UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Multiplexed real-time PCRs for the detection and differentiation of *Leishmania* utilising bisulphite conversion technology

by Ineka Gow

Thesis submitted in fulfilment of the requirements for
the degree of

Doctor of Philosophy

under the supervision of Em. Prof. John Ellis, Dr. Damien Stark, Prof. Nicholas Smith, Dr. Douglas Millar

University of Technology Sydney
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March 2023

Certificate Of Original Authorship

I, **Ineka Gow**, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Acknowledgments

I would like to thank my wonderful supervisor Emeritus Professor John Ellis, for the opportunity to undertake this incredible journey; it has been quite an honour to work as part of the Ellis research group and your expertise, patience and encouragement have been invaluable.

To my co-supervisors, Dr. Damien Stark, Professor Nick Smith and Dr. Doug Millar, thank you for all the fantastic advice, support and encouragement. I feel extremely lucky and humbled to have been guided and mentored by such an incredible group of experts.

To Dr. John Melki and all my colleagues at Genetic Signatures Ltd., you have allowed me the opportunity to complete this PhD and an incredible opportunity to grow and learn as a scientist.

To the staff at the Microbiology department at St. Vincent's Hospital, Sydney and the other members of the Ellis research group of the University of Technology Sydney thank you for all the support and assistance.

To my family, immediate and extended, thank you for all your love, support and encouragement over the years. To my two babies Elke and Ambrose, who came along on the way, the joy and happiness that you bring each and every day has sustained me through this journey.

But most of all, to Michael, my loving partner in life, to whom this thesis is dedicated, without your selfless support every step of the way, the steadfast confidence you hold in me and your ability to make me smile during the toughest PhD moments, thank you. I would not be where I am without you.

Format of Thesis

I, **Ineka Gow** declare the format of this manuscript is 'Thesis by compilation'.

Publications arising from thesis

Gow, I., Millar, D.S., Ellis, J., Melki, J., Stark, D. 2019., *Semi-Quantitative, Duplexed qPCR Assay for the Detection of Leishmania spp. Using Bisulphite Conversion Technology*. **Trop Med Infect Dis**, 4(4), 1-11.

Gow, I., Smith, N.C., Stark, D., Ellis, J. 2022., *Laboratory diagnostics for human Leishmania infections: a polymerase chain reaction-focussed review of detection and identification methods*. **Parasit Vectors**, 15(412), 1-24.

Gow, I., Smith, N., Stark, D. & Ellis, J., 2023., *Molecular Detection of Neglected Tropical Diseases: The Case for Automated Near Point-of-Care Diagnosis of Leishmaniasis.* **Am J Trop Med Hyg**, 108(1), 2-6.

Gow, I., Stark, D., Millar, D.S., Smith, N., Ellis, J., *Development of multiplexed real-time PCRs for the differentiation of Leishmania species targeting mini-exon DNA based on bisulphite technology,* under review.

Conference presentations arising from thesis

Gow, I., Stark, D., Millar, D.S., Smith, N., Ellis, J., *Development of multiplexed real-time PCRs for the differentiation of Leishmania species targeting mini-exon DNA based on bisulphite technology,* Australian Society for Parasitology Conference, Brisbane, Australia (2022).

Gow, I., Stark, D., Ellis, J., *Multiplexed qPCR panels to differentiate clinically relevant Leishmania species utilising bisulphite conversion technology,* 2nd LEAP Scientific Conference, virtual conference (2021).

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Thesis Abstract

Leishmaniasis, caused by protozoan parasites in the genus *Leishmania,* is a cause of serious morbidity and mortality around the globe but particularly in the world's poorest nations. It is for this reason that the disease and its various clinical forms is recognised as a neglected tropical disease (NTD). There are more than 20 species of *Leishmania* that cause human infection and all are transmitted by the bite of a female *Phlebotomus* or *Lutzomyia* sandfly. They can either elicit cutaneous, mucocutaneous or visceral disease; the cutaneous and mucocutaneous forms cause significant morbidity with disfiguring ulcers and resultant scarring, whilst the visceral form is fatal if left untreated. However, many human cases are asymptomatic and both humans and animals act as reservoir hosts, thereby contributing to the spread of the disease.

Aside from the devastating impact on impoverished communities, leishmaniasis is also defined as an NTD due to the relative lack of funding for research, interventions, tools, and diagnostics. The wide range of diagnostics currently used includes both traditional microbiological and serological techniques, but also molecular techniques which are not deemed appropriate for resource-constrained regions. The World Health Organisation (WHO) has identified the need for accurate and rapid diagnosis to reach elimination targets in addition to broader public health intervention programs. At the patient level, rapid detection informs dosage and case management and achieves differential diagnosis from circulating diseases with similar clinical presentations. As the gap between the need and availability of apposite *Leishmania* diagnostics currently stands, it raises the following questions: can modern diagnostics for leishmaniasis be developed commensurate to its context of endemicity; and, can the perceived inappropriateness of these molecular techniques be challenged within these contexts? These questions form the overarching focus of this thesis.

This thesis is one by compilation - consisting of one publication, one chapter in-press, two chapters under review and one conventional thesis chapter. Each of these chapters are separate studies aimed at addressing the aforementioned research questions and current gaps in knowledge. The results show that the development of a highly sensitive and specific *Leishmania* molecular diagnostic method, real-time PCR, appropriately solves these research questions. Sensitivity and specificity of 100% were observed for both the detection and the differentiation assays, against a reference method. Moreover, its adaptability in addressing specific priorities of test-of-cure and less invasive sampling was able to be achieved. A detection limit of 100 cells/mL was observed in whole peripheral blood and RNA/DNA ratios were determined in clinical sample nucleic acid. This method was developed on a platform that can easily be deployed as part of routine testing or outbreak response. Thus, it supports the discussion as to whether these methods are appropriate within the regions that need them most.

Exegesis

Leishmania is a genus of protozoan parasites, and the disease they cause, leishmaniasis, is transmitted by female sand fly bites. It is a global human disease of significance that is overrepresented in resource-poor regions. Leishmaniasis is a neglected tropical disease and the current laboratory techniques for diagnosis are insufficient, leading to poor individual and public health outcomes. The objective of this body of research, which represents a thesis by compilation, was to provide a diagnostic solution by the design and validation of real-time PCR based tests, utilising bisulphite technology, and to be appropriate for regions where this disease is endemic. The chapters of this thesis are distinct studies that address this primary, yet multi-faceted objective through literature review, methodologies, results and discussions.

Chapter 1 is a review of the literature of current laboratory-based techniques in the diagnosis of leishmaniasis, and the differentiation of the infecting species, with a focus on the PCR-based methods. The scope of *Leishmania* diagnostic methods ranges from widely-used traditional methods to emergent novel techniques, although each method and technology share drawbacks and challenges. PCR-based methods (including bisulphite-based methods) are continually moving into wider use and their advantages in terms of diagnostic performance and ease of use are becoming evident. The main findings of this chapter were the illustration of diagnostic gaps, particularly in PCR methods, which would aim to be addressed in chapters two and three.

Chapter 2 presents the development of a *Leishmania* detection system at the genus level through bisulphite conversion technology and real-time PCR. The conserved, multicopy 18S rRNA gene was targeted in a duplex assay and high specificity, high sensitivity and low detection limits were achieved when analysed alongside the reference method. The diagnostic system encompassed

target and control detection as well as the DNA extraction preceding it; all validated on semi-portable, automated platforms requiring a small laboratory footprint, that could be used in established laboratories and also deployed in outbreak scenarios. This is the first detection of *Leishmania* using bisulphite technology, and introduces its advantages of increasing sequence homology, establishing the methodology that would be used and built upon in chapters three and four.

Chapter 3 advances upon the preceding paper by differentiating clinically relevant species by multiplex detection, utilising the aforementioned bisulphite conversion-based diagnostic system. In these assays, the divergent, yet multicopy mini-exon gene target was employed and bisulphite conversion ensured the design of highly specific assays, upholding the high sensitivity and low detection limits observed in the previous study, and moreover, achieving complete concordance of species detection to the reference method. Although species within one subgenus (*Leishmania braziliensis, Leishmania panamensis, Leishmania peruviana* and *Leishmania guyanensis*) could not be distinguished, the genetic distinction of these species is contested in the literature.

Chapter 4 addresses current shortfalls in *Leishmania* diagnostics as communicated by the World Health Organisation's 2020 priorities to reach elimination targets. In order to further address these global requirements of the optimal *Leishmania* diagnostic test, technologies and assays were implemented building upon the previously validated platforms. Firstly, an assay for *Leishmania* parasite viability was established based on the Spliced Leader RNA; due to its lability and short half-life, the detection of RNA indicates transcription, and, therefore live parasites. This is particularly important in circumstances such as monitoring whether *Leishmania* carriers will act as reservoirs of disease, evaluating drug treatment efficacy and for a test-of-cure. Second, the need for less-invasive testing methods for visceral leishmaniasis

diagnosis was prioritised. *Leishmania* was detected at a clinically significant range in contrived clinical samples of whole peripheral blood spiked with *Leishmania* cells.

Chapter 5 discusses the common, but perhaps outdated perception that modern diagnostic options are not suitable for the resource-limited regions where leishmaniasis is endemic. Following WHO frameworks for diagnostic test requirements, including at various healthcare levels, the rationale that methods such as real-time PCR and automation are suitable is presented. Perceived barriers to the provision of these methods such as infrastructure, personnel and cost are increasingly being overcome with the advancement of technology and logistics. This discussion contextualises the current diagnostic landscape, emphasising that assays such as those developed in chapters two, three and four hold a place in resource-limited settings, now and into the future.

Chapter 1

Laboratory diagnostics for *Leishmania* infections: a PCR-focussed review of detection and identification methods

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Publication:

Gow, I., Smith, N.C., Stark, D., Ellis, J. 2022., *Laboratory diagnostics for human Leishmania infections: a polymerase chain reaction-focussed review of detection and identification methods*. **Parasit Vectors**, 15(412), 1-24.

Certificate:

I certify the following chapter is largely my own work although the contributions of other authors are duly recognised. The contributions of other authors are detailed as follows:

- By providing suggestions on topics to be reviewed
- By proof reading draft manuscripts
- By correcting spelling and grammatical errors in drafts
- By providing suggestion to improve writing style and language
- By providing suggestions to improve layout

Otherwise, the core composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

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Gow et al. Parasites & Vectors (2022) 15:412
https://doi.org/10.1186/s13071-022-05524-z

Parasites & Vectors

REVIEW Open Access

Laboratory diagnostics for human *Leishmania* infections: a polymerase chain reaction-focussed review of detection and identification methods

Ineka Gow[1*], Nicholas C. Smith[1], Damien Stark[2] and John Ellis[1]

Abstract

Leishmania infections span a range of clinical syndromes and impact humans from many geographic foci, but primarily the world's poorest regions. Transmitted by the bite of a female sand fly, *Leishmania* infections are increasing with human movement (due to international travel and war) as well as with shifts in vector habitat (due to climate change). Accurate diagnosis of the 20 or so species of *Leishmania* that infect humans can lead to the successful treatment of infections and, importantly, their prevention through modelling and intervention programs. A multitude of laboratory techniques for the detection of *Leishmania* have been developed over the past few decades, and although many have drawbacks, several of them show promise, particularly molecular methods like polymerase chain reaction. This review provides an overview of the methods available to diagnostic laboratories, from traditional techniques to the now-preferred molecular techniques, with an emphasis on polymerase chain reaction-based detection and typing methods.

Keywords: Leishmaniasis, Diagnostics, Laboratory, Polymerase chain reaction

Background

Among parasitic diseases, leishmaniasis is second only to malaria as a cause of human mortality [1]. Human leishmaniasis is considered a neglected tropical disease (NTD) by the World Health Organization (WHO). It is widespread, occurring in all continents except for Australia and Antarctica, but primarily impacts developing nations in the tropics. However, its endemicity in developed nations is changing due to human migration as a result of war, and expansion in sand fly habitats linked to changes in environmental factors that are often associated with climate change [2–5].

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Human leishmaniasis is caused by 20 or more species of the protozoan genus *Leishmania* (Kinetoplastida: Trypanosomatidae). These are often referred to as New World or Old World species based on their geographic localisation either in the Western Hemisphere (specifically, Mexico, Central and South America) or the Eastern Hemisphere (specifically, southern Europe, Africa, the Middle East and parts of Asia), respectively. New World species include *Leishmania infantum*, *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis*, *Leishmania peruviana*, *Leishmania lainsoni*, *Leishmania naiffi*, *Leishmania mexicana* (syn. *Leishmania pifanoi*) and *Leishmania amazonensis* (syn. *Leishmania garnhami*) [6]. Old World species include *Leishmania donovani* (syn. *Leishmania archibaldi*), *Leishmania infantum*, *Leishmania tropica* (syn. *Leishmania killicki*), *Leishmania major* and *Leishmania aethiopica*

[6, 7]. Other species that infect humans include *Leishmania shawi*, *Leishmania lindenbergi*, *Leishmania venezuelensis*, *Leishmania martiniquensis*, *Leishmania waltoni* (all in the New World), and *Leishmania arabica* and the newly described *Leishmania orientalis* (in the Old World) [7–9]. It must be acknowledged, and kept in mind, however, that the classification of *Leishmania* is problematic largely due to the use of different genetic markers in evolutionary relationship studies [10, 11]. There have been repeated calls for a consensus classification of the genus, but this has yet to be achieved [6, 12].

Humans become infected with *Leishmania* through the bite of female sand flies of the genera *Lutzomyia* (in the New World) or *Phlebotomus* (in the Old World) (Fig. 1). Transmission has also been documented through needle sharing, congenital transmission and sexually transmitted infection, albeit rarely [14–16]. Clinical presentation of leishmaniasis consists of two main forms: cutaneous leishmaniasis (CL), which includes manifestations such as mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis, disseminated leishmaniasis, leishmaniasis recidivans and post-kala-azar dermal leishmaniasis; and kala-azar or visceral leishmaniasis (VL) (Table 1). Each manifestation may be associated with certain species of *Leishmania*, although there is considerable overlap, exceptions, hybrid species and mixed infections that

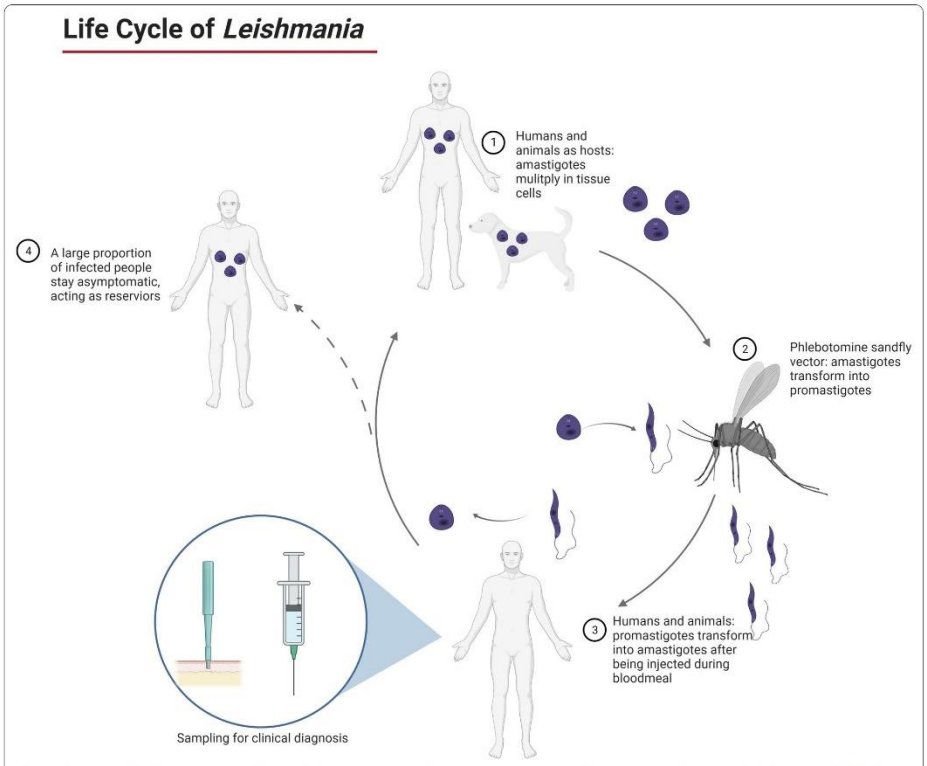

Fig. 1 Life cycle of *Leishmania* species. The sand flies inject metacyclic promastigote stages of the parasite as they take their blood meal [13]. These parasites transform into asexually reproducing amastigotes within macrophages and can affect different organs and tissues depending, in part, on the parasite species and the species and immune status of the host. The *Leishmania* species that infect humans are also found in a variety of mammalian reservoir hosts, including canids (particularly domestic dogs), rodents and marsupials [17]

