Socio-economic and environmental factors associated with Montenegro skin test positivity in an endemic area of visceral leishmaniasis in northern Morocco

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Abstract

In Morocco, many aspects of human asymptomatic visceral leishmaniasis (VL) still have to be clarified and little information is available about the factors that predispose individuals to asymptomatic infection. A prospective study was carried out in 889 healthy children under the age of 15 years living in two provinces in the endemic area in northern Morocco (the provinces of Taounate and My Yacoub) from April to May 2010. The aim of the study was to evaluate the prevalence and the socio-economic and environmental characteristics associated with infection by *Leishmania infantum*. The Montenegro skin test (MST) was used to detect asymptomatic infection. The prevalence of transmission of infection was 11.4% and approximately 2 times higher in Taounate than in My Yacoub, as measured by MST. Asymptomatic infection was associated with gender, age, presence of familial links, proximity to chickens, and the number of people in the house and locality, but it was not associated to education status, presence of dogs, livestock waste, sewage disposal, water supply system or use of insecticides.

Introduction

Leishmaniasis constitute a group of diseases caused by an obligatory, intracellular, protozoan parasite of the *Leishmania* genus that causes a spectrum of diseases, ranging from self-limiting, self-curing cutaneous leishmaniasis (CL) to visceral leishmaniasis (VL)

with fatal spontaneous evolution.¹ The domestic dog constitutes the principal reservoir of infection and is important in zoonotic transmission.² The worldwide incidence of VL is approximately 0.5 million cases per year.³

Visceral leishmaniasis is widespread in northern Morocco (Chefchaoun, Taounate, Taza, Fes, Moulay Yacoub, Meknes, Sefrou, AL houceima and Sidi Kacem) (Figure 1). Few epidemiological data are, however, available concerning its epidemiology and clinical features. However, a new type of cutaneous leishmaniasis due to *Leishmania infantum* was recently described.⁴ Before 1995, human visceral leishmaniasis was not an obligatory notifiable disease. The incidence of VL is about 152 cases per year⁵ predominantly affecting children under the age of 5 years. Extension of arid zones and an increase in temperatures have a synergic role on the risks of an increase in the number of cases of leishmaniasis in these regions.⁵ The sand fly vectors of *L. infantum* in these regions are: *Phlebotomus perniciosus*,⁶ *Phlebotomus longicuspis*⁷ and *P. arisi*.⁸ Dog is the main reservoir of *L. infantum* in Morocco.⁸⁻¹⁰ Zymodeme MON-1 is predominant in Morocco; however, zymodeme MON-24 has been occasionally isolated from a dog.¹¹ This zymodeme was considered a sporadic form of CL.

Epidemiological patterns are changing in the Mediterranean area due to several factors, such as the widespread migration from rural to urban and peri-urban areas, climatic changes increasing exposure to the sand fly and also, in urban areas, an increase in HIV infection. Human activity is the leading factor in the significant changes recently observed in the ecology and behavior of many vector-borne diseases.¹² Most parasite-driven processes are asymptomatic or subclinical; however, some result in severe visceral diseases that are lethal if not treated.¹³ It is not well understood why the parasite-driven processes remain asymptomatic in certain subjects and cause a lethal disease in others. It has been suggested that environmental factors that affect sand fly ecology,¹⁴ human activities that increase exposure to sand flies,¹⁵ and the presence of other animals permissive to the *Leishmania* life cycle all play critical roles in the development of the human disease.¹ Poor economic conditions,^{16,17} malnutrition¹⁸ and impaired reactivity of the immune system¹⁹ have also been shown to increase the risk of VL. Furthermore, certain age groups are at a greater risk of VL than others.²⁰ Field studies showed that asymptomatic infection may occur in endemic areas. In these areas of VL, only about 20% of the subjects infected by *Leishmania chagasi* will develop classical VL, the majority of the infected individuals have a subclinical infection that may remain completely asymptomatic

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Contributions: SH carried out the project, conducted the field work, collected and analyzed data, and prepared the final draft of the manuscript; AF, RE, helped in the statistical analysis; AL, HA, helped in the study design; MH, revised the manuscript; ML overviewed the study for intellectual content.

