a high proportion of healthy people from endemic regions of *Leishmania* present a positive result for antibodies for the parasite and/or PCR. Many of these individuals may have suffered asymptomatic *Leishmania* infection and will never progress to disease. A better understanding of the mechanism(s) responsible for this asymptomatic state may lead to an essential knowledge of the pathogenesis of leishmanial disease. At present, a major caveat consists in the precise diagnosis of healthy asymptomatic individuals for *Leishmania*. Thus, establishing a standard case definition for asymptomatic leishmanial infection in endemic regions should be a priority in the near future.

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Compliance with ethical standards

Conflict of interest The authors declare that they no competing interest.

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