Table 1 Clinical forms of leishmaniasis

Clinical form	Principal causative agents	Common symptoms and manifestations	Complications, confounding factors and prognosis
Cutaneous leishmaniasis (CL)	*Leishmania infantum, Leishmania donovani, Leishmania tropica, Leishmania major* and *Leishmania aethiopica* (Old World); *L. infantum, Leishmania braziliensis, Leishmania guyanensis, Leishmania panamensis, Leishmania peruviana, Leishmania mexicana* and *Leishmania amazonensis* (New World) [19, 36, 255]	Parasites remain localised, with patients mostly exhibiting non-healing ulcers on exposed regions of the body; a primary lesion (in New World cases) or lesions (in Old World cases) in the form of a red papule occurs 1 week to 3 months after infection, progressing into a larger plaque or nodule weeks after the development of the initial papule; an ulcer with a dark border and crusted base then forms between 1 and 6 months, occasionally paired with painless, rubbery nodules, papules or hardened masses around the site of the ulcer; surrounding lymph nodes can become enlarged and may itch, although pain is mild or absent [79, 161, 183, 254, 256, 271]	Lesions may heal spontaneously at between 1 and 36 months, leaving a discoloured scar with social and psychological consequences for the patient; complete immunity generally occurs; complications include bacterial supra-infection [257]
Mucocutaneous leishmaniasis (MCL)	*L. braziliensis*, *L. panamensis, L. guyanensis* and, occasionally, *L. major* and *L. infantum* (Old World); and *L. amazonensis* (New World) [272, 273]	Characterised by metastatic spread of parasites from the site of the sand fly bite to the upper respiratory tract mucosa, occurring concurrently with cutaneous lesions or up to 5 years after the lesions have healed; initially, reddening and ulceration around the nasal region occurs, followed by destruction of the nasal septum, pharynx and larynx and, rarely, the eyes and genitalia [24]	The disease does not heal spontaneously and healing post-treatment can leave devastating scarring with social and psychological consequences for the patient; complications include malnutrition and pneumonia [22, 260–263]
Kala-azar/visceral leishmaniasis (VL)	*L. donovani* in East Africa and the Indian subcontinent or *L. infantum* in Central and South America, Europe and North Africa [21, 32, 35, 104, 268]	Parasites spread to the liver, spleen and bone marrow from the site of the sand fly bite via macrophages travelling in the blood or lymphatic system; patients present with fever, fatigue, weakness, anorexia and enlargement of the liver and spleen; the incubation period is between 12 and 32 weeks [15, 39, 57, 136]	Complications include co-infections with human immunodeficiency virus, bacterial pneumonia, tuberculosis, dysentery; fatal if left untreated, often due to severe anaemia [258, 259]
Diffuse cutaneous leishmaniasis	*L. aethiopica*, *L. infantum* (Old World); *L. mexicana, L. amazonensis* (New World) [264, 270]	Presents as mixed lesions and plaques affecting limbs, buttocks and face due to an anergic response, usually in immunocompromised patients, producing non-ulcerative nodules that become chronic with a high parasite load [77, 264, 265]	A rare complication of cutaneous leishmaniasis; visually, very hard to distinguish from leprosy [264, 271]
Disseminated leishmaniasis	*L. braziliensis*, *L. mexicana* (New World only) [266]	Presents as mixed-type lesions on multiple sites of the body, often including the mucosal regions, and a low parasite load in skin [266]	A rare complication of cutaneous leishmaniasis [266]
Leishmaniasis recidivans	*L. braziliensis* (New World); *L. tropica* (Old World) [267]	Characterised by red papules arising from within the borders of healed cutaneous lesions and slowly progressing into chronic recurring nodules; may be a recurrence of a prior infection, occurring after months/years of dormancy; often affecting the face [18, 28, 267]	A rare complication of cutaneous leishmaniasis [24]

Table 1 (continued)

Clinical form	Principal causative agents	Common symptoms and manifestations	Complications, confounding factors and prognosis
Post-kala-azar dermal leishmaniasis	*L. donovani*, *L. infantum* [269, 274, 275]	Cases are rare and location-specific: in Africa, symptoms include a rash of papules on the face, ears and forearms, which may heal spontaneously after a few months; in India, small macules that progress into large irregular patches on chest, back, neck and both thighs and arms, before developing into soft, painless, non-ulcerating nodules on the face, ears, trunk and genitals or, sometimes, on the hands and feet [17]	A rare complication of VL; visually, very hard to distinguish from leprosy and confirmation by microscopy may be problematic due to the low parasite load associated with the condition [24]

are recognized [18, 19]. Furthermore, *Leishmania* has been shown to exhibit mosaic aneuploidy, which influences genetic diversity not only within a given species but within a single isolate [20, 21]. The clinical manifestations of *Leishmania* infection may also be affected by the presence of sand fly saliva and the host's genetic makeup and immune status [22, 23].

Despite being listed as a NTD in 2007, and being the subject of concerted global efforts for its control, the incidence of leishmaniasis remains significant, and the risk to vulnerable, mostly poor, populations remains lamentably high [24, 25]. In 2018, over 200,000 new cases were reported to the WHO, and it is now estimated that there are 1 billion people at risk of contracting *Leishmania* [26, 27]. These figures are probably underestimates, as *Leishmania* is not always a notifiable disease and treatment is not always sought due to financial or geographic constraints [28, 29]. Mortality rates are also under-reported, being confined mainly to official hospital deaths. VL has case fatality rates of between 1.5%, in Bangladesh, to 20%, in South Sudan; based on a global case fatality rate of 10%, these figures represent approximately 20,000–40,000 deaths per year [30]. In recent years (2006–2016), morbidity related to leishmaniasis, in terms of disability-adjusted life years, has risen by 12.5% for CL/MCL but decreased by 61.1% for VL [31]. Other burdens exist, such as psychological morbidity arising from the social stigma surrounding the disfiguring lesions or scarring caused by CL and MCL [17].

The importance of the detection and identification of *Leishmania*

Worldwide control of leishmaniasis is believed to be achievable, but it does present a multifactorial problem because transmission of *Leishmania* species takes place in a complex biological interplay involving a human host, a diversity of parasite species, sand fly vectors, and animal reservoirs of infection, and is affected by a number of factors, including climate change, deforestation and war. Asymptomatic infections (and paucisymptomatic infections) add a further challenge, as undiagnosed patients become unobserved reservoirs of the disease, contributing to further transmission and maintenance of leishmaniasis foci [24, 32, 33]. Mild cutaneous infection, such as *L. major* CL infection, often goes undiagnosed and untreated, especially in resource-limited settings, which increases the risk of spread to the community [34]. Asymptomatic infections have been identified in several screening studies, including those of blood donors, and some studies have found these to be more common than symptomatic infection [35–38]. This was quantified in an epidemiological survey of peripheral blood by using real-time polymerase chain reaction (PCR), where the authors were able to determine a threshold of five parasitic genomes per millilitre of blood at which asymptomatic disease progressed to symptomatic disease [39].

One particularly confounding factor for any leishmaniasis control program is that *Leishmania* infection induces a broad spectrum of disease states and the clinical presentation cannot be linked to individual species with certainty; moreover, geographic foci may harbor multiple species. Thus, diagnosis on clinical grounds alone, through physical examination and interrogation of patient travel history, is not sufficient for complete case assessment. Figure 2 shows the recommended diagnostic techniques for the various clinical forms of leishmaniasis given by the WHO, the Centers for Disease Control and Prevention, the Walter Reed Army Institute of Research, the National Reference Centre for Parasitology (Canada) guidelines, and relevant studies. For clinical case management, species-level data provide important information for educated prognosis and therapeutic decision-making. For instance, species-led treatment was found to be imperative for cutaneous leishmaniasis in Peru, as tolerance and susceptibility to antimony was dependent upon the infecting species [40]. At a national and global level, the epidemiological monitoring of individual species and their prevalence and transmission patterns guide public health responses, such as the VL elimination program launched in 2005 in India, Nepal and Bangladesh [41]. Moreover, tracking the presence of exotic species, e.g. *L. tropica* discovered in a returned traveller to Mexico, allows local health authorities to enact sanitary measures (such as indoor residual spraying) [42]. Consequently, a globally applicable technique that captures both genus- and species-level data has been suggested, to mitigate assay design complications, such as intraspecies heterogeneity and gene target copy number variations, and to detect asymptomatic and multiple-organism infections (including co-infection with multiple *Leishmania* species) [6]. Seven criteria have been proposed for species-typing tools: discrimination of species, global applicability, sensitivity, specificity, standardisation, applicability for particular settings, and validation [6].

Additionally, test availability and timeliness affect opportune treatment. Shortages of diagnostic materials and a long period of time needed to deliver diagnostic results impede patient outcomes [51, 52]. These limitations result in a lack of useful information for medical decision-making. Furthermore, many current diagnostic tests do not have the resolving power required to provide molecular epidemiological data for public health policy makers. Accurate and qualitative detection and identification of a *Leishmania* infection are, therefore, key to the diagnosis of leishmaniasis, and should be at the heart of any successful control program. This can lead to the

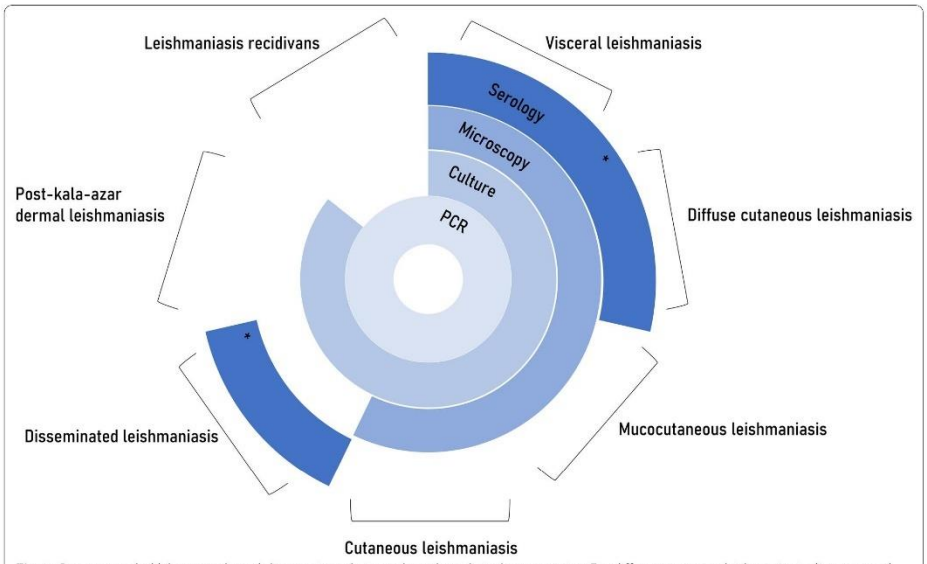

Fig. 2 Recommended laboratory-based diagnostic techniques based on clinical presentation. For diffuse cutaneous leishmaniasis, disseminated leishmaniasis and leishmaniasis recidivans, where recommendations are not available from a public health organisation or reference laboratory, guidance is taken from relevant studies [43–50]. The asterisk indicates the rK39 rapid immunochromatographic test kit only. *PCR* Polymerase chain reaction

achievement of early and improved treatment regimens, implementation of control measures leading to better patient outcomes, and a reduction of sustained reservoirs in the transmission cycle [53]. Additionally, optimised diagnostic tools would be important additions to the One Health approach for the control of leishmaniasis [54]. The comparative strengths and weaknesses of existing diagnostic approaches, with a focus on the widely used and robust PCR-based methods that address many of the requirements for an optimal diagnostic test, are reviewed below.

Methods for the detection and diagnosis of leishmaniasis

Conventional detection methods

Detection via microscopy, histology, culture and serology, and other methods, are commonly utilised by laboratories globally, and especially in endemic, resource-poor nations [55].

For microscopic detection of parasites, direct aspirate smears are used, often with staining; amastigotes appear round in shape and 2–4 µm in diameter, and cultured promastigotes range between 15 and 25 µm in length and are ellipsoid to slender in shape [56–58]. Staining methods help to clarify the cells and Giemsa and Leishman stains (both derivatives of Romanowsky stain) are the most widely used for this [59]. Upon staining, *Leishmania* amastigotes are generally observed within macrophages and have a pale blue cytoplasm, red nucleus and adjacent purple-pink-stained kinetoplasts [60, 61]. Parasitic load may be estimated using the modified Ridley's parasitic index (Table 2), which quantifies the number of amastigotes [62]. The sensitivity of detection varies (54.0–96.4%),

Table 2 Modified Ridley's parasitic index [76]

Parasitic index	Number of amastigotes per standard section
1+	≥ 1
2+	≥ 10
3+	≥ 100
4+	≥ 1000
5+	≥ 10,000
6+	≥ 100,000

and specificity as low as 46.0% has been reported, depending upon the primary sample taken, the quality of the reagent used for staining and technical expertise [43, 63–68]. The stage of infection can also greatly affect sensitivity; in CL, amastigote levels decrease as infection progresses, including in MCL infection, whereas in VL, parasitic load increases as infection becomes chronic [69–71]. Generally, in VL, the highest sensitivity when using microscopy is for more invasive specimens, such as those seen in splenic aspirates [70, 72, 73]. Microscopy is of use at the genus level, but cannot be used for species differentiation as all species of *Leishmania* are morphologically very similar [74]. Recently, machine learning has been incorporated into microscopical examination for leishmaniasis, with sensitivity and specificity of 83% and 35%, respectively, although efficacy and speed are dependent on image quality and the particular algorithm employed [75].

Histological examination for CL cases uses 4- to 5-µm tissue sections, stained with Giemsa or haematoxylin and eosin stains, and fixation to reveal histiocytes containing intracellular amastigotes, often near the epidermis [77]. The marquee sign, where organisms are located around the periphery of the dermal macrophage, is regarded as a typical characteristic of leishmaniasis [78]. Histological examination does, however, depend upon the disease stage, since the number of amastigotes decreases as CL progresses until they are undetectable [62]. Indeed, it is considered the least sensitive diagnostic method, with sensitivities of between 42.0% and 70.0%, although 100% specificity has been reported [79–81]. Furthermore, *Leishmania* cells may be mistakenly identified in histological sections as *Toxoplasma gondii*, *Mycobacterium leprae*, fungi, including *Histoplasma*, or even artifacts, and differential diagnosis requires alternative stains [81–84]. Histological examination is less used in VL, where clusters of histiocytes, amastigote presence or morphological changes may be observed [85]. Amastigotes are often unevenly distributed, as sections are of varying thickness, which results in lengthy analysis [86].

Culture of *Leishmania* promastigotes is a useful tool for increasing the sensitivity of downstream detection and identification by microscopy or molecular applications [87]. A variety of semi-solid, liquid or biphasic media are used to culture promastigotes, including sloppy Evans, Novy-MacNeil-Nicole (the reference medium for isolation), Tobie's, Schneider's *Drosophila* medium, Senekjie's, Medium 199, RPMI 1640, Grace's insect medium, brain–heart infusion medium, blood agar (including rabbit blood) and chocolate agar [88–90]. These media generate growth in differing ways; of note, Tobie's medium encourages the transformation of amastigotes to promastigotes, and cell density is increased on Grace's medium [73]. Novel culture methods include the microcapillary culture method, which concentrates the sample in capillary tubes, or liquid (single-phase) media, used to create the microaerophilic conditions that are optimal for amastigote transformation into promastigotes [91]. One study found an improvement from 69.2% sensitivity with traditional culture methods to 92.3% sensitivity with the newer microcapillary method, with a minimal change in specificity (98.9% vs 97.8%, respectively) [92]. It can take days to weeks to produce a result by culture methods, which are also expensive and labour-intensive to set up [86]. Furthermore, the distribution, transport and storage of cultures and culture material, including antibiotics used to prevent contamination from other microorganisms, make it an impractical method in many clinical settings [93, 94].

Serological methods test for *Leishmania* by detecting antigens or antileishmanial antibodies in the blood or, sometimes, urine or saliva. Antibody detection is primarily used in cases of VL rather than for CL, as the humoral response to the latter is poor [95]. Many antigens have been assessed for antibody detection, and recombinant antigens are preferred over natural antigens, as the latter often cause problems such as cross-reactivity and resultant false-positive results [61, 96]. Some immunoassays, such as enzyme-linked immunosorbent assay (ELISA) and western blot, require relatively sophisticated and expensive equipment and materials, which renders them less useful in endemic regions in poorer countries, despite their good sensitivities and robustness [97, 98]. However, ELISA is widely used as a serological method in countries where leishmaniasis is endemic or non-endemic, and it can provide detailed information on antibody responses [99, 100]. The sensitivity of ELISA depends on the antigen used to capture a specific antibody, with the commonly used crude soluble antigen (CSA), for example, providing sensitivities of between 80 and 100%. However, cross-reactions with trypanosomiasis, tuberculosis and toxoplasmosis have been observed with this method [101]. Flow cytometry for serological *Leishmania* detection is a recent development and can quantify antibodies rapidly with lower sample input volumes than other serological tests [102]. Despite the range of tests available and the ability of some of these tests to be used in the field, serological assays share the same limitations. The antigen load may not be sufficient in early infection for detection; conversely, because antigen-specific antibodies persist long after cure, active relapsed disease cannot always be discerned [61, 103]. Furthermore, these tests are less accurate for immunocompromised patients, and cross-reactivity is reported with other diseases endemic to *Leishmania*-affected areas, including Chagas disease [53, 88, 104–108].

