Suspected cutaneous leishmaniasis in a sample of Western Sicily residents: what correlation with occupation?

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SUMMARY

Background: Leishmaniasis is a widespread infectious disease, but there is not much information about its prevalence in high risk occupational categories. Objectives: The aim of this study is to assess the prevalence of Leishmania immunological positivity in human skin tissues collected from subjects living in Western Sicily, with suspected cutaneous Leishmania infection, in order to explore the risk possibly related to occupation. Methods: 318 consecutive subjects (M/F ratio=1.0, mean age=40±25.4 years), attending the Dermatology Department of the University of Palermo Hospital from 2013 to 2015, without any previous history of Leishmania infection and performing various occupations, were included. Parasite isolation and PCR-RT test on skin scrapings were performed to evaluate the immunological status; all data were analyzed by the chi square test, comparing all positive results from the different provinces. Results: 81 (50.9%) out of 159 females and 79 (49.7%) out of 159 males were found PCR-RT positive to Leishmania infantum, with a higher risk in the Agrigento district (p<0.001) and in subjects living in rural areas (p=0.0038), regardless of the type of work performed. The observed animal leishmaniasis prevalence in the same areas shows the endemic status of the disease in Sicily. Conclusions: Although based on a relatively small sample, our study shows that cutaneous leishmaniasis represents a health care problem with a medical and social impact in Western Sicily. An active surveillance system and the establishment of diagnosis and treatment centres could be useful in controlling this public health problem.

RIASSUNTO

«Studio trasversale su un campione di soggetti della Sicilia occidentale con sospetto di leishmaniosi cutanea: quale correlazione con l’attività lavorativa?». Introduzione: La leishmaniosi è una malattia infettiva diffusa in tutto
Introduction

Cutaneous leishmaniasis (CL) is an infectious disease that affects humans and animals, most often dogs (reservoir of infection). It is caused by protozoa of the Leishmania genus, widespread in the Mediterranean area and transmitted through the phlebotomine sandfly bite. In the Mediterranean basin two main types of disease are common: cutaneous leishmaniasis (CL), with single or multiple lesions spread all over the body, and visceral leishmaniasis (VL), with systemic spread and localization in the reticuloendothelial system (liver, spleen, bone marrow). The diagnosis of leishmaniasis, based primarily on clinical observations, needs nonspecific laboratory confirmation with blood counts, showing pancytopenia, and several laboratory tests as well as IgG antibody research in blood serum, protozoan observation on bone marrow aspirate, needle aspiration to perform isolation culture and the molecular tests (PCR-RT). The control of adult sandflies (phlebotomus in Old World and lutzomyia in the New World) requires the use of insecticides (mostly pyrethroids) for residual spraying of dwellings and animal shelters, space-spraying, insecticide-treated nets, impregnated dog-collars and personal protection through the application of repellents/insecticides for topical use (15). The disease is widespread all over the world, especially in temperate and tropical areas, including Europe, the Mediterranean basin and some bordering countries, such as Portugal (8, 9, 11, 14, 19, 22, 23, 25). In Italy, 72 cases of VL and 22 of CL were reported in 2009 by the last available Epidemiological Bulletin of the Italian Ministry of Health with a significant prevalence in Sicily, Campania and Latium (12). To date, no official nationwide data on this and similar diseases related to the different occupations are available. In rural areas workers such as farmers, herdsmen, forest rangers and veterinary surgeons belong to categories with a greater risk of exposure, because they are most likely to come into contact with vectors and infected animal reservoirs present in the territory. In Italy leishmaniasis, similarly to other infectious diseases, must be reported. In addition, the reporting of diseases possibly related to occupation is mandatory (6,7).

The objective of this cross sectional study is to assess the prevalence of Leishmania infection positivity in skin scrapings, bone marrow and blood samples from a population sample of Western Sicily. Age and occupation were analyzed in order to evaluate their contribution to the risk of Leishmania positivity.

Methods

In this cross sectional study, approved by the Local Ethic Committee, 318 consecutive patients...
who attended the Dermatology Department of the Palermo University Hospital from 2013 to 2015 were enrolled. All patients included in this study lived in different Western Sicilian provinces and had a CL diagnostic suspicion expressed by general practitioners and dermatologists.

