International Journal for Parasitology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

# Minimally invasive microbiopsies: a novel sampling method for identifying asymptomatic, potentially infectious carriers of *Leishmania donovani*

Oscar David Kirstein [a], Ibrahim Abbasi [a], Ben Zion Horwitz [a], Laura Skrip [b], Asrat Hailu [c], Charles Jaffe [a], Lynlee L. Li [d], Tarl W. Prow [d], Alon Warburg [a,*]

[a] Department of Microbiology and Molecular Genetics, The Institute for Medical Research Israel-Canada (IMRIC), The Kuvin Centre for the Study of Infectious and Tropical Diseases, The Hebrew University – Hadassah Medical School, The Hebrew University of Jerusalem, 91120, Israel
[b] Department of Biostatistics, School of Public Health, Yale University, 60 College Street, New Haven, CT 06520, USA
[c] Department of Microbiology, Immunology & Parasitology, Faculty of Medicine, Addis Ababa University, P.O. Box 9086, Addis Ababa, Ethiopia
[d] Dermatology Research Centre, The University of Queensland School of Medicine, Translational Research Institute, Brisbane, QLD 4012, Australia

ARTICLE INFO

*Article history:*
Received 6 October 2016
Received in revised form 20 February 2017
Accepted 24 February 2017
Available online xxxx

*Keywords:*
Asymptomatic carriers
*Leishmania donovani*
Microbiopsy
Phlebotomine sand flies
Visceral leishmaniasis
Xenodiagnosis

ABSTRACT

Visceral leishmaniasis (VL) is a potentially lethal, sand fly-borne disease caused by protozoan parasites belonging to the *Leishmania donovani* species complex. There are several adequate methods for diagnosing VL, but the majority of infected individuals remain asymptomatic, comprising potential parasite reservoirs for transmission of the disease. The gold standard for assessing host infectiousness to biting vector insects is xenodiagnosis (i.e. scoring infection rates among insectary-reared insects that had fed on humans suspected of being infected). However, when it comes to sand flies and leishmaniasis, xenodiagnosis is an intricate operation burdened by logistical hurdles and ethical concerns that prevent its effective application for mass screening of widely dispersed communities, particularly in rural regions of underdeveloped countries. Minimally invasive microbiopsy (MB) devices were designed to penetrate the skin to a depth of ~200 μm and absorb blood as well as skin cell lysates, mimicking the mode by which phlebotomine sand flies acquire blood meals, as well as their composition. MBs taken from 137 of 262 volunteers, living in endemic VL foci in Ethiopia, detected *Leishmania* parasites that could potentially be imbibed by feeding sand flies. Although the volume of MBs was 10-fold smaller than finger-prick blood samples, *Leishmania* DNA detection rates from MBs were significantly higher, implying that skin, more often than blood, was the source of parasites. Volunteers with histories of VL were almost as likely as healthy volunteers to test positive by MBs (southern Ethiopian focus: 95% CI: 0.35–2.59, $P = 1.0$. northern Ethiopian focus 0.87: 95% CI: 0.22–3.76, $P = 1$), suggesting the importance of asymptomatic patients as reservoirs of *L. donovani*. Minimally invasive, painless MBs should be considered for reliably and efficiently evaluating both *L. donovani* infection rates among large numbers of asymptomatic carriers and their infectiousness to blood-feeding sand flies.



## 1. Introduction

Visceral leishmaniasis (VL) is caused by disseminated infections with eukaryotic *Leishmania donovani* (complex) parasites. An estimated 390,000 VL cases occur annually, over 90% of which are concentrated in the Indian sub-continent, eastern Africa and Brazil (Alvar et al., 2012). Distinct modes of transmission characterize the two causative parasite species responsible for VL. *Leishmania donovani infantum* in Latin America, Europe, the Middle East and North Africa is transmitted zoonotically with dogs serving as the principal reservoir while *Leishmania donovani donovani* in the Indian subcontinent and eastern Africa is transmitted anthroponotically between humans (Chappuis et al., 2007). The worst affected African countries are Sudan and Ethiopia (Alvar et al., 2012; Gadisa et al., 2015). To understand the anthroponotic transmission dynamics of *L. donovani*, it is crucial to diagnose not only the VL cases that normally comprise a small minority of the infected population, but also to identify the asymptomatic carriers