Simplified assays have been introduced, such as the rK39 immunochromatographic assay (ICT), the direct agglutination test, the indirect fluorescent antibody test and latex agglutination testing [109]. They include the Food and Drug Administration-approved ICTs, CL Detect, based on the peroxiredoxin antigens, and Kalazar Detect, based on a 39 amino acid repeat recombinant leishmanial antigen, rK39. Although the rk39 ICT is widely used, its sensitivity has been found to be higher in some regions, with studies carried out in Southeast Asian countries and the Indian subcontinent documenting higher sensitivities than those undertaken in East Africa and Brazil [110–112]. Furthermore, because antibodies are present in asymptomatic individuals and remain present for several years after cure, antibody tests must be used in conjunction with strictly standardised clinical case definition (i.e., more than 2 weeks of fever, weight loss and splenomegaly) for VL diagnosis [24]. These limitations must be considered in a modern diagnostic setting, hence, serological methods are being reassessed as principal diagnostic options [86, 113].

Other, lesser-used, methods include the leishmanin (or Montenegro) skin test. In CL, this test can detect past and active cases with high sensitivity, whereas in active VL, the test shows negative as patients are anergic, so it is used in screening studies for an indication of past exposure only [57, 114]. It involves intradermal injection of antigen, whereby induration of an area of the skin of 5 mm or greater is considered a positive test result; sensitivity and specificity with this cut-off point have been reported as 97.4% and 93.9%, respectively [15, 115, 116]. When promastigote levels are too low for culture growth, xenodiagnosis can be used to detect *Leishmania*, through inoculation of the footpad of a hamster with a test sample; however, this approach is time-consuming and involves euthanasia of the hamster [56, 117, 118].

DNA-based detection methods

In DNA-based detection of *Leishmania* a multitude of genomic targets are used which vary in sensitivity and are influenced largely by the target's copy number in the genome of the organism. Table 3 summarises the targets that have been investigated for diagnostics. The design of molecular diagnostics is complex, however, and aside from the copy number of a chosen target, which is selected to increase the sensitivity of a given assay (for instance, the 18S rRNA gene), other attributes are sought in molecular assay design [119, 120]. For detection to the subgenus, species complex or species level, selection of gene targets exhibiting increased divergence may necessitate a loss of assay sensitivity by using a target with fewer copy numbers per cell (such as the mini-exon gene) [121]. For instance, polymorphisms, copy number variation and high copy number are attractive features of the kinetoplast DNA (kDNA) minicircle, whereas single copy genes, despite their decreased sensitivity, may be chosen for their stability or to normalise minicircle copy numbers [122, 123]. Moreover, detection of multiple species distinctly and concurrently requires multiplex PCR assays, and designing a single PCR cycling protocol to suit each primer pair can present assay constraints. The use of the novel bisulphite-conversion technique can mitigate such limitations [124].

DNA-based methods represent a new era in clinical *Leishmania* diagnostics, where the limitations of previous detection methods have been overcome in terms of sensitivity, specificity, rapidity, ease of use and access in endemic settings. Similarly, the prices of molecular methods are decreasing globally; Table 4 illustrates the varying costs associated with *Leishmania* diagnostics in low income and upper-middle income nations [125–128]. The costs associated with molecular methods are comparable to those of other detection methods; however, the methodologies used to collect the costing data varied between studies. Some studies compiled comprehensive costings, including that of the healthcare setting that the test may be performed in, to the basic supplier cost of the kit [125, 126]. Despite their differences, these cost analyses were performed at similar times, and their costing conclusions are similar. These advances have also provided new data on frequencies of asymptomatic carriage, and have been used in the monitoring of specific geographic disease burden and to measure the outcomes of intervention programs [109]. Techniques such as real-time PCR can give more information on parasite load and responsiveness to therapy than other methods [122]. It can be used to identify asymptomatic patients who carry the infection, or infected patients before the onset of symptoms, which is important for the development of control measures and for blood donor monitoring [129, 130].

DNA may be acquired from a vast range of clinical specimens, and each approach has varying advantages in terms of diagnostic sensitivity and specificity, ease of collection, transport and storage and invasiveness. For CL, the punch biopsy is the most commonly performed diagnostic procedure, but less invasive sampling can be achieved by using skin scrapings, fine needle aspiration and swabs, although sensitivity is sacrificed with these methods [131–134]. For post-kala-azar dermal leishmaniasis, split skin smears and skin biopsies are most commonly used [135]. In VL, splenic, bone marrow or lymph aspiration are used, and sensitivity increases with the invasive nature of the sampling method (93–99% for spleen, 53–86% for bone marrow, and 53–65% for lymph [136]). Specimens obtained from less invasive

Table 3 DNA targets investigated for the detection of *Leishmania* species in DNA-based methods

Gene	Location	Number of copies[a]	References
6-Phosphogluconate dehydrogenase (*6pgd*)	Chromosomal DNA	S	[276]
18S rRNA	Chromosomal DNA	M (200)	[124]
7SL RNA	Chromosomal DNA	M	[277]
A2 (5′A2 rel, 3′A2 rel, internal A2 rel)	Chromosomal DNA	S (CL)/M (VL)	[278]
Amino acid permease 3 (AAP3)	Chromosomal DNA	M	[279]
Calmodium intergenic spacer	Chromosomal DNA	M	[280]
Casein kinase	Chromosomal DNA	S	[281]
Catalytic subunit of DNA polymerase α (POLA)	Chromosomal DNA	S	[282]
Chitinase	Chromosomal DNA	S	[283]
Cysteine protease A (*cpA*)	Chromosomal DNA	S	[230, 284]
Cysteine protease B (*cpB*)	Chromosomal DNA	M	[230, 285]
Elongation factor-1α (EF-1α)	Chromosomal DNA	S	[286]
Glucose-6-phosphate dehydrogenase (*g6pd*)	Chromosomal DNA	S	[41]
Glucose phosphate isomerase (*gpi*)	Chromosomal DNA	S	[287]
Glyceraldehyde-3-phosphate dehydrogenase (GADPH)	Chromosomal DNA	M	[288]
Heat-shock proteins (HSP)—*hsp10, hsp40, hsp60, hsp70* (M 1–15), *hsp83, hsp90, hsp100, hsp110*; and small HSPs—*hsp20* (S) and *hsp23* (S)	Chromosomal DNA	S/M	[289]
Histones: H2A, H2B, H3, and H4) and linker histones (H1 and H5)	Chromosomal DNA	M	[290]
Hydrophilic acylated surface protein A and B (HASPA/HASPB)	Chromosomal DNA	S	[291]
Intergenic spacer (*igs*) rRNA	Chromosomal DNA	M	[66]
Internal transcribed spacer 1 (ITS1)	Chromosomal DNA	M	[232, 292]
Internal transcribed spacer 2 (ITS2)	Chromosomal DNA	M (50–350)	[232, 293]
Iso-citrate dehydrogenase (*icd*)	Chromosomal DNA	S	[41]
Large subunit rRNA (5.8S, 5S and 28S rRNA)	Chromosomal DNA	M	[232]
Leishmania-activated C-kinase antigen (LACK) gene	Chromosomal DNA	M (2)	[235]
Lipophosphoglycans (*lpg*)	Chromosomal DNA	S	[294]
Macrophage migration inhibitory factor (*mif*)	Chromosomal DNA	S	[232, 292, 295]
Major surface protease (*msp*)/glycoprotein 63 (*gp63*)/leishmanolysin	Chromosomal DNA	M (7–70)	[232, 293, 296]
Mannose phosphate isomerase (*mpi*)	Chromosomal DNA	S	[133]
Meta1/2	Chromosomal DNA	S/ M (3)	[297]
Mini-exon [or spliced leader (SL) RNA)	Chromosomal DNA	M (50–650)	[298]
Mitogen-activated protein kinase (MAPK): MAPK2, MAPK3, MAPK4, MAPK5 and MAPK7	Chromosomal DNA	S	[218, 296]
MSP associated gene (*mag*)	Chromosomal DNA	M (18)	[282]
N-acetylglucosamine-1-phosphate transferase (NAGT)	Chromosomal DNA	S	[289, 299]
Pteridine reductase 1 (PTR1)	Chromosomal DNA	S	[300, 301]
Repetitive nuclear DNA sequences (REPL)	Chromosomal DNA	M	[282, 302]
RNA polymerase II largest subunit (RPOIII.S)	Chromosomal DNA	S	[299, 303]
SIDER repeat	Chromosomal DNA	M	[282]
Small hydrophilic endoplasmic reticulum-associated protein (SHERPs)	Chromosomal DNA	S	[304]
Splice leader associated retrotransposons (SLACS)	Chromosomal DNA	M	[282]
Telomeric sequences	Chromosomal DNA	M	[305]
Tryparedoxin peroxidase	Chromosomal DNA	M (3)	[306]
Tubulins: alpha, beta, gamma, zeta and epsilon tubulin	Chromosomal DNA	M	[218, 282]
Triose-phosphate isomerase (TIM)	Chromosomal DNA	M (2)	[278, 307]
Topo isomerase II	Chromosomal DNA	S	[286, 308]
12S, 9S	Non-chromosomal DNA	M	[295, 309]
Conserved minicircle region (CSB-I, CSB-II and CSB-III)	Non-chromosomal DNA	M (10,000)	[162, 276]
Cytochrome oxidase (CO) I, II and III	Non-chromosomal DNA	M	[290, 310]
Cytochrome b (*cytb*)	Non-chromosomal DNA	M	[282, 311]
Maxicircle divergent region (DR)	Non-chromosomal DNA	M	[133, 312]
Variable minicircle region	Non-chromosomal DNA	M (10,000)	[303, 313]

rRNA Ribosomal RNA

[a] Single copy (*S*) or multi-copy (*M*) (approximate number, if available)

Table 4 Comparison of costs associated with *Leishmania* diagnostics

Country	World Bank income classification	Presentation	Costs (in parentheses)	Associated costs	Year for which costs were determined	References
Colombia	Upper-middle income	MCL	Biopsy + culture + stains + IFA + MST (USD 172.40); biopsy + culture + stains + IFA (USD 162.57); biopsy + stains + IFA (USD 128.91); PCR-mini-exon (USD 128.77); PCR-kDNA (USD 128.77)	Direct	2015	[128]
Afghanistan	Low income	MCL, CL	Microscopy (USD 53.79); RDT (USD 53.91); LAMP (USD 60.18)	Direct and indirect	2016	[127]
Iran	Upper-middle income	CL	PCR–RFLP (USD 5.72); PCR sequencing (USD 11.20); PCR -HRM (USD 4.46)	Basic kit tariff	2015	[126]
Brazil	Upper-middle income	VL	IT LEISH (USD 6.57); DAT-LPC (USD 4.92); Kalazar Detect (USD 7.45); IFAT (USD 11.39); bone marrow aspirate (ambulatory setting) (USD 27.10); PCR USD 32.72	Direct	2016	[125]

IFA Indirect immunofluorescence assay, *MST* Montenegro skin test, *RDT* CL Detect Rapid Test, *LAMP* (loop-mediated isothermal amplification) Loopamp *Leishmania* Detection Kit, *DAT* direct agglutination test, *PCR* polymerase chain reaction, *RFLP* restriction fragment length polymorphism; for other abbreviations, see Table 1

procedures, e.g., the taking of peripheral blood, are being explored for VL testing. Testing of peripheral blood by PCR is associated with a vast range of reported sensitivities, 62—93.2%, depending on the timing of sample collection during the infection process, or the fact that some *Leishmania* species may circulate at lower levels in the peripheral blood [137, 138]. The use of peripheral blood is also being explored as a diagnostic option for CL, and a limit of detection of 0.1 parasites per reaction for *Leishmania* (*Viannia*) spp. parasites has been achieved [139]. Optimal sampling, storage and transport to a receiving laboratory (such as the Centres for Disease Control and Prevention, US, or Fiocruz, Brazil) is critical. Before detection, DNA must first be extracted and purified; this is often performed by using commercial kits, such as the silica-based DNeasy Blood and Tissue Kit (Qiagen, Germany), NucliSENS easyMAG system (BioMerieux, France) or via in-house extraction protocols based on phenol extraction and ethanol precipitation [36, 140, 141]. Some rapid extraction methods have been developed recently, e.g., SpeedXtract (Qiagen, Hilden, Germany), which greatly reduce the time to test result [142]. These methods may be performed manually or through the use of automated systems, as discussed below.

Standardisation of protocols and quality control for assays that are used to detect *Leishmania* are important, particularly for molecular techniques [143]. Only few studies have compared sampling, extraction, gene target choice and primer design, and some of the findings differ from one report to an other [55]. These inconsistencies can be limited if experiments incorporate controls, which is of particular importance in settings where re-testing is expensive or the number of specimens limited. Poor DNA recovery due to losses during extraction and degradation during storage was determined by the addition of both an external and internal control to a conventional PCR (cPCR) assay for *Leishmania* [143]; DNA recovery was poor for 15.1% of samples, and a reliable result was not produced for up to 1/6 of the samples [143]. Furthermore, few multi-site studies have been undertaken to validate protocols and examine their reproducibility. An endogenous extraction control used in the measurement of a host sequence can help to account for sample quality and extraction efficacy—two major issues associated with PCR methods—and may also be used to normalise parasite load [122, 143]. For this, an exogenous internal control is spiked into a sample at a known concentration, and sample inhibition is indicated if the control is not detected or is detected at lower levels than expected. This is especially useful for potential inhibitors in samples, such as in peripheral blood; a human β-actin gene was used in a real-time PCR assay to control for this [144]. Other controls that may be included are external positive controls that are used to assess the performance of the PCR, negative template control for PCR contamination, or a negative process control for contamination of the extraction process. Laboratories should also enrol in some form of an external quality control testing program, such as the Pan American HealthOrganization's Regional External Quality Assessment Program (developed for microscopic diagnosis), as an indicator of performance [55, 145].

A molecular technique that can be easily used in a resource-limited setting is nucleic acid sequence-based amplification (NASBA), which is based on the isothermal amplification of nucleic acids by enzymatic action. However, NASBA is prone to contamination, as it is not a closed tube system, which potentially leads to false–positive results. It can be used as a quantitative test, targeting RNA in the DNA background, with one method targeting 18S rDNA showing a sensitivity of 79.8% and specificity of 100% [146]. Quantitative NASBA can be combined with electrochemiluminescence, although this is more expensive and time-consuming; however, as the reaction includes a fluorescent beacon, it may be used in a real-time, closed tube format [147]. Loop-mediated isothermal amplification is another alternative to PCR that may be used in an endemic setting, as only basic equipment is required, with no need for a thermal cycler, and a total amplification time of about 40 min [148]. It involves amplification in a water bath and a visible colour change, which can be detected by the naked eye or under blue light, and more recently, in real-time by fluorimetry [149–151]. Sensitivity of this technique has been reported as 80–90% with specificities of 94–100% for human *Leishmania* diagnosis [152]. Additionally, as it is a closed tube test there is no need for post-amplification handling, thus the risk of laboratory contamination is low [153]. Recombinase polymerase amplification (RPA) and recombinase-aided amplification (RAA) are isothermal amplifications wherein recombinase enzymes and proteins avoid the need for temperature cycling as used in PCR methods [154, 155]. In RPA, the recombinase is derived from a phage, whereas in RAA, it is derived from bacteria and/or fungi. RPA was paired with a rapid extraction method to detect *L. donovani* in two studies [142, 156]; the resultant detection systems were rapid, mobile and avoided the need for refrigerated reagents. The kDNAminicircle target was used in both assays, which had sensitivities and specificities of 100% and 100% [142] and 65.5% and 100% [156], respectively. However, there are challenges in the use of RPA and RAA, as robust design guidelines have yet to be published for either method, and both are time-consuming, labour intensive and expensive.