In addition to the collection of the clinical history, each patient was asked to compile a structured questionnaire, prepared and validated by the National Reference Centre for Leishmaniasis (C.Re.Na.L), Istituto Zooprofilattico Sperimentale di Sicilia. The questionnaire included information about age, gender, body lesion localization (single or multiple), pet ownership, residence (urban, rural areas or both), hobbies and occupation, with particular interest in occupations having direct contact with animals.

We evaluated the specific status against Leishmania through parasite isolation and PCR test. Parasite isolation was performed through several human samples: skin biopsies, bone marrow, blood, which were cultured in Tobie agar medium, Evans modified (EMTM medium) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 26°C for up to 30 days. The examined human samples (skin scrapings, bone marrow, blood) were extracted by “E.Z.N.A. Tissue DNA Kit (Omega biotech VWR)” according to the manufacturer’s instructions. The PCR test was targeted on a 123 bp fragment inner the constant region in the mini circle kinetoplast DNA (kDNA) (NCBI accession number AF291093) and was carried out as previously described. The primer sequences were: QLK2-U 5’-GGCGTTCTGCAAAAACCACG-3’; QLK2-D5’-AAAAATGGCATTTTCGGGCGC-3’; while the associated probe was: 5’-TGGGTGCA-GAAATCCCGTTC-3’ 5’FAM and 3’ BHQ labelled. Each amplification was performed in duplicate, in 20 μl reaction mixture containing 1× TaqMan Universal Master Mix (Applied Biosystem), 20 pmol/μl of the specific primers and 10 pmol/μl of labelled probe (Qleish 2), 1×EXO IPC Mix, 1×EXO IPC DNA according to the manufacturer’s instructions of the TaqMan Exogenous Internal Positive Control Reagents kit (Applied Biosystem). The thermal cycling conditions comprised an initial incubation for 2’ at 50°C for uracil-N-glycosylase activity. This step was followed by a 10’ denaturation at 95°C and 45 cycles at 95°C for 15’ and 60°C for 1’ each. Results were expressed as a parasite count for ml of the liquid matrices such as blood, according to the parasite charge per ml of the standard curve described below. Standard DNA was extracted from cultured IPT1 MON1, obtained from the collection of the Italian National Reference Centre for Leishmaniasis (C.Re.Na.L), counted at 1×10^6 cells/ml and homogenized in 1 ml of lysis mix, (1% Tween 20, 1% Non idet P-40, e 20% Chelex). Then decimal serial dilutions of the stock solution were performed to obtain the points of the curve ranging from the DNA equivalent of 1×10^6 cells to 1 cell for ml.

The IFAT (immunofluorescence antibody test) on several animal species was performed according to OIE Terrestrial Manual chapter 2.1.8. (24). The IPT1 ZMON1 strain was used as antigen fixed on multipot microscope slides (Bio-Merieux, Marcy L’Etoile, France) in acetone bath. The canine sera were serially diluted (1:40 to 1:5120) in phosphate-buffered saline (PBS), pH 7.2, and added to the antigen-coated wells. The slides were incubated for 30 min at 37°C. Positive and negative controls were included in each series of analysed samples. Fluorescent staining was performed using an anti-dog IgG labelled with fluorescein isothiocyanate (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:200 in PBS. The slides were examined by a Leica DM 4000B fluorescence microscope (Leica, Heerbrugg, Switzerland). The cutoff value, according to OIE Terrestrial Manual chapter 2.1.8., for a positive result was established at a serum dilution of 1:40. The positive control consisted of a known titre serum of a dog with positive cultural isolation. The negative control consisted of a serum from a dog resulting negative to the cultural test.

Statistical analysis of human data was performed by EpiInfo (version 7; Centers for Disease Control and Prevention, Atlanta, Ga) and R software, version 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria). Category variables were summarized as absolute and relative frequency (%) and compared by using Pearson Chi-square test or Chi-square test for linear trend as appropriate. Odds ratios (ORs) and 95% confidence intervals (CIs) were
estimated by logistic regression. All reported P values were 2-sided and P<.05 was considered significant.

