Transactions of the Royal Society of Tropical Medicine and Hygiene (2006) **100**, 527—532

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Pool screen PCR for estimating the prevalence of *Leishmania infantum* infection in sandflies (Diptera: Nematocera, Phlebotomidae)

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Received 21 April 2005; received in revised form 27 June 2005; accepted 3 August 2005
Available online 23 November 2005

KEYWORDS
Leishmania infantum;
Sandflies;
Vector infection;
Prevalence;
PCR—ELISA;
Spain

Summary Prevalence studies of infection in the sandfly vector can be used as an indicator of a change in the intensity of *Leishmania* transmission. However, these studies are difficult to carry out as prevalence in the vector is usually low and its estimation requires a large number of sandflies to be dissected. Our objective was to establish whether a *L. infantum*-specific PCR—ELISA applied to pools of female sandflies and a previously described algorithm could be useful tools to study the prevalence of infection by this parasite in natural vector populations. We collected sandflies from six collection points in two stable foci of leishmaniasis in southern ($N = 3$) and north-eastern ($N = 3$) Spain, following standard procedures. A fraction of the collected females was dissected and morphologically identified. Another fraction was used for pool screening. In total, 127 pools of 30 females (3810 specimens) were studied by PCR—ELISA and 1764 specimens were individually dissected. The prevalence of infection determined by dissection does not differ from that determined by pool screen PCR. The results suggest that pool screen PCR can be of practical use in the epidemiological surveillance of leishmaniasis in European countries of the western Mediterranean basin, associated with control interventions or global change.

1. Introduction

Leishmaniasis is a serious health concern and its repercussions on public health as well as its geographical distribution, far from declining, actually appear to be increasing (Desjeux, 2004). The protozoan is transmitted by

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doi:10.1016/j.trstmh.2005.08.005

the bite of an infected female sandfly (Diptera: Nematocera, Phlebotomidae) and causes a wide range of clinical forms of leishmaniasis ranging from a mere ulcer to a systemic disease that can be fatal if not treated correctly. The main leishmaniasis control strategy includes case finding and treatment, vector control and, in zoonotic foci, animal reservoir control. New and better disease control tools have recently been made available. These include: (i) new diagnostic tests; (ii) marketing of the first oral drug for leishmaniasis; (iii) the use of insecticide-impregnated bed nets for sandfly control; and (iv) pyrethroid-treated collars for dog control (Alexander and Maroli, 2003; Desjeux, 2004). These prevention and control measures must be evaluated in terms of cost effectiveness in the different epidemiological settings in which they are contemplated. In this regard, estimates of the prevalence of infection in the vector population may be important in order to measure the effect that control programmes can have on leishmaniasis transmission. It has also been suggested that environmental changes and, in particular, climatic change could alter the transmission patterns of vector-borne diseases (Patz et al., 2000). However, we do not know how, or to what extent, these changes will affect the geographical distribution of sandflies or the transmission of *Leishmania*. Sandflies are abundant in the Mediterranean region where they extend northwards to a latitude of 49°N (Lewis, 1982).

Prevalence studies of infection in the vector could serve as an indicator of a change in transmission intensity. However, these studies are difficult to perform as prevalences are generally low and can only be estimated by dissecting a large number of sandflies. PCR-based techniques can detect *Leishmania* in the sandfly vector but it would be costly and time consuming to use this technique on individual insects when hundreds or thousands must be counted. Katholi et al. (1995) showed that the prevalence of infection of *Onchocerca volvulus* can be estimated in vector populations using (i) a specific PCR for this nematode and pools of vector black flies and (ii) an algorithm that can estimate the prevalence of infection in a vector population based upon the size of the pools screened and on the percentage of negative pools found. These authors suggest that the algorithm they describe can be applicable to any disease for which a PCR assay is available. Our objective was to establish whether using a specific PCR for *L. infantum* (PCR–ELISA), pools of female sandflies and the above-mentioned algorithm could be a useful tool to study the prevalence of infection by this parasite in natural sandfly populations.

Other authors (Jorquera et al., 2005; Perez et al., 1994) have applied PCR techniques with different specificities for *Leishmania* detection in sandfly pools, but only one of them calculated the prevalence of infection. Miranda et al. (2002) determined the prevalence of *L. braziliensis* in groups of 20 *Lutzomyia* phlebotomines by assuming that there is only one infected sandfly in each positive pool, since it was not possible to establish whether there was more than one. In our case, application of the algorithm of Katholi et al. (1995) will enable us to calculate the prevalence of infection from the proportion of negative pools and to obtain more accurate prevalences of infection.

2. Material and methods

2.1. Collection of sandfly specimens

To evaluate the PCR pool screening assay, *Phlebotomus* spp. were obtained from six collection points from two stable foci of leishmaniasis in the south-east (Alfacar, Viznar and Torvizcon in Granada province) and north-east (Sant Just Desvern in Barcelona province, and Torroja del Priorat and Margalef de Montsant in Tarragona province) of Spain, following standard procedures. Sandflies were captured overnight in CDC miniature traps placed peridomestically near stables and houses (Table 1). Females were separated from males and a fraction of the females was dissected (Rioux et al., 1986) and identified to species level based on their spermathecae morphology. The number of sandflies carrying promastigotes of *Leishmania* in the midgut was recorded. A second fraction of female sandflies from each collection point and date was preserved at −20 °C until use.

2.2. PCR analysis of sandfly pools

Females were divided into pools of known size ($N = 30$). Application of the algorithm of Katholi et al. (1995) is not restricted to pools of a particular size. However, the calculated confidence interval is dependent upon the pool size and the number of pools studied. For a given number of pools gathered, the confidence interval narrows as the pool size increases. Similarly, for a given pool size, the confidence intervals decrease as the number of pools increases. A pool size of 30 specimens was selected on the basis of this consideration and also in an attempt to minimise the number of tests required.

DNA was isolated from the pools using the GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Uppsala, Sweden). In general, the protocol recommended by the manufacturer was followed. Briefly, sandflies were homogenized using a sterile pestle and liquid nitrogen. Then, 600 μl of cell lysis solution was added and incubated at 65 °C for 1 h. After incubation, 200 μl of protein precipitation solution was added, vortexed vigorously at high speed for 20 s and centrifuged at 13 000 rpm for 3 min. To ensure elimination of all the insect cuticle components that could inhibit *Taq* polymerase, protein precipitation was repeated. Then, 600 μl of 100% isopropanol was added and mixed by inverting the tube and centrifuged at 13 000 rpm for 1 min. The supernatant was removed and rinsed with 70% alcohol. Finally, the DNA pellet was re-suspended in 50 μl hydration solution and 3 μl and 5 μl were then used as a substrate in the *L. infantum* PCR–ELISA assay (Martin-Sanchez et al., 2001). In each PCR, DNA obtained from 30 males and from 1000 *L. infantum* promastigotes were used as negative and positive controls, respectively. The absorbance values obtained were always less than 0.1 for male sandfly DNA and more than 2.5 for the positive control. Absorbance values ≥1.0 were considered as positive (Martin-Sanchez et al., 2001).

The prevalence of infection in the population was then determined using the PoolScreen™ computer program (www.main.uab.edu/show.asp?durki=34764). PoolScreen™ 2.0 provides estimates of the prevalence of infection in the vector population together with user-selectable confidence