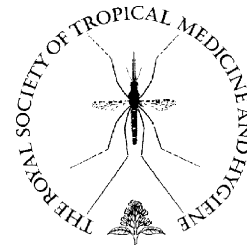




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A real-time PCR assay to estimate *Leishmania chagasi* load in its natural sand fly vector *Lutzomyia longipalpis*

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Summary *Leishmania chagasi*, transmitted mainly by *Lutzomyia longipalpis* sand flies, causes visceral leishmaniasis and atypical cutaneous leishmaniasis in Latin America. Successful vector control depends upon determining vectorial capacity and understanding *Leishmania* transmission by sand flies. As microscopic detection of *Leishmania* in dissected sand fly guts is laborious and time-consuming, highly specific, sensitive, rapid and robust *Leishmania* PCR assays have attracted epidemiologists' attention. Real-time PCR is faster than qualitative PCR and yields quantitative data amenable to statistical analyses. A highly reproducible *Leishmania* DNA polymerase gene-based TaqMan real-time PCR assay was adapted to quantify *Leishmania* in sand flies, showing intra-assay and inter-assay coefficient variations lower than 1 and 1.7%, respectively, and sensitivity to 10 pg *Leishmania* DNA (~120 parasites) in as much as 100 ng sand fly DNA. Data obtained for experimentally infected sand flies yielded parasite loads within the range of counts obtained by microscopy for the same sand fly cohort or that were around five times higher than microscopy counts, depending on the method used for data analysis. These results highlight the potential of quantitative PCR for *Leishmania* transmission studies, and the need to understand factors affecting its sensitivity and specificity.

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1. Introduction

The leishmaniases are a set of diseases caused by *Leishmania* parasites, which affect more than 2 million people in over 88 tropical and Mediterranean countries. Resistance to

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first- and second-line chemotherapy, particularly in regions of intense *Leishmania* transmission, has been reported (Mittal et al., 2007). *Leishmania* parasites are transmitted to sylvatic or peridomestic mammalian reservoir hosts and to humans by blood-feeding female sand flies.

Leishmania real-time PCR assays for estimating relative loads within vertebrate hosts have been developed, based upon *Leishmania* small ribosomal subunit, DNA polymerase or glucose-6 phosphatase genes (Bossolasco et al., 2003; Bretagne et al., 2001; Mary et al., 2004; Nicolas et al., 2002; Wortmann et al., 2001). These PCR studies have indicated that *Leishmania* load influences clinical outcome and that low levels of parasitaemia or clearance are associated with either cure or fewer relapses in HIV–*Leishmania* co-infection. In canine leishmaniasis, the quantity of *Leishmania* DNA correlates with parasite density in the bone marrow, blood, skin or urine, and often with severity of clinical symptoms (Manna et al., 2006; Solano-Gallego et al., 2007). Furthermore, Svodová et al. (2003) demonstrated *Le. tropica* transmission to its *Phlebotomus sergenti* sand fly vector from asymptomatic 'reservoir' black rats using quantitative PCR. Thus, real-time PCR offers a feasible approach to follow *Leishmania* infection time course, parasite clearance and tissue tropism.

Natural infection rates in sand flies are traditionally estimated by microscopic identification of *Leishmania* within dissected sand fly guts and/or parasite isolation from dissected sand flies in vitro or in vivo. However, these methods are time- and labour-consuming, especially when considering the low infection prevalence found in most endemic foci (Ashford et al., 1991). A number of *Leishmania*-DNA-based PCR assays, with differing sensitivities and specificities, have been applied to studies of sand fly natural infection rates (Aransay et al., 2000; Córdoba-Lanús et al., 2006). *Leishmania* species typing through RFLPs, hybridisation or sequencing of amplified *Leishmania* DNA, with species-specific PCR primers, has also been reported (Azizi et al., 2006; Garcia et al., 2007; Jorquera et al., 2005; Martín-Sánchez et al., 2006).