* Corresponding author at: Department of Microbiology and Molecular Genetics, The Institute for Medical Research Israel-Canada (IMRIC), The Kuvin Centre for the Study of Infectious and Tropical Diseases, Botnar Building, Floor -1, Room: A003, The Hebrew University – Hadassah Medical School, The Hebrew University of Jerusalem, 91120, Israel.
*E-mail address:* alonw@ekmd.huji.ac.il (A. Warburg).

http://dx.doi.org/10.1016/j.ijpara.2017.02.005
0020-7519/

with high parasitemias accessible and, therefore, potentially infectious to biting sand flies (Miller et al., 2014; Singh et al., 2014; Hirve et al., 2016).

Several serological assays for VL have been developed but none serve as "stand-alone" tests (Boelaert et al., 2008). Serological diagnosis is achieved using Freeze Dried – Direct Agglutination Tests (FD-DAT) or rK39 strip tests. Parasitological confirmation of VL is achieved by microscopic examination of Giemsa-stained splenic aspirate smears (96% sensitivity) (Boelaert et al., 2008; ter Horst et al., 2009). Targets for PCR-based diagnosis of *Leishmania* infections in humans include kinetoplast DNA (kDNA) minicircles (Abbasi et al., 2013), the ssrRNA gene (van Eys et al., 1992), the internal transcribed spacer 1 (ITS1) (el Tai et al., 2000) and the spliced leader sequence (van Eys et al., 1992). Further analysis of the PCR amplicon is required for species identification (e.g. restriction cut analysis of ITS1 (el Tai et al., 2000), high resolution melt analysis of kDNA and 7SL (Bensoussan et al., 2006) or DNA sequencing (el Tai et al., 2000; Bensoussan et al., 2006; Abbasi et al., 2013).

Some blood-feeding insects (e.g. triatomine bugs, vectors of Chagas disease) obtain blood by direct cannulation of blood vessels (vessel feeders). On the other hand, the mouthparts of the phlebotomine vectors of leishmaniasis are relatively short (250–350 μm) allowing penetration no deeper than the dermis (100–150 μm), which is relatively poor in blood vessels (Lewis, 1987; Brinson et al., 1993; Bates, 2007). Sand flies induce minute superficial hemorrhages by macerating skin cells and cutting dermal capillaries with their serrated mouthparts. They imbibe blood that drains into the resultant hematomas together with skin cell lysates (pool-feeding or telmophagous). *Leishmania* parasites are not blood-borne parasites per se, therefore pool-feeding makes sand flies particularly likely to ingest *Leishmania* amastigotes inhabiting resident dermal macrophages, despite the small volume of their blood meals (e.g. 0.59 μl on average for *Phlebotomus orientalis*, a vector of VL in Sudan and Ethiopia) (Seblova et al., 2013).

Host infectiousness is optimally assessed based on the infection rates of insectary-reared vectors that had fed on it (xenodiagnosis). Unfortunately, xenodiagnosis for leishmaniasis is an intricate operation encumbered by technical, logistical and ethical hurdles that preclude its mass application for screening large communities in remote localities (Hirve et al., 2016). Here we describe a novel approach for detecting *L. donovani* infections and determining the potential of humans to infect biting sand flies. We used absorbent microbiopsy (MB) devices that painlessly collect minute skin/blood samples similar in composition to sand fly blood meals. These MB devices were developed by two of the authors (T. Prow and L. Li), utilizing state-of-the-art computer modeling, feeding into subtractive and additive manufacturing techniques to generate a simple, albeit sophisticated tool for use under field conditions (Lin et al., 2013). We suggest that absorbent MBs can serve as surrogates for sand flies in the xenodiagnosis of VL. Similar MBs can potentially serve for the xenodiagnosis of other vector-borne diseases caused by skin-dwelling parasites such as onchocerciasis (river blindness).