Polymerase chain reaction-based detection methods

The use of polymerase chain reaction (PCR) as a molecular technique for *Leishmania* detection, in which purified nucleic acids of the pathogen are amplified, is becoming more widespread. A PCR product may be detected at the end of amplification (cPCR) by gel electrophoresis, amplified further before detection (nested PCR) or detected as amplification occurs (real-time PCR) [157–159]. PCR has the best-reported sensitivities and specificities of all the diagnostic methods, and has been suggested as the future gold standard for *Leishmania* detection [123, 132, 160, 161]. One systematic review and meta-analysis reported sensitivities of up to 100% and specificities up to 100% [136]. Another systematic review assessing assays designed for New World parasitic species regularly found limits of detection of less than one copy [119]. Several commercially available kits based on PCR amplification and detection of *Leishmania* genes have been developed and are given in Table 5. PCR performance depends on certain factors, such as the nucleic acid extraction method employed, the type of sample, the copy number of the gene target and the design of the primers that target these [53, 109]. Thus, in-house PCR methods, which are developed and used widely, have great differences in the types of DNA targets used, primer and probe design and PCR cycling protocol [129]. Technology transfer from the research and development phase does not always occur, and as these types of PCR assays are far from standardised, data comparison between laboratories is compromised [109, 162]. More recently, PCR has been used to monitor a subject of growing concern in *Leishmania* infections, particularly in endemic regions: the relapse of disease or resistance to chemotherapy. Both issues present major challenges for the control of leishmaniasis [163]. A PCR-based study that monitored parasite load in VL showed that the presence of 10 parasites/mL of blood after treatment indicated relapse, thus gave useful information for disease prognosis [164]. Four single nucleotide polymorphisms of cysteine protease B gene were identified and indicated resistance to a widely used drug, amphotericin-B; thus, detection of these could be incorporated into a PCR assay [165]. These techniques have yet to be standardised, thus are not widely available in clinical settings, but have great potential to aid public health responses [166].

cPCR: In this method, DNA is amplified using a thermal cycler, amplicons are separated by electrophoresis due to their molecular weight and detected by staining (usually ethidium bromide) and ultraviolet light (via a transilluminator) [167, 168]. This method can be time consuming, requires an array of equipment and, as the PCR tube containing amplicons must be opened for electrophoresis, is associated with a risk of contamination [169]. Clinical sensitivity and specificity of up to 100% each have been achieved for lesion samples, with detection of as low as 0.01–0.1 pg of cultured *Leishmania* promastigote DNA [170].

Nested PCR: This method is used to overcome poor sensitivity and specificity [171, 172]. Two sequential PCRs are used: first, an outer set of primers is used to amplify the target gene (first round), then the amplicons of this PCR are re-amplified with a set of inner primers

Table 5 Commercially available DNA-based diagnostic kits for detection of *Leishmania*

Product	Supplier	Technology	Gene	Reported sensitivity	Species detected	Regulatory approval (agency)
EasyScreen *Leishmania* Detection Kit	Genetic Signatures	qPCR	18S rRNA	10 Copies/PCR	Pan-*Leishmania*	No
Genesig*Leishmania* (all species)	Primer Design	qPCR	*cytb*	100 Copies/PCR	Pan-*Leishmania*	No
Leishmania infantum and *Leishmania donovani* PCR Kit	MyBioSource	qPCR	DNA pol I protein B	100 Copies/PCR	*L. infantum* and *L. donovani*	No
Leishmania OligoC-TesT	Coris BioConcept	NASBA-OC	18S ribosomal	1 Parasite/PCR	Pan-*Leishmania*	No
Leishmania major PCR Kit	MyBioSource	qPCR	ND1	100 Copies/PCR	*L. major*	No
Leishmania sp. PCR Detection Kit	BioKits	cPCR	NR	20 Copies/mL	Pan-*Leishmania*	No
Leishmania tropica PCR Kit	MyBioSource	qPCR	GP63	100 Copies/PCR	*L. tropica*	No
Loopamp *Leishmania* Detection Kit	Eiken	LAMP	18S rRNA/kDNA minicircle	NR	Pan-*Leishmania*	No
SMART Leish[a]	Cepheid/WRAIR	qPCR	16S rRNA/GPI gene	4 Copies/PCR	CL causative species	Yes (FDA)
STAT-NAT *Leishmania* spp.	Sentinel Diagnostics	qPCR	NR	NR	Pan-*Leishmania*	No

NR Not recorded, *FDA* Food and Drug Administration (USA), *qPCR* real-time PCR, *NASBA-OC* nucleic acid sequence-based amplification-oligochromatography, *WRAIR* Walter Reed Army Institute of Research, *cPCR* conventional PCR, *kDNA* kinetoplast DNA; for other abbreviations, see Table 1

[a] The SMART Leish Kit is intended for use only in US Department of Defense laboratories

(second round) [173]. The disadvantages of this approach are that the use of two PCRs is more time consuming and requires more reagent, and since the amplicon tube is opened for the second PCR, it is an open system, which poses a contamination risk [174]. Encouragingly, however, 100% sensitivity and 100% specificity were reported for a recently developed Leishmania spp.-specific modified version of a nested PCR that was created to reduce carryover and cross-contamination [175].

Real-time PCR: This method, in which fluorescent dyes give a visual indication of amplification as the reaction occurs, is a more recent advance than cPCR [122, 176]. Although limiting in some settings due to the equipment and expertise necessary, real-time PCR is faster than cPCR and is a closed system, so contamination risk is reduced [147, 177]. Real-time PCR is generally used with intercalating dyes, e.g. SYBR green due to its lower cost, but this method is prone to false–positives as it will visualise any amplified double-stranded DNA [178]. Use of probe-based real-time PCR increases specificity, thus avoids this issue, and targets can be multiplexed, whereby multiple assays occur in the same reaction, which saves reagents and time and increases throughput [179]. Real-time PCR was compared to cPCR, and higher sensitivity and specificity, 93.9% and 100%, respectively, for the former was reported compared to two cPCR assays tested (75.6% and 100% for kDNA, and 53.7% and 88.8% for ITS1, respectively) [180]. Higher sensitivity, specificity and reproducibility of real-time PCR have also been reported in other studies [181–184]. For example, using peripheral buffy coat from cases of VL, 100% sensitivity and 100% specificity were achieved with real-time PCR [103, 129, 185]. The technique can give data on parasite load, which in turn provides information for prognosis and treatment, and is useful for epidemiological studies [186, 187]. Bisulphite modification is a method for reducing the complexity of the genome prior to application of PCR that has been adapted to *Leishmania* detection in real-time PCR [124, 188]. This assay achieved an analytical sensitivity of 10 genomic copies per real-time PCR reaction, and clinical sensitivity of 97.0% and specificity of 100.0%. Through treatment with sodium bisulphite, cytosine is converted to uracil and ultimately thymine during the first round of PCR. This causes the genomes of subtypes to become more similar to each other, making genus-level primer and probe design possible for highly polymorphic gene targets [189]. The resulting simplified genome enables the design of simplified primers with unique characteristics, and the consequently fewer mismatches result in a similar melting temperature (Tm), allowing for the design of longer oligonucleotides, thereby increasing the specificity of the assay. Additionally, proximate Tms between species-level oligonucleotides can be achieved, making multiplexing more efficient, as a uniform PCR cycling protocol can be easily designated [124].

Droplet digital PCR: The advent of this method has enabled the absolute quantitative measurement of target DNA, negating the need for calibration curves in PCR assays [190]. DNA molecules are partitioned into tens of thousands of replicate PCR reactions, and amplification to endpoint PCR occurs, at which point each "droplet" has template or no template present [191]. Due to the vast numbers of binary (positive or negative) results, the number of target DNA molecules can be calculated precisely. A droplet digital PCR was developed based on 18S rDNA and validated for seven *Leishmania* species; however, despite the accurate quantification of DNA, the assay was marginally less sensitive and specific than an equivalent real-time PCR assay (84.0% for the former vs 85.0% for the latter) [192]. Moreover, the authors reported that the cost of droplet digital PCR is three times that of real-time PCR, thus is not suitable for the routine diagnosis of *Leishmania*.

Methods for the identification of species of *Leishmania*

Differentiation of *Leishmania* species to discriminate the species, or group of species, that cause disease is important, both clinically and epidemiologically, for disease prognosis, determining therapeutic options and surveillance of populations [122, 193]. As in *Leishmania* detection, no single differentiation method is considered to be a gold standard, although several techniques have been proposed, including PCR, multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) [122, 133, 193–198]. The lack of a definitive gold standard for species typing may be attributed to inherent issues related to a lack of standardisation, particularly with regard to the interpretation of the typed result. This pitfall is complicated by the closely related *Leishmania* species and inter-species hybrids that exist, and the novel species that continue to be discovered [199, 200]. Moreover, methods of interpretation differ between laboratories and, if species assignment is based on centralised programs like BLAST, results can be dependent upon the evaluation of resultant similarity scores [201]. Despite this, multiple robust methods exist and the results obtained with their use continue to strengthen the molecular epidemiological, taxonomic and clinical databases, even if these methods are not yet optimal for routine uses.

Multilocus studies are now used in preference to single locus analysis for population-wide studies as they achieve better resolution [129]. By combining genomic targets in parallel, multilocus schemes capture genetic relationships that may be missed by one genetic locus, which is particularly advantageous in intraspecific variation studies or for species typing within species complexes due to the amount of information gathered [6]. MLEE distinguishes between organisms through electrophoresis of enzymes, and is regarded by some as the gold standard for *Leishmania* typing and taxonomic studies; information acquired using MLEE led to the development of the first phylogenetic trees of *Leishmania* [18, 202, 203]. Differences in enzyme mobilities are due to differences in their protein structures, which comprise different amino acids, and lead to the creation of banding patterns from which zymodemes (populations with similar isoenzyme patterns) can be assigned [24]. MLEE is laborious as it requires a large volume of cell culture and can take 1–2 months to produce results, which cannot be compared, with confidence, between laboratories; e.g., different enzyme panels are used in Europe and South America [204, 205]. Furthermore, one zymodeme, MON-1, for *L. infantum*, the causative agent of most cases of VL in the Mediterranean Basin and South America, was shown to be heterogeneous and polymorphic [129].

DNA-based identification methods

MLST, which involves DNA sequencing sections of defined housekeeping genes (usually seven or more) and the many allelic combinations produced, results in unambiguous characterisation of isolates, giving both inter- and intra-species information on heterogeneity [206, 207]. This method is considered so powerful it has also been proposed as the new gold standard for taxonomic determination of *Leishmania* [208]. MLST has high reproducibility and can be compared between laboratories; however, it is technically demanding [209].

Like MLST, multilocus microsatellite typing (MLMT) uses co-dominant markers, and because of the relatively high mutation rate of microsatellites, comparison of closely related organisms is possible [209, 210]. It works by the amplification of repeat sequences found in microsatellites, where polymorphisms in the copy number of repeats define the type assigned [211]. For instance, MLMT has been used to analyse *L. donovani* strains. In one study, the identification of heterogeneous genotypes by MLMT negated the usefulness of MLEE determining genetic relationships in zymodeme MON-37. Not only were the isolates genetically diverse but, geographically, they were spread globally, leading the authors to surmise that the discriminatory power of MLMT adds depth to both diagnostic and population genetic studies [212].

DNA sequencing is based on the classic Sanger sequencing (chain termination) method and, more recently, next generation sequencing (NGS), both of which identify the precise order of nucleotide bases in a targeted DNA locus [213]. DNA sequencing provides important information for genetic, clinical and epidemiological studies. For instance, gene sequence analysis

has been used to detect *Leishmania* hybrids using the cytochrome b gene, to study differences in genetic composition between healed and non-healed patients using the ITS1, 7SL RNA and heat-shock protein 70 regions, and to illuminate the diversity of kDNA minicircle classes [213–215]. It is viewed currently as an impractical technique for endemic areas, as it is technically demanding, although simplified techniques such as nanopore sequencing are being developed [198, 216].

NGS involves the extremely high-throughput sequencing of nucleotides in parallel [217]. It provides deep genome sequencing and whole genome sequencing (WGS) of organisms, and provides population-level data in a clinical context, although it is too costly for routine typing [218, 219]. The first complete genome sequence of any *Leishmania* parasite (i.e., *L. major*) was completed in 2005 and, since then, the complete genomes of *L. mexicana*, *L. tropica*, *L. amazonensis*, *L. donovani*, *L. infantum*, *L. panamensis*, *L. braziliensis*, *L. guyanensis*, *L. naiffi*, *L. peruviana*, *L. lainsoni*, *L. martiniquensis* and *L. orientalis* have been sequenced [220, 221]. One type of NGS couples WGS with MLST, and is a methodological advance for the provision of standardised epidemiological, molecular evolution and pathogenicity data [222]. Aneuploidy, which is observed in laboratory cultivated samples, as well as mosaicism, can be challenging in WGS, and thus read depth is critical for correct interpretation of *Leishmania* NGS data [223–225].

PCR-based identification methods

Restriction fragment length polymorphism (RFLP) technology: This methodology is used after amplification by PCR, and is widely applied in species identification [199]. Post-PCR amplicons are digested with a restriction enzyme and the products are detected on a gel. The banding pattern produced can be used to identify a particular species, depending on the presence or absence of a restriction enzyme site in the PCR product derived from the target [226]. It is a relatively inexpensive technique compared to real-time PCR, and a multitude of gene targets can be utilised, including heat-shock protein 70, glycoprotein 63, kDNA, cysteine protease B, miniexon or NADH dehydrogenase subunit 7 [121, 227–231].

An ITS1-RFLP reported by Schönian et al. [231] was able to differentiate most *Leishmania* species but was less useful for those species within the *L. braziliensis* complex [232, 233]. Such findings can be problematic, as the clinical presentation of the MCL tropism is caused by more than one of these species, but the response to treatment differs between them [129]. Improvements in species discrimination using RFLP were achieved recently using the NADH dehydrogenase subunit 7 maxicircle gene target, where it was possible to discriminate *L. braziliensis* from other species within the *L. braziliensis* complex (Fig. 3) [231]. RFLP presents multiple difficulties, as it needs a relatively high parasite load, and is thus often paired with cell culture, and multiple restriction enzymes may have to be employed depending on the DNA target. Additionally, RFLP can be difficult to compare between laboratories as banding patterns may be dissimilar due to differing gel size or concentration [234].

Melt curve analysis: This method is used to differentiate species following real-time PCR. Its effectiveness relies on the fact that the temperature at which a sequence of double-stranded DNA dissociates (or "melts") is a function of the GC/AT ratio and the length of an amplicon [235]. Different species exhibit different melting points, which allows for discrimination [236]. In a study that used Tms to group infecting species, species that caused different clinical presentations (i.e., CL/MCL and diffuse cutaneous leishmaniasis) could be differentiated [234]. Either a standard melt curve or high resolution melt can be use for analysis, the latter being a method that is able to detect more subtle differences in temperature, which potentially gives better species discrimination [237–239].

Biosensors

Biosensors lead the emerging field of nanodiagnostics, spanning target detection of DNA/RNA, proteins and even volatile organic compounds from exhaled breath. They are devices that, put simply, convert a biological signal into an electrical signal via a recognition element; they are reported to be low-cost and portable, with high sensitivity and specificity of performance documented [240, 241]. Biosensors use one of several modes of signal generation, like electrochemiluminescence or optical signals, or are based on surface plasmon resonance (SPR). Genosensors, recognising DNA (or RNA), dominate biosensor diagnostics for *Leishmania.* Other recognition elements may be antibodies, antigens or the newer aptamer-based sensors [242]. Aptamers show great promise in binding biological targets with high affinity; they are short, single-stranded nucleic acids that form unique three-dimensional structures and recognise and bind target molecules in a similar fashion to antibodies, such as those targeting the *L. infantum* histone H3 or poly(A) binding protein [243, 244]. A DNA-based biosensor using SPR that targeted the kDNA detected *L. major* and *L. tropica* [245]. Also using SPR biosensing techniques, Ferreira et al. [245] developed an immunosensor based on circulating antibodies against *L. infantum*, which achieved antibody detection within 7 min [246]. Another DNA detection biosensor, which uses fluorescent probes based on the kDNA of *L. infantum* and nanostructured films as sensing platforms, provided sensitive results (1.1 nM of target DNA) even