**RESULTS**

One hundred and fifty-nine males and one hundred and fifty-nine females (average age 40 ± 25.4 years old) were enrolled in the study; they lived and worked in the Western Sicilian provinces of Palermo, Agrigento, Trapani, Caltanissetta, and Enna, and had no direct contact with animals at work (table 1). The characteristics regarding the age groups, area of residence, pet ownership and occupational risk (farmers) are in the table.

Their occupations were: 4 lawyers (1.3%), 11 building contractors (3.5%), 34 civil servants (10.7%), 9 teachers (2.8%), 12 housewives (3.8%), 7 bricklayers (2.2%), 8 car mechanics (2.5%), 65 pensioners (20.4%), 97 in pre-working age (30.5%) and

| Table 1 - Socio-demographic characteristics of subjects enrolled in the study and prevalence odds ratio of Leishmania PCR test positivity calculated by risk factors |
|-----------------------------------------------|-----------------|-------------------------------|-----------------|-----------------|-----------------|
| Risk factors                               | Total N. (%)    | Leishmania PCR positives, N/Tot (%) | OR (95% CI)    | P-value        |
|-----------------------------------------------|-----------------|-------------------------------|-----------------|-----------------|-----------------|
| Sex                                           |                 |                               |                 |                 |
| Female                                        | 159 (50.0)      | 81/159 (50.9)                 | Ref.            | 0.82<sup>a</sup>|
| Male                                          | 159 (50.0)      | 79/159 (49.7)                 | 0.95 (0.61-1.48)|                 |
| Age in years                                 |                 |                               |                 |                 |
| 0 to 18                                       | 97 (30.5)       | 55/97 (56.7)                  | Ref.            | 0.07<sup>b</sup>|
| 19 to 44                                      | 61 (19.2)       | 31/61 (50.8)                  | 0.79 (0.42-1.50)|                 |
| 45 to 64                                      | 95 (29.9)       | 46/95 (48.4)                  | 0.72 (0.41-1.26)|                 |
| 65 or more                                    | 65 (20.4)       | 28/65 (43.08)                 | 0.58 (0.31-1.09)|                 |
| Living area                                   |                 |                               |                 |                 |
| Urban                                         | 221 (69.5)      | 100/221 (45.2)                | Ref.            | 0.006<sup>a</sup>|
| Rural                                         | 83 (26.1)       | 49/83 (59.0)                  | 1.96 (1.21-3.20)|                 |
| Both                                          | 14 (4.4)        | 11/14 (78.6)                  |                 |                 |
| Pet                                           |                 |                               |                 |                 |
| None                                          | 212 (66.7)      | 100/212 (47.2)                | Ref.            | 0.039<sup>a</sup>|
| Dogs                                          | 71 (22.3)       | 45/71 (63.4)                  | 1.94 (1.11-3.37)|                 |
| Cats                                          | 13 (4.1)        | 0                              |                 |                 |
| Dogs, cats and others                         | 22 (6.9)        | 15/35 (42.9)                  | 0.84 (0.41-1.72)|                 |
| Farmworker                                    |                 |                               |                 |                 |
| No                                            | 271 (85.2)      | 136/271 (50.2)                | Ref.            | 0.91<sup>a</sup>|
| Yes                                           | 47 (14.8)       | 24/47 (51.1)                  | 0.97 (0.52-1.79)|                 |
| Residency                                     |                 |                               |                 |                 |
| Palermo                                       | 155 (48.7)      | 57/155 (36.8)                 | Ref.            | <0.001<sup>a</sup>|
| Agrigento                                     | 131 (41.2)      | 85/131 (64.9)                 | 3.18 (1.95-5.16)|                 |
| Other                                         | 32 (10.1)       | 18/32 (56.2)                  | 2.2 (1.02-4.78) |                 |

<sup>a</sup> Chi square test; <sup>b</sup> Chi square for linear trend
24 unemployed (7.5%); only farmers (47, 14.8%) were considered as potentially exposed to occupational risks. No one was an animal breeder.

None of our patients had travelled to foreign Countries in the previous months and only 5% of men had outdoor hobbies such as gardening, without statistical differences among those resulting positive and negative to PCR tests.