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or have an oligosymptomatic form of the disease.²¹ Although the extent of this phenomenon has not been fully evaluated, people without a definite history of disease may have evidence of infection as demonstrated by a positive Delayed-Type Hypersensitivity (DTH) reaction assessed by the Leishmanin skin test, also called the Montenegro skin test (MST). This test is currently used to assess the prevalence of *Leishmania* infection in the human population.²² The MST is considered the most complementary test in diagnosis, and is also used in epidemiological studies and as an indicator of unapparent infection with *Leishmania*.²³

There are no statistical data in Morocco concerning the force of transmission of visceral leishmaniasis in endemic areas. Studies of a marker of cellular immune response, such as the Montenegro skin test, might be useful since the response rarely disappears with time. Therefore, the objective of this study was to identify socio-economic and environmental factors associated with Montenegro skin test

positivity in an endemic area in northern Morocco, and estimate the force of transmission and the frequency of healthy carriers.

Material and Methods

Study area

This study was undertaken from April to May 2010 in two rural endemic areas in northern Morocco, Zouagha Moulay Yacoub and Taounate provinces (Figure 1 and Table 1). This is a mountainous region with variable relief and it is, therefore, common to find a wide range of vegetation and bioclimatic variation. We studied 8 localities at altitudes of 300-600 m with a semi-arid climate, a mild winter and yearly precipitation of 400-800 mm in Zouagha Moulay Yacoub and 900-1000 mm in Taounate. Rural populations under the age of 15 years number 20,600 and 51,000 in Taounate and My Yacoub, respectively.⁵

Study population

A questionnaire that included socio-economic and demographic characteristics was given to 889 healthy children. Data were obtained on age, gender, familial links, literacy, history of migration, number of people in household, history of VL in the family, style of the house, water supply, sewage disposal, age and number of dogs, presence of other animals (chickens, horses, rabbits and cats), animal sheds, sewage in the peri-domestic area, and exposure to insecticide in the home.

Measurements and test

The main outcome of the study is the result of the MST using 0.1 mL of *Leishmania* antigen injected intradermally.²⁴ The antigen was prepared and provided by the Department of Immunology of the Institut Pasteur of Iran, using a strain of *Leishmania major* (MRHO/IR/75/ER strain). Reactions were measured at 48-72 h, and induration of 5 mm or more in diameter was considered positive. The test was carried out on 889 school children aged 3-15 years.

Ethical approval

The study protocol was approved by the Committee on Research Ethics of the Institut Pasteur du Maroc.

Statistical Analysis

Associations between variables were expressed as prevalence ratios (PR) and their respective 95% confidence intervals (95% CI), examined by univariate analysis (χ^2 and t-test

where appropriate) and multivariate analysis (logistic regression). Statistical analysis was performed using STATA 11.0 software (Stata Corporation, College Station, TX, USA).

Results

Force of transmission of visceral leishmaniasis in northern Morocco

A total of 889 healthy children were included in the study. All were from the northern region where visceral leishmaniasis is endemic. The mean age of the population studied was 8.5 ± 2.23 years; the median age was eight years (81% under the age of ten years); 417 females (47%) and 472 males (53%). The prevalence of MST positivity was 11.4% and approximately two times greater in Taounate than in My Yacoub. The number of asymptomatic cases was 66 males (66.34%) and 34 females (33.66%).

Table 2 shows the prevalence of MST positivity according to the variables investigated and the crude prevalence ratios and respective

95% confidence intervals for the association with outcome. Most variables studied (Tables 2 and 3) were associated with a significant prevalence of MST positivity. There was a significant difference in exposure to infection with *L. infantum* between males and females. Risk factor of infection was approximately two times greater for males than for females. On the other hand, this risk increases with age (PR=1 for children under the age of eight years and PR=1.85 for children over the age of ten years), presence or absence of familial links (PR=2.87 for children tested with a familial link), and according to province and locality. Exposure to risk infection is more prevalent in Taounate (PR=1.72) than in My Yacoub. Bouadel, a site in Taounate, has the highest prevalence of infection (PR=2.34) in this study, even though the difference was not significant.