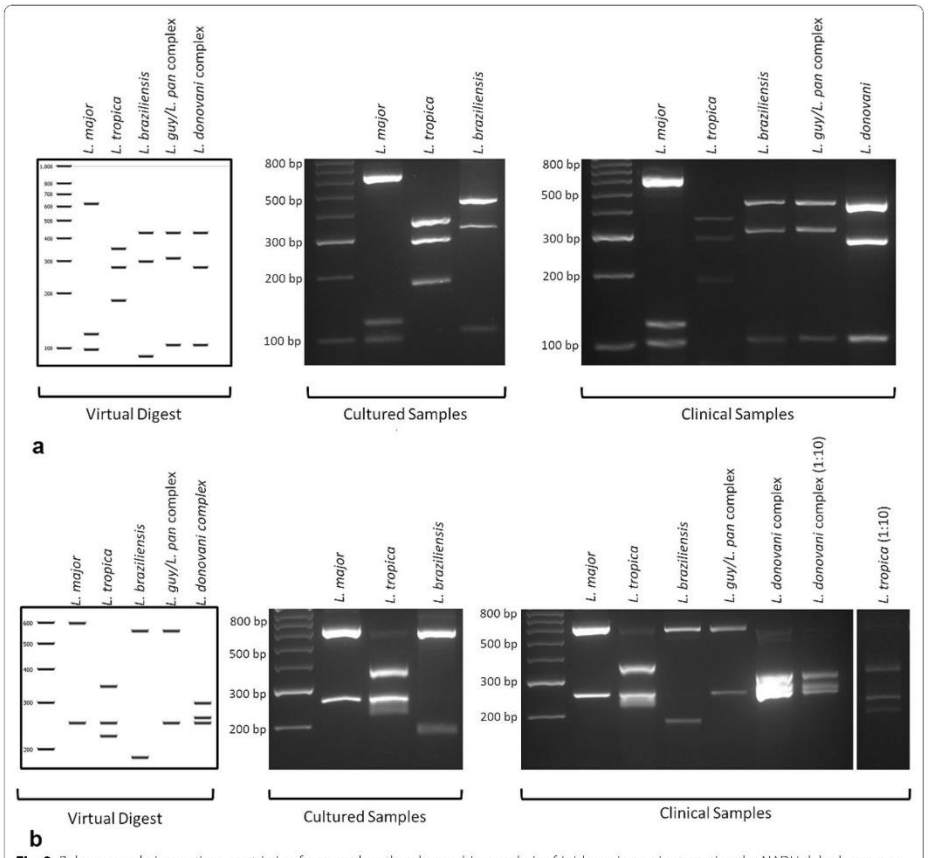

Fig. 3 Polymerase chain reaction—restriction fragment length polymorphism analysis of *Leishmania* species targeting the NADH dehydrogenase subunit 7 gene, digested with *Nla*III (**a**) or *Hpy*CH4IV (**b**) run alongside 50-base pair (*bp*) molecular weight markers. Digestion of in silico, culture and direct clinical samples enabled differentiation of *Leishmania* species. Reprinted with permission of Kaufer et al. [231]

for complex sample types such a human blood [247]. To determine selectivity for the target molecule, the authors measured fluorescence recovery intensity when a target DNA sequence with a single base mismatch was introduced, and observed a reduction of 32% when compared to a fully complementary sequence [247]. Most recently, another genosensor based on the recognition of a single-stranded DNA sequence of *L. infantum* on cadmium sulfide nanosheets was described; the detection limit for *L. infantum* DNA was 1.2 ng/uL without reaction with *L. major* and *L. tropica* DNA [248]. Biosensor development for *Leishmania* detection is in its early stages and requires more research to improve efficiencies and standardisation. Despite this, recent publications on their use in NTDs have indicated the potential for their increased performance as well as a reduction in interactions with interfering substances, good stability and the miniaturisation of devices, allowing their portability [249]. Furthermore, this detection method has been widely integrated into smartphone technology, simplifying the interpretation of results and allowing for multiplexing of targets, such as multiple species [250, 251]. Biosensor

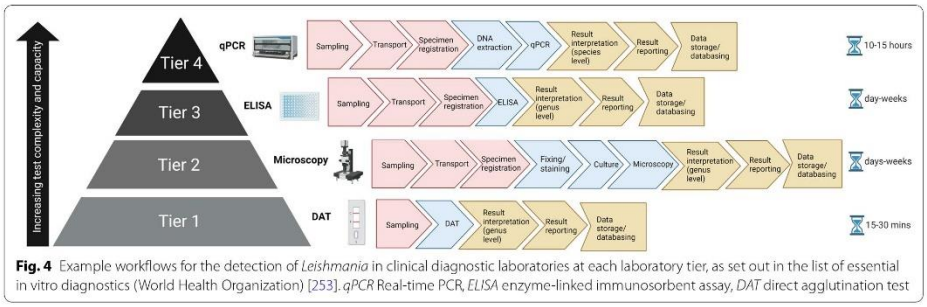

Fig. 4 Example workflows for the detection of *Leishmania* in clinical diagnostic laboratories at each laboratory tier, as set out in the list of essential in vitro diagnostics (World Health Organization) [253]. *qPCR* Real-time PCR, *ELISA* enzyme-linked immunosorbent assay, *DAT* direct agglutination test

technology, though in itsinfancy with regard to leishmaniasisdetection, may be a good solution to the challenge of providing a cost-effective, fast and portable detection method [252].

Conclusions

A multitude of diagnostic assays exist for the detection of *Leishmania* species, but there is no widely accepted gold standard [122, 195, 196]. There have been, however, huge developments in the speed and accuracy of methods with advances in technology, and different approaches may be better suited to different diagnostic health care settings, ranging from primary health care centres (where technical staff perform point-of-care or single-use tests for outpatients) to district hospital laboratories (where limited numbers of staff perform selected routine tests), regional or provincial hospital laboratories (where high numbers of laboratory staff are present to cover many pathology disciplines) and, ultimately, to national reference laboratories (providing highly specialised tests, education and training in research or for teaching hospitals) [253]. All the techniques for the detection and identification of *Leishmania*, of which there is a vast array, have their own strengths and limitations, but high sensitivity, high specificity, low turnaround times and affordability are the critical features of an ideal test. Also important is discrimination between *Leishmania* species, which is vital for epidemiological studies, disease prognosis and for the implementation of patient treatment regimens. Figure 4 illustrates typical workflows for the techniques used in diagnostic laboratories, and highlights that, whilst more traditional protocols can be used to identify *Leishmania* to the genus level relatively speedily when in the hands of trained, experienced professionals, only molecular-based techniques can give species-specific diagnostic information. As global efforts increase to control and eliminate NTDs, there is a need to develop, validate and standardise novel diagnostics for the detection and differentiation of *Leishmania* spp. With the successful implementation of such methods, the global burden of this disease could be reduced dramatically, with positive outcomes being seen for the people that need them the most.

Abbreviations

CL: Cutaneous leishmaniasis; cPCR: Conventional polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; ICT: Immunochromatography test; MCL: Mucocutaneous leishmaniasis; MLEE: Multilocus enzyme electrophoresis; MLMT: Multilocus microsatellite typing; MLST: Multilocus sequence typing; NASBA: Nucleic acid sequence-based amplification; NGS: Next-generation sequencing; NTD: Neglected tropical disease; PCR: Polymerase chain reaction; RAA: Recombinase-aided amplification; RFLP: Restriction fragment length polymorphism; RPA: Recombinase polymerase amplification; SPR: Surface plasmon resonance; Tm: Melting temperature; VL: Visceral leishmaniasis; WGS: Whole genome sequencing; WHO: World Health Organization.

Acknowledgements

We would like to acknowledge BioRender.com for the software used to create Figs. 1 and 4.

Author contributions

IG was responsible for the conception and design of the review, and drafted and substantively revised the review. NCS, DS and JE substantively revised the review. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 28 July 2022 Accepted: 2 October 2022
Published online: 05 November 2022

References

1. Savoia D. Recent updates and perspectives on leishmaniasis. J Infect Dev Ctries. 2015;9:588–96.
2. World Health Organization. First WHO report on neglected tropical diseases 2010: working to overcome the global impact of neglected tropical diseases. 2010.
3. Gonzalez C, Wang O, Strutz SE, Gonzalez-Salazar C, Sanchez-Cordero V, Sarkar S. Climate change and risk of leishmaniasis in North America: predictions from ecological niche models of vector and reservoir species. PLoS Negl Trop Dis. 2010;4:e585.
4. Alawieh A, Musharrafieh U, Jaber A, Berry A, Ghosn N, Bizri AR. Revisiting leishmaniasis in the time of war: the Syrian conflict and the Lebanese outbreak. Int J Infect Dis. 2014;29:115–9.
5. Curtin JM, Aronson NE. Leishmaniasis in the United States: emerging issues in a region of low endemicity. Microorganisms. 2021;9:578.
6. Van der Auwera G, Dujardin JC. Species typing in dermal leishmaniasis. Clin Microbiol Rev. 2015;28:265–94.
7. Espinosa OA, Serrano MG, Camargo EP, Teixeira MM, Shaw JJ. An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as *Leishmania* and *Endotrypanum*. Parasitology. 2016;145:430–42.
8. Akhoundi M, Downing T, Votypka J, Kuhls K, Lukes J, Cannet A, et al. *Leishmania* infections: molecular targets and diagnosis. Mol Aspects Med. 2017;57:1–29.
9. Sereno D. *Leishmania* (*Mundinia*) spp.: from description to emergence as new human and animal *Leishmania* pathogens. New Microbes New Infect. 2019;30:100540.
10. Schönian G, Lukeš J, Stark O, Cotton JA. Molecular evolution and phylogeny of *Leishmania*. In: Ponte-Sucre A, editor. Drug resistance in *Leishmania* parasites. Cham: Springer; 2018.
11. Fraga J, Montalvo AM, De Doncker S, Dujardin J-C, Van der Auwera G. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. Infect Genet Evol. 2010;10:238–45.
12. Schönian G, Mauricio I, Cupolillo E. Is it time to revise the nomenclature of *Leishmania*? Trends Parasitol. 2010;26:466–9.
13. Fernandes Shimabukuro PH, de Andrade AJ, Bianchi Galati EA. Checklist of American sand flies (Diptera, Psychodidae, Phlebotominae): genera, species, and their distribution. ZooKeys. 2017;660:67–106.
14. Haque A, Ekram ARMS, Sharmin LS, Belaluddin M, Salam MA. Congenital visceral leishmaniasis. Pak J Med Sci. 2010;26:485–7.
15. Magill AJ, Meyers WM, Klassen-Fischer MK, Neafie RC. Visceral leishmaniasis. In: Topics on the pathology of protozoan and invasive arthropod diseases. Edited by Sciences USUotH. Bethesda, MD, USA: Uniformed Services University of the Health Sciences; 2011: 1–11.
16. Guedes DL, van Henten S, Cnops L, Adriaensen W, van Griensven J. Sexual transmission of visceral leishmaniasis: a neglected story. Trends Parasitol. 2020;36:950–2.
17. Bern C, Maguire JH, Alvar J. Complexities of assessing the disease burden attributable to leishmaniasis. PLoS Negl Trop Dis. 2008;2:e313.
18. Bañuls A-L, Hide M, Prugnolle F. *Leishmania* and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. Adv Parasitol. 2007;64:1–109.
19. Raja M, Gonzales Zamora JA. Visceral leishmaniasis with cutaneous involvement caused by *Leishmania infantum-chagasi*. IDCases. 2018;11:16–7.
20. Sterkers Y, Lachaud L, Bourgeois N, Crobu L, Bastien P, Pages M. Novel insights into genome plasticity in eukaryotes: mosaic aneuploidy in *Leishmania*. Mol Microbiol. 2012;86:15–23.
21. Prieto Barja P, Pescher P, Bussotti G, Dumetz F, Imamura H, Kedra D, et al. Haplotype selection as an adaptive mechanism in the protozoan pathogen *Leishmania donovani*. Nat Ecol Evol. 2017;1:1961–9.
22. Pace D. Leishmaniasis. J Infect. 2014;69:S10–8.
23. Chagas AC, Oliveira F, Debrabant A, Valenzuela JG, Ribeiro JM, Calvo E. Lundep, a sand fly salivary endonuclease increases *Leishmania* parasite survival in neutrophils and inhibits XIIa contact activation in human plasma. PLoS Pathog. 2014;10:e1003923.
24. World Health Organization. Control of the leishmaniases. 2010. https://www.who.int/publications/i/item/WHO-TRS-949.
25. Hotez P, Molyneux D, Fenwick A, Ottesen E, Ehrlich Sachs S, Sachs J. Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria. PLoS Med. 2006;3:e102.
26. World Health Organization. Global leishmaniasis surveillance update, 1998–2016. In: Global leishmaniasis surveillance update, 1998–2016. World Health Organization. 2018.
27. World Health Organization. Leishmaniasis. https://www.who.int/health-topics/leishmaniasis. 2022. Accessed August 2022.
28. Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis. 2004;27:305–18.
29. Okwor I, Uzonna J. Social and economic burden of human Leishmaniasis. Am J Trop Med Hyg. 2016;94:489–93.
30. Wamai RG, Kahn J, McGloin J, Ziaggi G. Visceral leishmaniasis: a global overview. J Glob Health Sci. 2020;2.
31. Collaborators GDH. Global, regional, and national disability-adjusted life-years (DALYs) for 333 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet. 2017;390:1260–344.
32. Ostyn B, Gidwani K, Khanal B, Picado A, Chappuis F, Singh SP, et al. Incidence of symptomatic and asymptomatic *Leishmania donovani* infections in high-endemic foci in India and Nepal: a prospective study. PLoS Negl Trop Dis. 2011;5:e1284.
33. Das VNR, Bimal S, Siddiqui NA, Kumar A, Pandey K, Sinha SK, et al. Conversion of asymptomatic infection to symptomatic visceral leishmaniasis: a study of possible immunological markers. PLoS Negl Trop Dis. 2020;14:e0008272.
34. Kamink S, Abdi A, Kamau C, Ashraf S, Ansari MA, Qureshi NA, et al. Failure of an innovative low-cost, noninvasive thermotherapy device for treating cutaneous leishmaniasis caused by *Leishmania tropica* in Pakistan. Am J Trop Med Hyg. 2019;101:1373–9.
35. Foroutan M, Dalvand S, Khademvatan S, Majidiani H, Khalkhali H, Masoumifard S, et al. A systematic review and meta-analysis of the prevalence of *Leishmania* infection in blood donors. Transfus Apher Sci. 2017;56:544–51.
36. Aliaga L, Ceballos J, Sampedro A, Cobo F, Lopez-Nevot MA, Merino-Espinosa G, et al. Asymptomatic *Leishmania* infection in blood donors from the south of Spain. Infection. 2019;47:739–47.
37. Mannan SB, Elhadad H, Loc TTH, Sadik M, Mohamed MYF, Nam NH, et al. Prevalence and associated factors of asymptomatic leishmaniasis: a systematic review and meta-analysis. Parasitol Int. 2021;81:102229.
38. Andrade-Narvaez FJ, Loria-Cervera EN, Sosa-Bibiano EI, Van Wynsberghe NR. Asymptomatic infection with American cutaneous leishmaniasis: epidemiological and immunological studies. Mem Inst Oswaldo Cruz. 2016;111:599–604.
39. Sudarshan M, Sundar S. Parasite load estimation by qPCR differentiates between asymptomatic and symptomatic infection in Indian visceral leishmaniasis. Diagn Microbiol Infect Dis. 2014;80:40–2.
40. Arevalo J, Ramirez L, Adaui V, Zimic M, Tulliano G, Miranda-Verastegui C, et al. Influence of *Leishmania* (*Viannia*) species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. J Infect Dis. 2007;195:1846–51.
41. Mondal D, Singh SP, Kumar N, Joshi A, Sundar S, Das P, et al. Visceral leishmaniasis elimination programme in India, Bangladesh, and Nepal: reshaping the case finding/case management strategy. PLoS Negl Trop Dis. 2009;3:e355.
42. Fernandez-Figueroa EA, Sanchez-Montes S, Miranda-Ortiz H, Mendoza-Vargas A, Cervantes-Sarabia R, Cardenas-Ovando RA, et al. Relevance of epidemiological surveillance in travelers: an imported case of *Leishmania tropica* in Mexico. Rev Inst Med Trop Sao Paulo. 2020;62:e41.
43. Zijlstra EE, Alvar J. The post-kala-azar dermal leishmaniasis (PKDL) atlas: a manual for health workers. World Health Organization. 2012.

