Transactions of the Royal Society of Tropical Medicine and Hygiene (2008) **102**, 891—897

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevierhealth.com/journals/trst

Two *Leishmania* species circulating in the Kaleybar focus of infantile visceral leishmaniasis, northwest Iran: implications for deltamethrin dog collar intervention

P. Parvizi[a,b], A.S. Mazloumi-Gavgani[c], C.R. Davies[d], O. Courtenay[e], P.D. Ready[b,*]

[a] *Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran*
[b] *Department of Entomology, Natural History Museum, Cromwell Road, London SW7 5BD, UK*
[c] *Department of Parasitology, University of Tabriz Medical Sciences, Tabriz, Iran*
[d] *Department of Infectious Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK*
[e] *Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK*

Received 20 December 2007; received in revised form 15 April 2008; accepted 18 April 2008
Available online 12 June 2008

KEYWORDS
Visceral leishmaniasis;
Leishmania infantum;
Leishmania tropica;
Phlebotomus perfiliewi transcaucasicus;
ITS-rDNA;
PCR;
Iran

Summary *Leishmania infantum* is the causative agent of infantile visceral leishmaniasis (IVL) in the Mediterranean Basin and, based on isoenzyme typing of a few isolates from patients and domestic dogs, this parasite was considered to predominate in the Kaleybar focus of IVL in northwest Iran. However, in the current investigation only one out of five sandfly infections was found to be *L. infantum*, based on PCR detection and sequencing of parasite internal transcribed spacer (ITS) rDNA infecting *Phlebotomus perfiliewi transcaucasicus*. The four other infections were of haplotypes of *L. tropica*, the causative agent of anthroponotic cutaneous leishmaniasis in the Middle East and a parasite occasionally detected in the viscera of dogs and patients in Iran and elsewhere. The widespread distribution of *L. tropica* in the Kaleybar focus suggests that this parasite is not a transient introduction. Kaleybar has been used for a deltamethrin dog collar intervention to reduce the biting rates of the vectors of *L. infantum* and this has significantly reduced the incidence of *Leishmania* infections both in children and the domestic dog, the usual reservoir host of IVL. The implications of finding *L. tropica* widespread in the heart of the intervention area are discussed. Extensive and intensive typing of natural *Leishmania* infections is a characteristic of epidemiological investigations in the Neotropics and the current report indicates that this will also be necessary in some regions of the Old World.

* Corresponding author. Tel.: +44 20 7942 5622; fax: +44 20 7942 5229.
E-mail address: p.ready@nhm.ac.uk (P.D. Ready).

0035-9203/$ — see front matter © 2008 Royal Society of Tropical Medicine and Hygiene. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.trstmh.2008.04.026

1. Introduction

Much of northwest Iran to the east of Turkey has a Mediterranean climate and, like the Mediterranean Basin, some parts are endemic for infantile visceral leishmaniasis (IVL) caused by *Leishmania infantum* (Nadim et al., 1978). The prevalence of human disease and infections is relatively high in only two districts of northwest Iran, the Meshkin-Shahr district of Ardabil province (Mohebali et al., 2005; Nadim et al., 1992) and, just to the west, in the Kaleybar district of East Azerbaijan province (Davies and Mazloumi-Gavgani, 1999; Gavgani et al., 2002a, 2002b). Both districts appear to have a transmission cycle typical of the Mediterranean, with domestic dogs incriminated as the reservoir hosts of *L. infantum* zymodeme MON-1 in rural villages and farms (Gavgani, 1998; Gavgani et al., 2002a, 2002b; Mohebali et al., 2005). Kaleybar district was chosen for an intervention trial using deltamethrin-impregnated dog collars to reduce the biting rates of female sandfly vectors on the reservoir host (Gavgani et al., 2002a). This intervention was successful based on a significant reduction in the incidence both of IVL and canine leishmaniasis (CanL). In the customary way, incidence was estimated using a serological test not specific for any one *Leishmania* species, namely the direct agglutination test (DAT) (Gavgani et al., 2002b), and so some of the infections detected might not have involved *L. infantum*, the specific causative agent of IVL.

The current report establishes that *L. infantum* is not the only causative agent of human leishmaniasis circulating in Kaleybar district. Our detection of *L. tropica* in sandflies from four Kaleybar villages raises the possibility that, following the intervention, some of the reduction in the incidence of IVL and CanL might have resulted from changes in the transmission rate of *L. tropica* and not of *L. infantum*.