Table 3 shows the association between nodule size and gender, age, familial links and provinces. There was a significant difference between nodule size and familial link and province. Nodule size increased with familial links (PR=3.20) and this difference was more common in Taounate (PR=3.95).

Figure 1. Endemic areas with visceral leishmaniasis in northern Morocco.

Table 1. Number of people injected by leishmanin according to locality.

Province	Locality	No. injections
My Yacoub	Ain Allah	137
	labrarcha	50
	karyet ba Mhammed	52
	Hamria	209
Taounate	Ain Abdoune	58
	Bouadel (Almarkaz)	82
	Bouadel bab zarouki	140
	Ain Barda (Rkiba)	34

Socio-economic and environmental factors associated to the Montenegro skin test

Some socio-economic and environmental factors associated with MST in the two endemic foci of VL are shown in Table 4. Variables included in the analysis were: literacy status of the head of household, number of people in the household, water supply system, livestock waste, housing, history of migration (whether they had ever lived outside My Yacoub/Taounate), presence of dogs and their age, presence of other animals, use of insecticides and knowledge of VL.

Few people came from other sites (10.41%), the majority were original inhabitants with the same type of housing and life-style (culture, work, life activities, etc.). On the other hand, only 2 cases with a familial history of VL were found. Most people were unaware of VL. Statistical analysis shows that in general there is no significant relationship between all these components and the risk of transmission of VL in these areas.

Discussion

Results show that 11.4% of children in the two provinces were asymptomatic, as measured by the MST. This percentage was comparable to that found in Spain (11.5%)²⁵ and in Brazil, where the incidence of the infection was 10.8% when determined by MST on children aged 0-5 years.²⁶ Studies conducted in high-risk areas of VL in southern Europe using MST indicated a high frequency of subclinical leishmaniasis: 9.7% in Sardinia (Italy),²⁷ 15.3% in Tuscany (Italy),²⁸ and 46.8% in the Alpes Maritimes (France).²⁹ There were no or very low incidence of positive reactions in children under the age of 15 years but this increased in older populations.^{27,29} The highest percentage of asymptomatic carriers (14.05%) and the highest nodule size was found in Taounate compared to My Yacoub, where the percentage of asymptomatic carriers was only 8.7%. In contrast, the Ministry of Health 2006 report⁵ confirms that the incidence of visceral leishmaniasis per 100,000 children under 15 years of age is higher in My Yacoub (33%) than in Taounate (12.1%). We suggest that children in Taouante are more protected against the disease. The difference between VL cases and asymptomatic forms may lie in the type of host cellular immune response.³⁰ Another hypothesis is that genetic factors are involved in the development of the clinical disease in these areas. The evidence of VL cluster in families could be an indication that genetic factors may predispose individuals to the development of the disease.³¹ This

idea is supported by the significant difference in results between related and non-related subjects. On the other hand, parasite exposure was higher than expected, despite the small number of clinical cases, suggesting factors other than infection could influence clinical outcome.

Analysis showed that the risk of transmission was related to gender; it was approximately 50% higher among males than females with a male/female sex ratio of 1.97. In France, adult males were more frequently found to be asymptomatic carriers than females.²⁹ A study carried out in Brazil had shown the same results with a male/female sex ratio of 1.34.³² Epidemiological data in Morocco also showed male predominance for VL⁵ with a sex ratio of 1.15. The predominance of visceral leishmaniasis among males has also been described in an epidemiological study in the northeast region of Brazil in which the male to female ratio was 1.7.³³ In Italy, the leishmanin skin

test positivity rate of males was only slightly higher than that of females, the tendency of women to work indoors during the time of day when transmission occurs, together with the exophily of the vector *P. perniciosus*, may explain this difference.²⁷

We suggest that males are more exposed to infectious bites due to socio-cultural factors. Males can stay outdoors later and spend more time outside on the whole. In hot weather they may not wear a shirt therefore exposing more of their body areas to the vector, especially at dusk, the time of highest sand fly activity. Another argument for the importance of gender in the transmission of *Leishmania* is reflected by the higher incidence of the disease in immunodepressed males (85.04%) compared to females.¹⁶ These patients had no history of the disease but lived in or visited visceral leishmaniasis endemic areas and the parasite may have persisted in the host for a long period of time.