44. World Health Organization. Post-kala-azar dermal leishmaniasis: a manual for case management and control: report of a WHO consultative meeting. World Health Organization. 2012.
45. Ganguly S, Saha P, Chatterjee M, Roy S, Ghosh TK, Guha SK, et al. PKDL–a silent parasite pool for transmission of leishmaniasis in kala-azar endemic areas of Malda District, West Bengal, India. PLoS Negl Trop Dis. 2015;9:e0004138.
46. Hashiguchi Y, Gomez EL, Kato H, Martini LR, Velez LN, Uezato H. Diffuse and disseminated cutaneous leishmaniasis: clinical cases experienced in Ecuador and a brief review. Trop Med Health. 2016;44:2.
47. Pan American Health Organization. Manual of procedures for leishmaniases surveillance and control in the Americas. Pan American Health Organization. 2019.
48. Gitari JW, Nzou SM, Wamunyokoli F, Kinyeru E, Fujii Y, Kaneko S, et al. *Leishmaniasis recidivans* by *Leishmania tropica* in Central Rift Valley Region in Kenya. Int J Infect Dis. 2018;74:109–16.
49. Walter Reed Army Institute of Research. Cutaneous leishmaniasis scrapings procedures. Edited by Laboratory LD. Silver Spring, USA: Walter Reed Army Institute of Research; 2022: 1–3.
50. Centres for Disease Control and Prevention. Diagnosis. https://www.cdc.gov/parasites/leishmaniasis/diagnosis.html. 2022. Accessed 1 July 2022.
51. Coulborn RM, Gebrehiwot TG, Schneider M, Gerstl S, Adera C, Herrero M, et al. Barriers to access to visceral leishmaniasis diagnosis and care among seasonal mobile workers in western Tigray, northern Ethiopia: a qualitative study. PLoS Negl Trop Dis. 2018;12:e0006778.
52. van Henten S, Adriaensen W, Fikre H, Akuffo H, Diro E, Hailu A, et al. Cutaneous leishmaniasis due to *Leishmania aethiopica*. EClinicalMedicine. 2018;6:69–81.
53. de Paiva-Cavalcanti M, de Morais RC, Pessoa ESR, Trajano-Silva LA, Goncalves-de-Albuquerque Sda C, Tavares Dde H, et al. Leishmaniases diagnosis: an update on the use of immunological and molecular tools. Cell Biosci. 2015;5:31.
54. Hong A, Zampieri RA, Shaw JJ, Floeter-Winter LM, Laranjeira-Silva MF. One health approach to leishmaniases: understanding the disease dynamics through diagnostic tools. Pathogens. 2020;9:809.
55. Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: current status and future applications. J Clin Microbiol. 2007;45:21–5.
56. Sakkas H, Gartzonika C, Levidiotou S. Laboratory diagnosis of human visceral leishmaniasis. J Vector Borne Dis. 2016;53:8–16.
57. Torres-Guerrero E, Quintanilla-Cedillo M, Ruiz-Esmenjaud J, Arenas R. Leishmaniasis: a review. F1000 Research. 2017;6:1–15.
58. Ogden G, Melby P. *Leishmania*. In: MS, editor. Encyclopedia of microbiology, third edn. San Diego: Academic Press; 2009; 663–73.
59. Barratt JL, Harkness J, Marriott D, Ellis JT, Stark D. Importance of nonenteric protozoan infections in immunocompromised people. Clin Microbiol Rev. 2010;23:795–836.
60. Barcia J. The Giemsa stain: its history and applications. Int J Surg Pathol. 2007;15:292–6.
61. Ejazi SA, Ali N. Developments in diagnosis and treatment of visceral leishmaniasis during the last decade and future prospects. Expert Rev Anti Infect Ther. 2016;11:79–98.
62. Yehia L, Adib-Houreih M, Raslan WF, Kibbi AG, Loya A, Firooz A, et al. Molecular diagnosis of cutaneous leishmaniasis and species identification: analysis of 122 biopsies with varied parasite index. J Cutan Pathol. 2012;39:347–55.
63. Mukhtar M, Ali SS, Boshara SA, Albertini A, Monnerat S, Bessell P, et al. Sensitive and less invasive confirmatory diagnosis of visceral leishmaniasis in Sudan using loop-mediated isothermal amplification (LAMP). PLoS Negl Trop Dis. 2018;12:e0006264.
64. Da Silva MRB, Stewart JM, Costa CHN. Sensitivity of bone marrow aspirates in the diagnosis of visceral leishmaniasis. Am J Trop Med Hyg. 2005;72:811–4.
65. Zijlstra EE, Ali MS, El-Hassan AM, El-Toum IA, Satti M, Ghalib HW, et al. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. Trans R Soc Trop Med Hyg. 1992;86:505–7.
66. de Goes TC, de Morais RCS, de Melo MG, Rezende AM, Rezende AM, de Paiva-Cavalcanti M. Analysis of the IGS rRNA region and applicability for *Leishmania* (V.) *braziliensis* characterization. J Parasitol Res. 2020;2020:8885070.
67. Abd El-Salam NM, Ayaz S, Ullah R. PCR and microscopic identification of isolated *Leishmania tropica* from clinical samples of cutaneous leishmaniasis in human population of Kohat region in Khyber Pakhtunkhwa. Biomed Res Int. 2014;2014:861831.
68. Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL. Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. J Clin Microbiol. 2006;44:1435–9.
69. Daboul MW. Is the amastigote form of *Leishmania* the only form found in humans infected with cutaneous leishmaniasis? Lab Med. 2008;39:38–41.
70. Goto H, Lindoso JA. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev Anti Infect Ther. 2010;8:419–33.
71. Rodrigues MM, Verma S, Kumar R, Katara GK, Singh LC, Negi NS, et al. Quantification of parasite load in clinical samples of leishmaniasis patients: IL-10 level correlates with parasite load in visceral leishmaniasis. PLoS ONE. 2010;5:e10107.
72. Siddig M, Ghalib H, Shillington D, Petersen E, Khidir S. Visceral leishmaniasis in Sudan. Clinical features. Trop Geogr Med. 1990;42:107–12.
73. Zijlstra E, Siddig Ali M, El-Hassan A, Isam K, El-Tourn A, Satti M, et al. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. Trans R Soc Trop Med Hyg. 1992;86:505–7.
74. Stark D, van Hal S, Lee R, Marriott D, Harkness J. Leishmaniasis, an emerging imported infection: report of 20 cases from Australia. J Travel Med. 2008;15:351–4.
75. Zare M, Akbarialiabad H, Parsaei H, Asgari Q, Alinejad A, Bahreini MS, et al. A machine learning-based system for detecting leishmaniasis in microscopic images. BMC Infect Dis. 2022;22:48.
76. Ridley DS, Ridley MJ. The evolution of the lesion in cutaneous leishmaniasis. Pathology. 1983;141:83–96.
77. Von Stebut E. Leishmaniasis. J Dtsch Dermatol Ges. 2015;13:191–200
78. Oetken T, Hiscox B, Orengo I, Rosen T. Cutaneous leishmaniasis mimicking squamous cell carcinoma. Dermatol Online J. 2017;23.
79. Aronson NE, Joya CA. Cutaneous leishmaniasis: updates in diagnosis and management. Infect Dis Clin North Am. 2019;33:101–17.
80. Ranawaka R, Abeygunasekara P, Weerakoon H. Correlation of clinical, parasitological and histopathological diagnosis of cutaneous leishmaniasis in an endemic region in Sri Lanka. Ceylon Med J. 2013;57:149–52.
81. Daneshbod Y, Oryan A, Davarmanesh M, Shirian S, Negahban S, Aledavood A, et al. Clinical, histopathologic, and cytologic diagnosis of mucosal leishmaniasis and literature review. Arch Pathol Lab Med. 2011;135:478–82.
82. Roiko MS, Schmitt BH, Relich RF, Meyer TL, Zhang S, Davis TE. An unusual presentation of leishmaniasis in a human immunodeficiency virus-positive individual. JMM Case Rep. 2016;3:e005011.
83. Masia R, Misdraji J. Liver and bile duct infections. In: Kradin R, editor. Diagnostic pathology of infectious disease. Amsterdam: Elsevier; 2018.
84. Venkataram M, Moosa M, Devi L. Histopathological spectrum in cutaneous leishmaniasis: a study in Oman. Indian J Dermatol Venereol Leprol. 2001;67:294–8.
85. Hermida MD, de Melo CVB, Lima IDS, Oliveira GGS, Dos-Santos WLC. Histological disorganization of spleen compartments and severe visceral leishmaniasis. Front Cell Infect Microbiol. 2018;8:394.
86. Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol. 2002;9:951–8.
87. Nasiri V. An overview of the recent findings in the cultivation of *Leishmania*. Rev Med Microbiol. 2017;28:34–42.
88. Antinori S, Calattini S, Longhi E, Bestetti G, Piolini R, Magni C, et al. Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and Revi. Clin Infect Dis. 2007;44:1602–10.
89. Castelli G, Galante A, Lo Verde V, Migliazzo A, Reale S, Lupo T, et al. Evaluation of two modified culture media for *Leishmania infantum* cultivation versus different culture media. J Parasitol. 2014;100:228–30.
90. Muniaraj M, Das P. *Leishmania donovani* promastigotes on "chocolate" agar. Ann Trop Med Parasitol. 2008;102:451–3.
91. Allahverdiyev A, Bagirova M, Uzun S, Alabaz D, Aksaray N, Kocabas E, et al. The value of a new microculture method for diagnosis of visceral

leishmaniasis by using bone marrow and peripheral blood. Am J Trop Med Hyg. 2005;73:276–80.
92. Aberra L, Abera A, Belay T, Kebede A, Gadisa E, Tasew G. Evaluation of microcapillary culture method for the isolation of *Leishmania aethiopica* parasites from patients with cutaneous lesions in Ethiopia. Diagn Progn Res. 2019;3:4.
93. Mäser P, Grether-Bühler Y, Kaminsky R, Brun R. An anti-contamination cocktail for the in vitro isolation and cultivation of parasitic protozoa. Parasitol Res. 2014;88:172–4.
94. Pratlong F, Balard Y, Lami P, Talignani L, Ravel C, Dereure J, et al. The Montpellier *Leishmania* Collection, from a laboratory collection to a biological resource center: a 39-year-long story. Biopreserv Biobank. 2016;14:470–9.
95. de Vries HJ, Reedijk SH, Schallig HD. Cutaneous leishmaniasis: recent developments in diagnosis and management. Am J Clin Dermatol. 2015;16:99–109.
96. Dias DS, Ribeiro PAF, Salles BCS, Santos TTO, Ramos FF, Lage DP, et al. Serological diagnosis and prognostic of tegumentary and visceral leishmaniasis using a conserved *Leishmania* hypothetical protein. Parasitol Int. 2018;67:344–50.
97. Maciel M, Soares MF, Costa SF, Bragato JP, de Freitas JH, Venturin GL, et al. Development of plasmonic ELISA for the detection of anti-*Leishmania* sp. IgG antibodies. J Immunol Methods. 2019;474:112664.
98. Srivastava P, Dayama A, Mehrotra S, Sundar S. Diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2011;105:1–6.
99. Maia Z, Lirio M, Mistro S, Mendes CM, Mehta SR, Badaro R. Comparative study of rK39 *Leishmania* antigen for serodiagnosis of visceral leishmaniasis: systematic review with meta-analysis. PLoS Negl Trop Dis. 2012;6:e1484.
100. Abeijon C, Alves F, Monnerat S, Mbui J, Viana AG, Almeida RM, et al. Urine-based antigen detection assay for diagnosis of visceral leishmaniasis using monoclonal antibodies specific for six protein biomarkers of *Leishmania infantum/Leishmania donovani*. PLoS Negl Trop Dis. 2020;14:e0008246.
101. Kumar A, Pandey SC, Samant M. A spotlight on the diagnostic methods of a fatal disease visceral leishmaniasis. Parasite Immunol. 2020;42:e12727.
102. da Silva ED, de Oliveira BC, Pereira AMS, Guedes DL, de Melo Neto OP, Costa CHN, et al. A flow cytometry-based serological assay to detect visceral leishmaniasis in HIV-infected patients. Front Med. 2021;8:553280.
103. Hossain F, Ghosh P, Khan MAA, Duthie MS, Vallur AC, Picone A, et al. Real-time PCR in detection and quantitation of *Leishmania donovani* for the diagnosis of visceral leishmaniasis patients and the monitoring of their response to treatment. PLoS ONE. 2017;12:e0185606.
104. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol. 2007;5:873–82.
105. Ready PD. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 2014;6:147–54.
106. Oliveira GG, Magalhaes FB, Teixeira MC, Pereira AM, Pinheiro CG, Santos LR, et al. Characterization of novel *Leishmania infantum* recombinant proteins encoded by genes from five families with distinct capacities for serodiagnosis of canine and human visceral leishmaniasis. Am J Trop Med Hyg. 2011;85:1025–34.
107. Caballero ZC, Sousa OE, Marques WP, Saez-Alquezar A, Umezawa ES. Evaluation of serological tests to identify *Trypanosoma cruzi* infection in humans and determine cross-reactivity with *Trypanosoma rangeli* and *Leishmania* spp. Clin Vaccine Immunol. 2007;14:1045–9.
108. Ortalli M, Lorrai D, Gaibani P, Rossini G, Vocale C, Re MC, et al. Serodiagnosis of visceral leishmaniasis in northeastern Italy: evaluation of seven serological tests. Microorganisms. 2020;8:1847.
109. Sundar S, Singh OP. Molecular diagnosis of visceral leishmaniasis. Mol Diagn Ther. 2018;22:443–57.
110. Chappuis F, Rijal S, Soto A, Menten J, Boelaert M. A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. Br Med J. 2006;333:723.
111. Reimao JQ, Coser EM, Lee MR, Coelho AC. Laboratory diagnosis of cutaneous and visceral leishmaniasis: current and future methods. Microorganisms. 2020;8:1632.
112. World Health Organization. Visceral leishmaniasis rapid diagnostic test performance. In: Visceral leishmaniasis rapid diagnostic test performance. World Health Organization. 2011
113. Varani S, Ortalli M, Attard L, Vanino E, Gaibani P, Vocale C, et al. Serological and molecular tools to diagnose visceral leishmaniasis: 2-years' experience of a single center in northern Italy. PLoS ONE. 2017;12:e0183699.
114. Carstens-Kass J, Paulini K, Lypaczewski P, Matlashewski G. A review of the leishmanin skin test: a neglected test for a neglected disease. PLoS Negl Trop Dis. 2021;15:e0009531.
115. Bettaieb J, Toumi A, Ghawar W, Chlif S, Nouira M, Belhaj-Hamida N, et al. A prospective cohort study of cutaneous leishmaniasis due to *Leishmania major*: dynamics of the leishmanin skin test and its predictive value for protection against infection and disease. PLoS Negl Trop Dis. 2020;14:e0008550.
116. Skraba CM, de Mello TF, Pedroso RB, Ferreira EC, Demarchi IG, Aristides SM, et al. Evaluation of the reference value for the Montenegro skin test. Rev Soc Bras Med Trop. 2015;48:437–44.
117. Cardo LJ. *Leishmania*: risk to the blood supply. Transfusion. 2006;46:1641–5.
118. Singh OP, Hasker E, Boelaert M, Sacks D, Sundar S. Xenodiagnosis to address key questions in visceral leishmaniasis control and elimination. PLoS Negl Trop Dis. 2020;14:e0008363.
119. Conter CC, Mota CA, dos Santos BA, de Souza BL, de Souza TM, Navasconi TR, et al. PCR primers designed for New World *Leishmania*: a systematic review. Exp Parasitol. 2019;207:107773.
120. Deborggraeve S, Boelaert M, Rijal S, De Doncker S, Dujardin JC, Herdewijn P, et al. Diagnostic accuracy of a new *Leishmania* PCR for clinical visceral leishmaniasis in Nepal and its role in diagnosis of disease. Trop Med Int Health. 2008;13:1378–83.
121. Marfurt J, Niederwieser I, Makia N, Beck H, Felger I. Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP. Diagn Microbiol Infect Dis. 2003;46:115–24.
122. Galluzzi L, Ceccarelli M, Diotallevi A, Menotta M, Magnani M. Real-time PCR applications for diagnosis of leishmaniasis. Parasit Vectors. 2018;11:273.
123. Mary C, Faraut F, Lascombe L, Dumon H. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. J Clin Microbiol. 2004;42:5249–55.
124. Gow I, Millar D, Ellis J, Melki J, Stark D. Semi-quantitative, duplexed qPCR assay for the detection of *Leishmania* spp. using bisulphite conversion technology. Trop Med Infect Dis. 2019;4:135.
125. Machado de Assis TS, Azeredo-da-Silva AL, Werneck GL, Rabello A. Cost-effectiveness analysis of diagnostic tests for human visceral leishmaniasis in Brazil. Trans R Soc Trop Med Hyg. 2016;110:464–71.
126. Mohammadi M, Bamorovat M, Fasihi Harandi M, Karimi T, Sharifi I, Aflatoonian M. Comparison of three PCR-based methods for simplicity and cost effectiveness identification of cutaneous leishmaniasis due to *Leishmania tropica*. Iran J Parasitol. 2017;12:215–23.
127. Aerts C, Vink M, Pashtoon SJ, Nahzat S, Picado A, Cruz I, et al. Cost effectiveness of new diagnostic tools for cutaneous leishmaniasis in Afghanistan. Appl Health Econ Health Policy. 2019;17:213–30.
128. Castillo-Rodriguez L, Ovalle-Bracho C, Diaz-Jimenez D, Sanchez-Vanegas G, Muvdi-Arenas S, Castaneda-Orjuela C. Cost-effectiveness analysis of mucosal leishmaniasis diagnosis with PCR-based vs parasitological tests in Colombia. PLoS ONE. 2019;14:e0224351.
129. Tsokana CN, Athanasiou LV, Valiakos G, Spyrou V, Manolakou K, Billinis C. Molecular diagnosis of leishmaniasis, species identification and phylogenetic analysis. In: Claborn DM, editor. Leishmaniasis- trends in epidemiology diagnosis & treatment. London: In Tech Open. 2014. p. 161–93.
130. Ortalli M, De Pascali AM, Longo S, Pascarelli N, Porcellini A, Ruggeri D, et al. Asymptomatic *Leishmania infantum* infection in blood donors living in an endemic area, northeastern Italy. J Infect. 2020;80:116–20.
131. Hosseinzadeh M, Omidifar N, Lohrasb MH. Use of fine needle aspiration cytology in the diagnosis of cutaneous leishmaniasis: a comparison with the conventional scraping method. Trop Doct. 2012;42:112–3.
132. Saab M, Hage HE, Charafeddine K, Habib RH, Khalifeh I. Diagnosis of cutaneous leishmaniasis: why punch when you can scrape? Am J Trop Med Hyg. 2015;92:518–22.