The analysis of PCR tests found positivity for *Leishmania infantum* in 160 (50.3%) subjects, equally distributed between men and women, with a higher prevalence among subjects living in Agrigento (table 1). The median value of positivity found was 2,300 PCR L/mL. We observed very high levels also in very young children. There was a statistically significant difference between those who lived in rural areas, even far from the sea coasts, or those who moved in the winter and the summer months from urban to rural areas, and city dwellers (OR=1.96; 95% CI=1.21-3.20). Subjects who lived both in cities and in the country have declared a rural stay long enough to be considered at increased risk of exposure. Therefore, in the statistical analysis they have been merged with those continuatively living in rural areas.

Dog ownership was another factor significantly associated with infection (OR=1.94; 95% CI=1.11-3.37).

There was no difference between positive result among farmers and other individuals who carried out other occupations, mainly indoors (p=0.91).

Finally, the infection prevalence showed a downward trend with age, although this difference was only marginally statistically significant (p=0.07).

**DISCUSSION AND CONCLUSIONS**

Although based on a limited sample of the general population, our results suggest that the disease is widespread in Western Sicily, considering that we tested only subjects attending one hospital in Western Sicily, although the main one.

Living in non-urban areas, having dogs and mainly living in the Agrigento area, as observed in the examined sample, are factors related to a higher risk of infection with *Leishmania*. On the other hand, in this study the occupations performed have not been found to be a risk factor for cutaneous leishmaniasis, even if this could be explained, at least in part, as a consequence of the small number of farmers enrolled in the sample.

A further interesting consideration is that Leishmania infections have been reported in all age groups, even in children a few months old. This is probably due to the high circulation of the protozoan and carriers in our regional context and, more widely, in Southern Italy. As reported by other authors, the positivity in children seems to suggest an active transmission of the infection (1-5, 10, 13, 16-18, 21).

Moreover, it cannot be excluded that the disease is more widespread than the official data show, probably due to underreporting, despite the reporting obligation in Italy, and because of the lack of a diagnosis in the absence of appropriate laboratory testing (molecular tools, parasite isolation, immunofluorescence antibody test).

In fact, in 2009 in Italy, including Sicily, only 22 cases of cutaneous leishmaniasis were reported.

Furthermore, the analysis conducted by the Istituto Zooprofilattico Sperimentale of Sicily in 2015 in animals shows that the infection is widespread. Of 13,148 tested dogs from the provinces of Western Sicily (Palermo, Agrigento, Trapani, Caltanissetta and Enna) 5,815 (44%) were positive to specific tests for leishmaniasis.

Leishmaniasis is a challenging disease to the immune system, requires long treatment, and has a less favourable prognosis in immunosuppressed subjects. For this reason, it is necessary to implement strategies to prevent its spread, both at work for those exposed (farmers, veterinary surgeons, etc.) and for the general population, by trying to counter the carriers and also by using protective clothing when working outdoors, especially in endemic areas like Southern Italy. At the present moment, there is no specific prophylaxis, such as vaccines, which could frustrate the onset of the disease after infection, and no treatment for infected animals has proven to be a useful tool to fight its spread.

In recent years it seems that the disease, which is traditionally endemic in Southern Italy, is spreading to the North, probably because of climate change, increasing the risk that it may turn into a more dif-
fused problem both for the animal and the human health (20).

Although the coast with its hot and humid climate is the place traditionally preferred by the carriers, it is interesting to observe that in our sample some of patients with cutaneous leishmaniasis lived in the drier and cooler hinterland, far from the coast. This may be explained by changes in climate.

Furthermore, the diagnosis, carried out not only with the determination of antibodies but also with the isolation of the parasite and by the PCR method, unveils apparently negative cases increasing the diagnostic accuracy.

Although the relatively small sample size could be considered a major limitation, nevertheless our results suggest that cutaneous leishmaniasis is widespread in Western Sicily and that its prevalence could even be higher than observed in this study.

The widespread presence of microbiological agents in a certain geographical area creates unfavourable conditions not only for those involved in risky occupations but for those who spend time outdoors as well.

Closer collaboration and an exchange of information between medical doctors and veterinary surgeons and an active surveillance system, with the establishment of diagnosis and treatment centres, seem to be the best public health strategy to fight the spread of Leishmania infection.

No potential conflict of interest relevant to this article was reported

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