Table 2. Number (N), percentage (P) and crude prevalence ratios (PR), and their 95% confidence intervals (95% CI) for the association between result of Montenegro skin test and gender, age, familial links, provinces and locality in two endemic areas of *L. infantum* infection in northern Morocco, 2010.

Variables	N ni	N a	Pni (%)	Pa (%)	PR	95% CI	P
Gender							
Female	383	34	46.91	33.66	1.00		0.0042
Male	405	67	53.9	66.34	1.86	1.20 - 1.19	
Age							
<8 yrs	373	39	91.35	8.65	1.00		
8-10 yrs	294	38	85.81	14.19	1.73	1.07 - 2.78	< 0.035
≥10 yrs	94	20	82.46	17.54	1.85	1.03 - 3.31	
Familial links							
no links	707	76	89.72	75.25	1.00		0.0001
links	81	25	10.28	24.75	2.87	1.73 - 4.77	
Province							
My Yacoub	409	39	51.9	38.61	1.00		0.013
Taounat	379	62	48.1	61.39	1.72	1.12 - 2.62	
Locality of taounat							
Ain abdoun	57	6	12.93	9.68	1.00		0.18
Bouadel	218	47	49.43	75.81	2.34	0.94 - 5.82	
Ghefssay	166	9	37.64	14.51	0.48	0.16 - 1.44	

Ni, non infected; a, asymptomatic.

Table 3. Association between nodule size and gender, age, familial links and province. Prevalence ratios (PR) and their 95% confidence intervals (95% CI).

Variables	PR	95% CI	P
Gender			
Female	1.00		0.39
Male	1.32	0.7 - 2.47	
Age			
≤8 yrs	1.00		0.45
>8 yrs	1.91	1.002 - 3.66	
Familial links			
no links	1.00		0.0007
links	3.20	1.78 - 6.504	
Province			
My Yacoub	1.00		0.001
Taounat	3.95	1.85 - 8.40	

Asymptomatic infection was also associated with age. Children over the age of eight years had a higher relative risk for the infection than those under the age of eight; suggesting a cumulative exposure to the parasite (parasitic load) for an asymptomatic infection. This finding is consistent with those from other studies.^{27,29,32,34,35} It is important to note that the prevalence of the infection tends to increase with age, since this test detects a type of hypersensitivity that rarely disappears with time.³⁶ Age, in this case, might be a proxy for time of individual exposure to the infection.

Most studies so far have focused on the risk of clinical disease and few have investigated the role of educational status on the risk of leishmaniasis infection. Our study showed that there was no correlation between the literacy level of the head of household and the infection. However, in Brazil, many studies reported that a higher literacy level of the head of household was inversely associated with the infection.^{26,32}

Divergent results were found when comparing the risk of infection and the number of people in the household. A Brazilian study found that risk of infection increased the more people there were in the household.³⁰ In the present study, the infection decreased when the number of people increased in the family, suggesting that with more people available as sources for a blood meal, the individual chance of being bitten by an infected vector would be reduced. The same result were found by Gouvêa *et al.*³²

The presence of dogs in the house did not seem to be a significant determining factor of human asymptomatic infection. This is similar to results found in Brazil.³⁷ This could be explained by the fact that although the subjects did not own dogs, they were exposed to them in the surrounding neighbourhood.