133. Zhang WW, Miranda-Verastegui C, Arevalo J, Ndao M, Ward B, Llanos-Cuentas A, et al. Development of a genetic assay to distinguish between *Leishmania viannia* species on the basis of isoenzyme differences. Clin Infect Dis. 2006;42:801–9.
134. Daoui OAKM, Mhaidi I, El Kacem S, Hjiyej Andaloussi L, Akarid K, Lemrani M. The role of sampling by cotton swab in the molecular diagnosis of cutaneous leishmaniasis. Transbound Emerg Dis. 2021;68:2287–94.
135. Adams ER, Versteeg I, Leeflang MM. Systematic review into diagnostics for post-kala-azar dermal leishmaniasis (PKDL). J Trop Med. 2013;2013:150746.
136. De Ruiter CM, Van Der Veer C, Leeflang MMG, Deborggraeve S, Lucas C, Adams ER. Molecular tools for diagnosis of visceral leishmaniasis: systematic review and meta-analysis of diagnostic test accuracy. J Clin Microbiol. 2014;52:3147–55.
137. Eberhardt E, Van den Kerkhof M, Bulte D, Mabille D, Van Bockstal L, Monnerat S, et al. Evaluation of a pan-*Leishmania* spliced-leader RNA detection method in human blood and experimentally infected Syrian golden hamsters. J Mol Diagn. 2018;20:253–63.
138. Deborggraeve S, Laurent T, Espinosa D, Van der Auwera G, Mbuchi M, Wasunna M, et al. A simplified and standardized polymerase chain reaction format for the diagnosis of leishmaniasis. J Infect Dis. 2008;198:1565–72.
139. Saldarriaga OA, Castellanos-Gonzalez A, Porrozzi R, Baldeviano GC, Lescano AG, de Los Santos MB, et al. An innovative field-applicable molecular test to diagnose cutaneous *Leishmania viannia* spp. Infect PLoS Negl Trop Dis. 2016;10:e0004638.
140. Suzuki RB, Cabral AD, Tonhosolo R, Marcili A, de Oliveira Campos Camargo Sanches C, Martins LPA, et al. A highly sensitive and specific conventional molecular diagnosis for *Leishmania infantum chagasi* based on a single copy gene. J Mol Biomark Diagn. 2016;07:1–4.
141. Beldi N, Mansouri R, Bettaieb J, Yaacoub A, Souguir Omrani H, Saadi Ben Aoun Y, et al. Molecular characterization of *Leishmania* parasites in Giemsa-stained slides from cases of human cutaneous and visceral leishmaniasis. Eastern Algeria. Vector Borne Zoonotic Dis. 2017;17:416–24.
142. Gunaratna G, Manamperi A, Bohlken-Fascher S, Wickremasinge R, Gunawardena K, Yapa B, et al. Evaluation of rapid extraction and isothermal amplification techniques for the detection of *Leishmania donovani* DNA from skin lesions of suspected cases at the point of need in Sri Lanka. Parasit Vectors. 2018;11:665.
143. da C Gonçalves-de-Albuquerque S, Pessoa-e-Silva R, Trajano-Silva LA, de Morais RC, Brandão-Filho SP, de Paiva-Cavalcanti M. Inclusion of quality controls on leishmaniases molecular tests to increase diagnostic accuracy in research and reference laboratories. Mol Biotechnol. 2015;57:318–24.
144. Franca AO, Pompilio MA, Pontes E, de Oliveira MP, Pereira LOR, Lima RB, et al. *Leishmania* infection in blood donors: a new challenge in leishmaniasis transmission? PLoS ONE. 2018;13:e0198199.
145. Pan American Health Organisation. Leishmaniasis regional health program. In: Communicable diseases and health analysis neglected, tropical, and vector borne diseases panaftosa - Veterinary Public Health; 2017. https://www.paho.org/en/documents/leishmaniasis-regional-program-americas-2010-2017-2017. Accessed 21 Mar 2019.
146. Basiye FI., Mbuchi M, Magiri C, Kirigi G, Deborggraeve S, Schoone GJ, et al. Sensitivity and specificity of the *Leishmania* OligoC-TesT and NASBA-oligochromatography for diagnosis of visceral leishmaniasis in Kenya. Trop Med Int Health. 2010;15:806–10.
147. van der Meide W, Guerra J, Schoone G, Farenhorst M, Coelho L, Faber W, et al. Comparison between quantitative nucleic acid sequence-based amplification, real-time reverse transcriptase PCR, and real-time PCR for quantification of *Leishmania* parasites. J Clin Microbiol. 2008;46:73–8.
148. Adams ER, Schoone G, Versteeg I, Gomez M, Diro E, Mori Y, et al. Development and evaluation of a novel loop-mediated isothermal amplification assay for diagnosis of cutaneous and visceral leishmaniasis. J Clin Microbiol. 2018;56:1–8.
149. Ibarra-Meneses AV, Cruz I, Chicharro C, Sanchez C, Bieler S, Broger T, et al. Evaluation of fluorimetry and direct visualization to interpret results of a loop-mediated isothermal amplification kit to detect *Leishmania* DNA. Parasit Vectors. 2018;11:250.
150. Sukphattanaudomchoke C, Siripattanapipong S, Thita T, Leelayoova S, Piyaraj P, Mungthin M, et al. Simplified closed tube loop mediated isothermal amplification (LAMP) assay for visual diagnosis of *Leishmania* infection. Acta Trop. 2020;212:105651.
151. Dixit KK, Ramesh V, Gupta R, Negi NS, Singh R, Salotra P. Real-time fluorimetry loop-mediated isothermal amplification for diagnosis of leishmaniasis and as a tool for assessment of cure for post-kala-azar dermal leishmaniasis. Am J Trop Med Hyg. 2021;104:2097–107.
152. Nzelu CO, Kato H, Peters NC. Loop-mediated isothermal amplification (LAMP): an advanced molecular point-of-care technique for the detection of *Leishmania* infection. PLoS Negl Trop Dis. 2019;13:e0007698.
153. Castellanos-Gonzalez A, White AC, Melby P, Travi B. Molecular diagnosis of protozoan parasites by recombinase polymerase amplification. Acta Trop. 2018;182:4–11.
154. Lin H, Zhao S, Liu YH, Shao L, Ying QJ, Yang K. Development of a fluorescent recombinase-aided isothermal amplification-based nucleic acid assay for detection of *Leishmania*. Zhongguo Xue Xi Chong Bing Fang Zhi Za Zhi. 2021;33:452–6.
155. Khan MAA, Faisal K, Chowdhury R, Ghosh P, Hossain F, Weidmann M, et al. Development of quantitative rapid isothermal amplification assay for *Leishmania donovani*. Diagnostics. 2021;11:1963.
156. Mondal D, Ghosh P, Khan MA, Hossain F, Bohlken-Fascher S, Matlashewski G, et al. Mobile suitcase laboratory for rapid detection of *Leishmania donovani* using recombinase polymerase amplification assay. Parasit Vectors. 2016;9:281.
157. Cruz I, Millet A, Carrillo E, Chenik M, Salotra P, Verma S, et al. An approach for interlaboratory comparison of conventional and real-time PCR assays for diagnosis of human leishmaniasis. Exp Parasitol. 2013;134:281–9.
158. Conter CC, Lonardoni MVC, Aristides SMA, Cardoso RF, Silveira TGV. New primers for the detection of *Leishmania* species by multiplex polymerase chain reaction. Parasitol Res. 2017;117:501–11.
159. Rosales-Chilama M, Diaz-Moreno N, Prieto MD, Giraldo-Parra L, Martinez-Valencia AJ, Gomez MA. Comparative assessment of DNA targets and amplification methods for *Leishmania* (*Viannia*) detection in human samples. Am J Trop Med Hyg. 2020;102:1323–7.
160. Antinori S, Calattini S, Piolini R, Longhi E, Bestetti G, Cascio A, et al. Is real-time polymerase chain reaction (PCR) more useful than a conventional PCR for the clinical management of leishmaniasis? Am J Trop Med Hyg. 2009;81:46–51.
161. Vega-Lopez F. Diagnosis of cutaneous leishmaniasis. Curr Opin Infect Dis. 2003;16:97–101.
162. Lachaud L, Marchergui-Hammami S, Chabbert E, Dereure J, Dedet JP, Bastien P. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. J Clin Microbiol. 2002;40:210–5.
163. Hasker E, Singh SP, Malaviya P, Singh RP, Shankar R, Boelaert M, et al. Management of visceral leishmaniasis in rural primary health care services in Bihar. India Trop Med Int Health. 2010;15:55–62.
164. Sudarshan M, Weirather JL, Wilson ME, Sundar S. Study of parasite kinetics with antileishmanial drugs using real-time quantitative PCR in Indian visceral leishmaniasis. J Antimicrob Chemother. 2011;66:1751–5.
165. Srivastava P, Prajapati VK, Rai M, Sundar S. Unusual case of resistance to amphotericin B in visceral leishmaniasis in a region in India where leishmaniasis is not endemic. J Clin Microbiol. 2011;49:3088–91.
166. Ponte-Sucre A, Gamarro F, Dujardin JC, Barrett MP, Lopez-Velez R, Garcia-Hernandez R, et al. Drug resistance and treatment failure in leishmaniasis: a 21st century challenge. PLoS Negl Trop Dis. 2017;11:e0006052.
167. Khatun M, Alam SMS, Khan AH, Hossain MA, Haq JA, Alam Jilani MS, et al. Novel PCR primers to diagnose visceral leishmaniasis using peripheral blood, spleen or bone marrow aspirates. Asian Pac J Trop Med. 2017;10:753–9.
168. Srivastava P, Mehrotra S, Tiwary P, Chakravarty J, Sundar S. Diagnosis of Indian visceral leishmaniasis by nucleic acid detection using PCR. PLoS ONE. 2011;6:e19304.
169. Hu Y. Regulatory concern of polymerase chain reaction (PCR) carryover contamination polymerase chain reaction for biomedical applications. London: Intech; 2016. p. 57–68.
170. Nateghi Rostami M, Darzi F, Farahmand M, Aghaei M, Parvizi P. Performance of a universal PCR assay to identify different *Leishmania* species causative of Old World cutaneous leishmaniasis. Parasit Vectors. 2020;13:431.

171. Pandey N, Siripattanapipong S, Leelayoova S, Manomat J, Mungthin M, Tan-Ariya P, et al. Detection of *Leishmania* DNA in saliva among patients with HIV/AIDS in Trang Province, southern Thailand. Acta Trop. 2018;185:294–300.
172. Salam MA, Mondal D, Kabir M, Ekram AR, Haque R. PCR for diagnosis and assessment of cure in kala-azar patients in Bangladesh. Acta Trop. 2010;113:52–5.
173. Namazi MJ, Dehkordi AB, Haghighi F, Mohammadzadeh M, Zarean M, Hasanabad MH. Molecular detection of *Leishmania* species in northeast of Iran. Comp Clin Path. 2018;27:729–33.
174. De Silva NL, De Silva VNH, Deerasinghe ATH, Rathnapala UL, Itoh M, Takagi H, et al. Development of a highly sensitive nested PCR and its application for the diagnosis of cutaneous leishmaniasis in Sri Lanka. Microorganisms. 2022;10:990.
175. Deepachandi B, Weerasinghe S, Soysa P, Karunaweera N, Siriwardana Y. A highly sensitive modified nested PCR to enhance case detection in leishmaniasis. BMC Infect Dis. 2019;19:623.
176. Castelli G, Bruno F, Reale S, Catanzaro S, Valenza V, Vitale F. Molecular diagnosis of leishmaniasis: quantification of parasite load by a real-time PCR assay with high sensitivity. Pathogens. 2021;10:865.
177. Moreira OC, Yadon ZE, Cupolillo E. The applicability of real-time PCR in the diagnostic of cutaneous leishmaniasis and parasite quantification for clinical management: current status and perspectives. Acta Trop. 2018;184:29–37.
178. Nath-Chowdhury M, Sangaralingam M, Bastien P, Ravel C, Pratlong F, Mendez J, et al. Real-time PCR using FRET technology for Old World cutaneous leishmaniasis species differentiation. Parasit Vectors. 2016;9:255.
179. Trajano-Silva LAM, Pessoa ESR, Goncalves-de-Albuquerque SDC, Morais RCS, Costa-Oliveira CND, Goes TC, et al. Standardization and evaluation of a duplex real-time quantitative PCR for the detection of *Leishmania infantum* DNA: a sample quality control approach. Rev Soc Bras Med Trop. 2017;50:350–7.
180. Mohammadiha A, Mohebali M, Haghighi A, Mahdian R, Abadi AR, Zarei Z, et al. Comparison of real-time PCR and conventional PCR with two DNA targets for detection of *Leishmania (Leishmania) infantum* infection in human and dog blood samples. Exp Parasitol. 2013;133:89–94.
181. Leon CM, Munoz M, Hernandez C, Ayala MS, Florez C, Teheran A, et al. Analytical performance of four polymerase chain reaction (PCR) and real time PCR (qPCR) assays for the detection of six *Leishmania* species DNA in Colombia. Front Microbiol. 2017;8:1907.
182. Pourmohammadi B, Motazedian M, Hatam G, Kalantari M, Habibi P, Sarkari B. Comparison of three methods for diagnosis of cutaneous leishmaniasis. Iran J Parasitol. 2010;5:1–8.
183. Eroglu F, Uzun S, Koltas IS. Comparison of clinical samples and methods in chronic cutaneous leishmaniasis. Am J Trop Med Hyg. 2014;91:895–900.
184. Filgueira CPB, Moreira OC, Cantanhede LM, de Farias HMT, Porrozzi R, Britto C, et al. Comparison and clinical validation of qPCR assays targeting Leishmania 18S rDNA and HSP70 genes in patients with American tegumentary leishmaniasis. PLoS Negl Trop Dis. 2020;14:e0008750.
185. Diotallevi A, Buffi G, Ceccarelli M, Neitzke-Abreu HC, Gnutzmann LV, da Costa Lima MS, et al. Real-time PCR to differentiate among *Leishmania* (*Viannia*) subgenus, *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis*: application on Brazilian clinical samples. Acta Trop. 2020;201:105178.
186. Dantas-Torres F, da SilvaSales KG, GomesdaSilva L, Otranto D, Figueredo LA. Leishmania-FAST15: a rapid, sensitive and low-cost real-time PCR assay for the detection of *Leishmania infantum* and *Leishmania braziliensis* kinetoplast DNA in canine blood samples. Mol Cell Probes. 2017;31:65–9.
187. Sevilha-Santos L, Dos Santos Junior ACM, Medeiros-Silva V, Bergmann JO, da Silva FF, Segato I F, et al. Accuracy of qPCR for quantifying *Leishmania* kDNA in different skin layers of patients with American tegumentary leishmaniasis. Clin Microbiol Infect. 2019;25:242–7.
188. Frommer M, McDonald L, Millar D, Collis C, Watt F, Grigg G, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA. 1992;89:1827–31.
189. Siah SP, Merif J, Kaur K, Nair J, Huntington PG, Karagiannis T, et al. Improved detection of gastrointestinal pathogens using generalised sample processing and amplification panels. Pathology. 2014;46:53–9.
190. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem. 2011;83:8604–10.
191. Roy M, Sarkar D, Chatterjee M. Quantitative monitoring of experimental and human leishmaniasis employing amastigote-specific genes. Parasitology. 2022;149:1085–93.
192. Ramirez JD, Herrera G, Muskus C, Mendez C, Duque MC, Butcher R. Development of a digital droplet polymerase chain reaction (ddPCR) assay to detect *Leishmania* DNA in samples from cutaneous leishmaniasis patients. Int J Infect Dis. 2019;79:1–3.
193. Satow MM, Yamashiro-Kanashiro EH, Rocha MC, Oyafuso LK, Soler RC, Cotrim PC, et al. Applicability of kDNA-PCR for routine diagnosis of American tegumentary leishmaniasis in a tertiary reference hospital. Rev Inst Med Trop Sao Paulo. 2013;55:393–9.
194. Rodriguez-Cortes A, Ojeda A, Francino O, Lopez-Fuertes L, Timon M, Alberola J. *Leishmania* infection: laboratory diagnosing in the absence of a "gold standard." Am J Trop Med Hyg. 2010;82:251–6.
195. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S. Asymptomatic *Leishmania* infection: a new challenge for *Leishmania* control. Clin Infect Dis. 2014;58:1424–9.
196. Izadi S, Mirhendi H, Jalalizand N, Khodadadi H, Mohebali M, Nekoeian S, et al. Molecular epidemiological survey of cutaneous leishmaniasis in two highly endemic metropolises of Iran, application of FTA Cards for DNA extraction from Giemsa-stained slides. Jundishapur J Microbiol. 2016;9:17–21.
197. Gomes CM, Mazin SC, Raphael E, Cesetti MV, Albergaria G, Bächtold B, et al. Accuracy of mucocutaneous leishmaniasis diagnosis using polymerase chain reaction: systematic literature review and meta-analysis. Mem Inst Oswaldo Cruz. 2015;110:157–65.
198. Van der Auwera G, Ravel C, Verweij J, Bart A, Schönian G, Felger I. Evaluation of four single-locus markers for *Leishmania* species discrimination by sequencing. J Clin Microbiol. 2014;52:1098–104.
199. Brodskyn CI, Kato H, Gomez EA, Seki C, Furumoto H, Martini-Robles L, et al. PCR-RFLP analyses of *Leishmania* species causing cutaneous and mucocutaneous leishmaniasis revealed distribution of genetically complex strains with hybrid and mito-nuclear discordance in Ecuador. PLoS Negl Trop Dis. 2019;13:e0007403.
200. Shaw J, Pratlong F, Floeter-Winter L, Ishikawa E, El Baidouri F, Ravel C, et al. Characterization of *Leishmania* (*Leishmania*) *waltoni* n.sp. (Kinetoplastida: Trypanosomatidae), the parasite responsible for diffuse cutaneous leishmaniasis in the Dominican Republic. Am J Trop Med Hyg. 2015;93:552–8.
201. Van der Auwera G, Bart A, Chicharro C, Cortes S, Davidsson L, Di Muccio T, et al. Comparison of *Leishmania* typing results obtained from 16 European clinical laboratories in 2014. Euro Surveill. 2016;21:30418.
202. Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Ann Parasitol Hum Comp. 1990;65:111–25.
203. Hide M, Banuls A, Tibayrenc M. Genetic heterogeneity and phylogenetic status of *Leishmania (Leishmania) infantum* zymodeme MON-1: epidemiological implications. Parasitology. 2001;123:425–32.
204. Ovalle-Bracho C, Camargo C, Diaz-Toro Y, Parra-Munoz M. Molecular typing of *Leishmania (Leishmania) amazonensis* and species of the subgenus *Viannia* associated with cutaneous and mucosal leishmaniasis in Colombia: a concordance study. Biomedica. 2018;38:86–95.
205. Cupolillo E, Grimaldi G Jr, Momen H. A general classification of New World *Leishmania* using numerical zymotaxonomy. Am J Trop Med Hyg. 1994;50:296–311.
206. Herrera G, Hernandez C, Ayala MS, Florez C, Teheran AA, Ramirez JD. Evaluation of a multilocus sequence typing (MLST) scheme for *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) panamensis* in Colombia. Parasit Vectors. 2017;10:236.
207. Hosseini M, Nateghi Rostami M, Hosseini Doust R, Khamesipour A. Multilocus sequence typing analysis of *Leishmania* clinical isolates from cutaneous leishmaniasis patients of Iran. Infect Genet Evol. 2020;85:104533.