Two drawbacks of end-point, compared with real-time, PCR are that the former is only qualitative and that it is still time-consuming, as PCR cycling time adds to the time required for visualisation of PCR products run in agarose gels. In real-time PCR assays, the PCR products are 'visualised' in real time and are also quantifiable, allowing statistical testing of the reproducibility and significance of results obtained. Furthermore, although initially more costly (i.e. for equipment and reagents), than end-point PCR, real-time PCR is significantly less time-demanding, reducing the overall research cost in the long term.

It is important to quantify *Leishmania* in sand flies to evaluate relative *Leishmania* development efficiencies between different sand fly species that transmit the same parasite species. These differences may account for vectorial capacity differences, which in turn could contribute to observed epidemiological differences between visceral leishmaniasis foci (Montoya-Lerma et al., 2003). It is also recognised that one major difference between natural and experimental infections is that the true parasite infective dose probably consists of 1–1000 metacyclics in natural conditions but several million in experimental infections (Rogers et al., 2004; Warburg and Schlein, 1986). Consequently, real-time

PCR would allow more accurate determination of natural infection doses. Numerous reports have established that effective parasite dose ingested at the vertebrate host biting site determines antigen concentration and distribution, and these in turn influence the timing and type of immune responses and hence clinical outcome (Lira et al., 2000). However, to date there are no published studies in which *Leishmania* load has been estimated within sand flies using as accurate a method as real-time PCR (Gómez-Saladín et al., 2005). This article reports the application of a TaqMan real-time PCR assay to quantify *Leishmania* within sand flies based on the *Le. infantum* single copy DNA polymerase α and the *Lutzomyia longipalpis* periodicity genes.

2. Materials and methods

2.1. *Leishmania* and sand fly maintenance

Lutzomyia longipalpis sand flies (Diptera: Psychodidae) from Jacobina, Bahia State, Brazil, were reared at 22–27°C, 60–70% relative humidity and 12:12 (L:D) photoperiod, as described by Modi and Tesh (1983). Newly emerged flies were fed on 70% (w/v) sucrose solution ad libitum before processing for DNA extraction. *Leishmania infantum* (MHOM/BE/67/ITMAP263), a reference strain used in other PCR assay development studies (Noyes et al., 1996), was selected to develop the real-time PCR assay within sand flies. Promastigotes were cultured in HOMEM at 26°C as described (Berens et al., 1976).

2.2. Sand fly experimental infection

Female *Lu. longipalpis* sand flies (~125 flies in a cage, 5 d after mating) were fed on fresh rabbit blood seeded with *Le. infantum* amastigotes (2×10^6 /ml) through a chick skin membrane feeding apparatus (Rogers et al., 2002). Amastigotes were obtained from *Le. infantum*-infected BALB/c mouse spleen homogenates in M199 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, B and E vitamins (Gibco, Invitrogen Corp., Paisley, UK) and 25 µg/ml gentamycin sulphate (Sigma-Aldrich Co., Cambridge, UK) at pH 7.2. To prevent premature mortality, flies were allowed to defecate onto filter paper inside the cage but were prevented from laying eggs by continuous saturated sucrose feeding and by withdrawing oviposition substrates. Twelve infected flies [13 d post-infection to allow metacyclogenesis (Rogers and Bates, 2007)] were dissected and parasite numbers were estimated by microscopic examination of gut homogenates using a haemocytometer, as detailed by Rogers et al. (2002). Six infected flies from the same infection cohort were individually stored in 2 ml 96% (v/v) ethanol at room temperature, and were used for *Leishmania* quantitation by real-time PCR.

2.3. DNA isolation

Cultured *Leishmania* promastigotes (10^8) were washed in buffered saline before DNA isolation, as described by Campos-Ponce et al. (2005). Individual female sand flies were placed onto 3MM Whatmann filter paper to allow the

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