## 2. Materials and methods

### 2.1. Study sites

The field studies were conducted in two relatively well-studied VL foci in Ethiopia where clinical, epidemiological and entomological studies have been conducted in the past (Gebre-Michael and Lane, 1996; Hailu et al., 2009; Abbasi et al., 2013). The first study site was Aba Roba (5°18′42.53″N/37°24′31.12″E), Konso district, southwestern Ethiopia. The incriminated vectors of *L. donovani* in Konso are *Phlebotomus martini* and *Phlebotomus celiae* (Gebre-Michael and Lane, 1996). The second study area was the villages around the town of Sheraro (14°23′41″N/37°46′15″E), Tahtay Adiyabo district, Tigray region, northern Ethiopia where the vector species of *L. donovani* is *Ph. orientalis* (Gebresilassie et al., 2015).

### 2.2. Ethical considerations

Informed consent was sought from all the adults recruited for the study. Consent for inclusion of young children was obtained from parents or guardians. Study procedures were approved by the ethical review committees of the Medical Faculty, Addis Ababa University, Ethiopia and the National Research Ethics Review Committee (NRERC) at the Ethiopian Ministry of Science and Technology.

### 2.3. MB devices

We evaluated two prototypes of MB devices made of 50 μm medical grade stainless steel plates (Fig. 1). The first type, MB1 (Fig. 1D), comprised two pointed outer plates and a central bifurcated one (Lin et al., 2013). The second type, MB2 (absorbent, Fig. 1D), consisted of two external plates produced by photo-etching, with a central absorbent layer made of either Polyethersulfone (PES) membrane (Supor®, Sigma–Aldrich, Australia) or Whatman (USA) paper No. 1. The cutting bits were fitted into a disposable spring-loaded plunger mechanism for accurate deployment (Fig. 1B). The MB devices were sealed in Stericlin® Tyvek® 1073 B pouches (Med-Con Pty. Ltd, Australia) and sterilized with gamma irradiation. MB samples were extracted from the arm, the nape of the neck and the cheek.

Prior to extraction of MB samples, the skin was wiped with 70% ethanol. The spring mechanism was compressed, the device was pressed against the skin, the plunger was released and the device held firmly in place for approximately 30 s to assure efficient absorption of blood and skin cell lysates.

### 2.4. Capillary blood samples

Blood samples were obtained by finger pricks (FPs) using disposable blood lancets and blotted on Whatman 3MM filter papers that were kept dry at room temperature.

### 2.5. DNA extraction

The cutting plates of MB devices were removed from the applicator immediately after use, placed in 1.5 ml micro-centrifuge tubes containing 200 μl of DNA extraction buffer and stored at room temperature.

Two paper-punch disks ($r = 3$ mm, calculated to have been saturated with approximately 20 μl of FP blood each) of Whatman No. 1 filter papers were placed in 1.5 ml micro-centrifuge tubes with 200 μl of DNA extraction buffer. Subsequent DNA extraction and purification were performed as described previously (Abbasi et al., 2013). DNA concentrations were measured using a full spectrum micro-volume UV/Vis spectrophotometer (NanoDrop 2000c, Thermo Scientific, Surrey, UK).

### 2.6. Quantitative real-time kinetoplast DNA PCR (qRT-kDNA PCR)

Real-Time hot-start PCR was performed with an Absolute Blue qPCR kit (Thermo Scientific) based on SYBR green detection using a real-time PCR thermocycler (Rotor-Gene 6000, Qiagen, Hilden, Germany). The qRT-PCR mixture contained 10 μl of the 2x concentrated absolute blue solution with 1 μM each of *Leishmania* kDNA minicircle-specific primers JW11 and JW12, and template DNA



Please cite this article in press as: Kirstein, O.D., et al. Minimally invasive microbiopsies: a novel sampling method for identifying asymptomatic, potentially infectious carriers of *Leishmania donovani*. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.02.005