The primary aim of screening Kaleybar sandflies for *Leishmania* infections was to help incriminate the regional vectors of *L. infantum*. In Mediterranean environments, infection rates in sandflies should be greatest towards the end of the summer season of adult activity, when few nulliparous female flies are emerging (Dye et al., 1987). Therefore, we began our survey by screening female sandflies caught in September of 2005 and 2006. Screening was performed using nested PCRs (Parvizi and Ready, 2007; Parvizi et al., 2005) targeting both sections of the internal transcribed spacer (ITS1 and ITS2) of the multicopy nuclear rRNA gene array (rDNA) of *Leishmania*, and parasite species were identified by phylogenetic analysis of the nucleotide haplotypes obtained by directly sequencing the amplicons.

2. Materials and methods

2.1. Sandfly collections within the area of the intervention trial

Sandflies were sampled from many of the villages used for the intervention trial in northeast Kaleybar province (Gavgani et al., 2002a), including control villages in which no dogs were collared and intervention villages in which deltamethrin-impregnated collars (Scalibor; InterVet, Boxmeer, The Netherlands) were put on all dogs annually from 2001 to 2006. In September 2005, the only Kaleybar collections came from two miniature CDC light traps (Sudia and Chamberland, 1962) placed in Garavanlu (intervention village) and one such trap in Goyogaj (control village). In September 2006, 30 villages were systematically sampled by placing one string of five sticky papers (A4 white paper soaked in castor oil) inside and outside each of three animal shelters per village for two 4-day periods.

All sandflies were stored in 80% analytical grade ethanol at −20 °C and processed for identification and DNA extraction as reported by Parvizi et al. (2005). Morphological identifications were based on characters of the head and abdominal terminalia slide-mounted in Berlese fluid (Lewis, 1982; Nadim and Javadian, 1976), following dissection with sterilized forceps and micro-needles.

2.2. Nested PCR detection and identification of *Leishmania* infections in sandflies

All female sandflies from these collections were screened for infections of *Leishmania* species by nested PCR of one or two fragments of ITS rDNA. Each PCR was carried out in two separate tubes (Parvizi et al., 2005). The first-stage PCR used the forward primer IR1 (5′-GCTGTAGGTGAACCTGCAGCAGCTGGATCATT-3′; at the 3′ end of the *small subunit rRNA* gene) with the reverse primer IR2 (5′-GCGGGTAGTCCTGCCAAACACTCAGGTCTG-3′; at the 5′ end of the *large subunit rRNA* gene). One of two fragments was amplified in the second-stage PCR: (i) the ITS1-5.8S fragment was amplified using the nested forward primer ITS1F (5′-GCAGCTGGATCATTTTCC-3′; overlapping the 3′ end of the *small subunit rRNA* gene and ITS1) with the nested reverse primer ITS2R4 (5′-ATATGCAGAAGAGAGGAGGC-3′; at the 5′ end of ITS2); and (ii) the ITS2m fragment was amplified with the nested forward primer ITSmF1 (5′-GTGTGGAAGCCAAGAGGAGG-3′, near the 5′ end of ITS2) and reverse primer ITSmR2 (5′-GCAAGCACCAGAGAGGAGT-3′; within ITS2) (Parvizi and Ready, 2007).

PCR products were directly sequenced to identify *Leishmania* haplotypes infecting individual female sandflies (Parvizi et al., 2005) and all haplotypes were identified to species level by phylogenetic analysis. For this, DNA sequences were edited and aligned using Sequencher 3.1.1 software (Gene Codes Corp., Ann Arbor, MI, USA) and the multiple alignments of new DNA haplotypes and homologous GenBank sequences were exported into PAUP* software (Swofford, 2002) for phylogenetic analysis. The homologous GenBank sequences were those representative of the main branches of the molecular phylogenetic tree for the *L. donovani* complex (*L. infantum*, *L. archibaldi*, *L. donovani*) as reported by Lukes et al. (2007) and for all available haplotypes of *L. major*, *L. tropica*, *L. turanica* and *L. gerbilli*. In Iran, the causative agent of most human visceral leishmaniasis is *L. infantum* and the causative agents of most human cutaneous leishmaniasis are *L. major* and *L. tropica* (Mohebali et al., 2004; Nadim and Seyedi-Rashti, 1971).