In this study, the presence of chickens in the house was significantly associated with the risk of infection by increasing the population density of sand flies.²⁶ Indeed it is known that transmission of asymptomatic visceral leishmaniasis occurs through the bite of a sand fly. This finding is consistent with the result of Caldas *et al.*²⁶ who explained that the presence of these insects in the peri-domestic areas was a risk factor for infection by *L. chagasi*, but contrary to another study carried out by Gouvêa *et al.*³² who found a weak or a non-significant association with the presence of chicken sheds in the peri-domestic environment. Thus, the role that chickens play in the epidemiology of VL is not clear and probably involves a balance between zooprophyllaxy, maintenance of a sand fly population, and attracting reservoir hosts.³⁰

On the other hand, in this study, use of insecticides was found not to reduce the risk of

infection. Probably the phlebotomies were present in the peri-domestic areas of the households, thus stressing that the vector control had been ineffective in reducing the transmission of parasites.

Livestock waste, sewage disposal and water supply system were also not associated with the prevalence of the infection. These results can be explained by the fact that the inhabitants of these areas live in the same kind of living conditions, so it was very difficult to find a

significant difference with all these factors and VL infection.

Most epidemiological studies were conducted in Brazil and in southern Europe. Strikingly the prevalence of asymptomatic carriers using a single test was highly variable (0.6-71.3%) according to the type of test used for detection.³⁸ The most frequently used technique was MST, followed by ELISA, with crude *Leishmania* antigens while recent studies using direct tests such as PCR confirm the

Table 4. Study of same socio-economic and environmental factors associated to risk of transmission of *Leishmania infantum* in two endemic areas in northern Morocco (Taounate and My Yacoub). Prevalence ratios (PR) and their 95% confidence intervals (95% CI).

Variable	PR	95% CI	P
Literacy of head of household			
Analphabetic	1.00	0.15 - 1.13	0.074
Only elementary	0.4		
Number of people in household			
03 - 04	1.00		0.005
05 - 07	0.48	0.097 - 2.39	
08+	0.17	0.033 - 0.94	
Water supply system			
Tap water	1.00		0.0985
Spring water	2.22	0.84 - 5.87	
Sewage disposal			
Public sanitation system	0.15	0.026 - 0.834	0.0137
Pit latrines	0.36	0.114 - 1.19	
Open air	1.00		
Age of dogs			
Do not have a dog	1.00		0.5212
≤04 years	1.60	0.17 - 14.95	
05 or more years	1.49	0.37 - 5.9	
Presence of a kennel in the peri-domestic environment			
No	1.00		0.5117
Yes	1.32	0.56 - 3.12	
Other animals in house or in the peri-domestic environment			
Presence of chickens			
No	1.00		0.0039
Yes	3.40	1.40 - 8.24	
Presence of bovines			
No	1.00		0.0683
Yes	2.24	0.92 - 5.45	
Presence of horses			
No	1.00		0.7321
Yes	0.84	0.31 - 2.25	
Presence of cats			
No	1.00		0.5657
Yes	1.29	0.53 - 3.15	
Presence of goats			
No	1.00		0.5809
Yes	1.82	0.21 - 15.99	
Presence of rabbits			
No	1.00		0.9641
Yes	0.96	0.25 - 3.81	
Livestock waste			
Peri-domestic environment	1.00		0.7371
Domestic environment	1.20	0.39 - 3.65	
Use of insecticide in house			
No	1.00	0.14 - 1.06	0.0560
Yes	0.39		

high proportion of asymptomatic carriage in endemic zones. Riera *et al.*³⁹ showed that MST is more sensitive than the other diagnostic techniques. This test has been used extensively in epidemiological studies in southwestern Europe and it is considered a good indicator of endemicity.^{25,27-29} Molecular methods such as nested PCR should be considered as an alternative for detecting asymptomatic *L. infantum* infection in blood donors.³⁹

To understand the dynamics of the spread of the disease, it would be interesting to study other factors that might be related to the infection and the transmission of VL in Morocco. In addition, recent data obtained in studies carried out on blood donors from southern Europe have revealed a high rate of infection in asymptomatic subjects,³⁹ so it would also be interesting to study the risk of transmission by blood transfusion in Morocco.

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