208. Lauthier JJ, Ruybal P, Barroso PA, Hashiguchi Y, Marco JD, Korenaga M. Development of a multilocus sequence typing (MLST) scheme for pan-*Leishmania*. Acta Trop. 2020;201:105189.
209. Schonian G, Kuhls K, Mauricio IL. Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. Parasitology. 2011;138:405–25.
210. Botilde Y, Laurent T, Quispe Tintaya W, Chicharro C, Cañavate C, Cruz I, et al. Comparison of molecular markers for strain typing of *Leishmania infantum*. Infect Genet Evol. 2006;6:440–6.
211. Kuhls K, Keilonat L, Ochsenreither S, Schaar M, Schweynoch C, Presber W, et al. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. Microb Infect. 2007;9:334–43.
212. Alam MZ, Haralambous C, Kuhls K, Gouzelou E, Sgouras D, Soteriadou K, et al. The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. Microbes Infect. 2009;11:707–15.
213. Kocher A, Valiére S, Banuls A-L, Murienne J. High-throughput sequencing of kDNA amplicons for the analysis of *Leishmania* minicircles and identification of Neotropical species. Parasitology. 2017;145:585–94.
214. Kato H, Caceres AG, Seki C, Silupu Garcia CR, Holguin Mauricci C, Castro Martinez SC, et al. Further insight into the geographic distribution of *Leishmania* species in Peru by cytochrome b and mannose phosphate isomerase gene analyses. PLoS Negl Trop Dis. 2019;13:e0007496.
215. Bamorovat M, Sharifi I, Mohammadi MA, Eybpoosh S, Nasibi S, Aflatoonian MR, et al. *Leishmania tropica* isolates from non-healed and healed patients in Iran: a molecular typing and phylogenetic analysis. Microb Pathog. 2018;116:124–9.
216. Imai K, Tarumoto N, Amo K, Takahashi M, Sakamoto N, Kosaka A, et al. Non-invasive diagnosis of cutaneous leishmaniasis by the direct boil loop-mediated isothermal amplification method and MinION nanopore sequencing. Parasitol Int. 2018;67:34–7.
217. Calarco L, Barratt J, Ellis J. Detecting sequence variants in clinically important protozoan parasites. Int J Parasitol. 2020;50:1–18.
218. Downing T, Imamura H, Decuypere S, Clark TG, Coombs GH, Cotton JA, et al. Whole genome sequencing of multiple *Leishmania donovani* clinical isolates provides insights into population structure and mechanisms of drug resistance. Genome Res. 2011;21:2143–56.
219. Morrison DA. Next generation systematics. Cambridge: Cambridge University Press; 2016.
220. Anuntasomboon P, Siripattanapipong S, Unajak S, Choowongkomon K, Burchmore R, Leelayoova S, et al. Comparative draft genomes of *Leishmania orientalis* isolate PCM2 (formerly named *Leishmania siamensis*) and *Leishmania martiniquensis* isolate PCM3 from the southern province of Thailand. Biology. 2022;11:515.
221. Carvalho KSS, da Silva Junior WJ, da Silveira Regueira Neto M, Silva VC, de Sa Leitao Paiva Junior S, Balbino VQ, et al. Application of next generation sequencing (NGS) for descriptive analysis of 30 genomes of *Leishmania infantum* isolates in middle-north Brazil. Sci Rep. 2020;10:12321.
222. Banu SS, Meyer W, Ferreira-Paim K, Wang Q, Kuhls K, Cupolillo E, et al. A novel multilocus sequence typing scheme identifying genetic diversity amongst *Leishmania donovani* isolates from a genetically homogeneous population in the Indian subcontinent. Int J Parasitol. 2019;49:555–67.
223. Imamura H, Dujardin JC. A guide to next generation sequence analysis of leishmania genomes. Methods Mol Biol. 2019;1971:69–94.
224. Maljkovic Berry I, Melendrez MC, Bishop-Lilly KA, Rutvisuttinunt W, Pollett S, Talundzic E, et al. Next generation sequencing and bioinformatics methodologies for infectious disease research and public health: approaches, applications, and considerations for development of laboratory capacity. J Infect Dis. 2019;221:1–16.
225. Imamura H, Jara M, Monsieurs P, Sanders M, Maes I, Vanaerschot M, et al. Evaluation of whole genome amplification and bioinformatic methods for the characterization of *Leishmania* genomes at a single cell level. Sci Rep. 2020;10:15043.
226. Montalvo AM, Fraga J, Monzote L, Montano I, De Doncker S, Dujardin JC, et al. Heat-shock protein 70 PCR-RFLP: a universal simple tool for *Leishmania* species discrimination in the New and Old World. Parasitology. 2010;137:1159–68.
227. Espada CR, Ortiz PA, Shaw JJ, Barral AMP, Costa JML, Uliana SRB, et al. Identification of *Leishmania (Viannia)* species and clinical isolates of *Leishmania (Leishmania) amazonensis* from Brazil using PCR-RFLP of the heat-shock protein 70 gene reveals some unexpected observations. Diagn Microbiol Infect Dis. 2018;91:312–8.
228. Victoir K, Dujardin J. How to succeed in parasitic life without sex? Asking *Leishmania*. Trends Parasitol. 2002;18:81–5.
229. Ghatee MA, Mirhendi H, Marashifard M, Kanannejad Z, Taylor WR, Sharifi I. Population structure of *Leishmania tropica* causing anthroponotic cutaneous leishmaniasis in southern Iran by PCR-RFLP of kinetoplastid DNA. Biomed Res Int. 2018;2018:6049198.
230. Quispe-Tintaya K, Laurent T, Decuypere S, Hide M, Bañuls A, De Doncker S, et al. Fluorogenic assay for molecular typing of the *Leishmania donovani* complex: taxonomic and clinical applications. J Infect Dis. 2005;192:685–92.
231. Kaufer A, Ellis J, Stark D. Identification of clinical infections of *Leishmania* imported into Australia: revising speciation with polymerase chain reaction-RFLP of the kinetoplast maxicircle. Am J Trop Med Hyg. 2019;101:590–601.
232. Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig H, Presber W, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003;47:349–58.
233. Roberts T, Barratt J, Sandaradura I, Lee R, Harkness J, Marriott D, et al. Molecular epidemiology of imported cases of leishmaniasis in Australia from 2008 to 2014. PLoS ONE. 2015;10:1–11.
234. de Morais RC, da Costa Oliveira CN, de Albuquerque C, Mendonca Trajano Silva LA, Pessoa ESR, Alvesda Cruz HL, et al. Real-time PCR for *Leishmania* species identification: evaluation and comparison with classical techniques. Exp Parasitol. 2016;165:43–50.
235. Kuang Z, Zhang C, Pang H, Ma Y. A rapid high-resolution melting method for differentiation of *Leishmania* species targeting *lack* gene. Acta Trop. 2018;178:103–6.
236. Schulz A, Mellenthin K, Schonian G, Fleischer B, Drosten C. Detection, differentiation, and quantitation of pathogenic *Leishmania* organisms by a fluorescence resonance energy transfer-based real-time PCR assay. J Clin Microbiol. 2003;41:1529–35.
237. Sirekbasan S, Polat E. Real-time PCR using high-resolution melting analysis technology for diagnosis of *Leishmania* and determination of types of clinical samples. Turk J Med Sci. 2018;48:1358–63.
238. Ceccarelli M, Diotallevi A, Buffi G, De Santi M, Fernandez-Figueroa EA, Rangel-Escareno C, et al. Differentiation of *Leishmania* (L.) *infantum*, *Leishmania* (L.) *amazonensis* and *Leishmania* (L.) *mexicana* using sequential qPCR assays and high-resolution melt analysis. Microorganisms. 2020;8:818.
239. Wittwer CT. High-resolution DNA melting analysis: advancements and limitations. Hum Mutat. 2009;30:857–9.
240. Perinoto Â, Maki R, Colhone M, Santos F, Migliaccio V, Daghastanli K, et al. Biosensors for efficient diagnosis of leishmaniasis: innovations in bioanalytics for a neglected disease. Anal Chem. 2010;82:9763–8.
241. Jain S, Santana W, Dolabella SS, Santos ALS, Souto EB, Severino P. Are nanobiosensors an improved solution for diagnosis of *Leishmania*? Pharmaceutics. 2021;13:491.
242. Gedda MR, Madhukar P, Shukla A, Mudavath SL, Srivastava ON, Singh OP, et al. Nanodiagnostics in leishmaniasis: a new frontiers for early elimination. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2021;13:e1675.
243. Frezza V, Pinto-Diez C, Fernandez G, Soto M, Martin ME, Garcia-Sacristan A, et al. DNA aptamers targeting *Leishmania infantum* H3 protein as potential diagnostic tools. Anal Chim Acta. 2020;1107:155–63.
244. Guerra-Perez N, Ramos E, Garcia-Hernandez M, Pinto C, Soto M, Martin ME, et al. Molecular and functional characterization of ssDNA aptamers that specifically bind *Leishmania infantum* PABP. PLoS ONE. 2015;10:e0140048.
245. Sattarahmady N, Movahedpour A, Heli H, Hatam G. Gold nanoparticles-based biosensing of *Leishmania major* kDNA genome: visual and spectrophotometric detections. Sensors Actuators B: Chem. 2016;235:723–31.
246. Ferreira E, Lima J, Alves-Balvedi RP, Bonan P, Medeiros E, Goulart L, et al. *Leishmania* spp. detection using a surface plasmon resonance biosensor. Proceedings. 2017;1(4):536.

247. Pedro GC, Gorza FDS, da Silva RJ, do Nascimento KTO, Medina-Llamas JC, Chavez-Guajardo AE, et al. A novel nucleic acid fluorescent sensing platform based on nanostructured films of intrinsically conducting polymers. Anal Chim Acta. 2019;1047:214–24.
248. Nazari-Vanani R, Heli H, Sattarahmady N. An impedimetric genosensor for *Leishmania infantum* based on electrodeposited cadmium sulfide nanosheets. Talanta. 2020;217:121080.
249. de Freitas Borges P, Fiel W, Vasconcellos V, Dorledo de Faria R. Current progresses in the development of biosensors for the diagnosis of neglected tropical diseases. Syst Biosci Eng. 2020;1:41–52.
250. Jarockyte G, Karabanovas V, Rotomskis R, Mobasheri A. Multiplexed nanobiosensors: current trends in early diagnostics. Sensors. 2020;20:6893.
251. Quesada-Gonzalez D, Merkoci A. Nanomaterial-based devices for point-of-care diagnostic applications. Chem Soc Rev. 2018;47:4697–709.
252. Martins BR, Barbosa YO, Andrade CMR, Pereira LQ, Simao GF, de Oliveira CJ, et al. Development of an electrochemical immunosensor for specific detection of visceral leishmaniasis using gold-modified screen-printed carbon electrodes. Biosensors. 2020;10:81.
253. World Health Organization. Second WHO model list of essential in vitro Diagnostics. World Health Organization. 2019
254. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE. 2012;7:e35671.
255. Reithinger R, Dujardin J-C, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. Lancet Infect Dis. 2007;7:581–96.
256. Elmahallawy E, Sampedro Martinez A, Rodriguez-Granger J, Hoyos-Mallecot Y, Agil A, Navarro Mari J, et al. Diagnosis of leishmaniasis. J Infect Dev Ctries. 2014;8:961–72.
257. Adaui Sicheri V. Molecular epidemiological approach to the understanding of the emergence and spreading of drug resistance in Neotropical *Leishmania*. Dissertation. Antwerp: Universiteit Antwerpen; 2011.
258. Mock DJ, Hollenbaugh JA, Daddacha W, Overstreet MG, Lazarski CA, Fowell DJ, et al. *Leishmania* induces survival, proliferation and elevated cellular dNTP levels in human monocytes promoting acceleration of HIV co-infection. PLoS Pathog. 2012;8:e1002635.
259. Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet. 2018;392:951–70.
260. Escobar MA, Martinez F, Scott Smith D, Palma GI. American cutaneous and mucocutaneous leishmaniasis (tegumentary): a diagnostic challenge. Trop Doct. 1997;27:69–78.
261. Pavli A, Maltezou HC. Leishmaniasis, an emerging infection in travelers. Int J Infect Dis. 2010;14:e1032–9.
262. de Paulo LF, Rocha GF, Luisi CM Jr, Rosa RR, Durighetto AF Jr. Mucocutaneous leishmaniasis: mucosal manifestations in an endemic country. Int J Infect Dis. 2013;17:e1088–9.
263. Yanik M, Gurel M, Simsek Z, Kati M. The psychological impact of cutaneous leishmaniasis. Clin Exp Dermatol. 2004;29:464–7.
264. Morrison B, Mendoza I, Delgado D, Reyes Jaimes O, Aranzazu N, Paniz Mondolfi AE. Diffuse (anergic) cutaneous leishmaniasis responding to amphotericin B. Clin Exp Dermatol. 2010;35:e116–9.
265. Alcover MM, Rocamora V, Guillen MC, Berenguer D, Cuadrado M, Riera C, et al. Case report: diffuse cutaneous leishmaniasis by *Leishmania infantum* in a patient undergoing immunosuppressive therapy: risk status in an endemic Mediterranean area. Am J Trop Med Hyg. 2018;98:1313–6.
266. Turetz M, Machado P, Ko A, Alves F, Bittencourt A, Almeida R, et al. Disseminated leishmaniasis: a new and emerging form of leishmaniasis observed in northeastern Brazil. J Infect Dis. 2002;186:1829–34.
267. Dassoni F, Daba F, Naafs B, Morrone A. *Leishmaniasis recidivans* in Ethiopia: cutaneous and mucocutaneous features. J Infect Dev Ctries. 2017;11:106–10.
268. Asfaram S, Fakhar M, Mohebali M, Mardani A, Banimostafavi ES, Ziaei Hezarjaribi H, et al. Asymptomatic human blood donors carriers of *Leishmania infantum*: potential reservoirs for visceral leishmaniasis in northwestern Iran. Transfus Apher Sci. 2017;56:474–9.
269. Stark D, Pett S, Marriott D, Harkness J. Post-kala-azar dermal leishmaniasis due to *Leishmania infantum* in a human immunodeficiency virus type 1-infected patient. J Clin Microbiol. 2006;44:1178–80.
270. Steverding D. The history of leishmaniasis. Parasit Vectors. 2017;10:82.
271. Garrido-Jareno M, Sahuquillo-Torralba A, Chouman-Arcas R, Castro-Hernandez I, Molina-Moreno JM, Llavador-Ros M, et al. Cutaneous and mucocutaneous leishmaniasis: experience of a Mediterranean hospital. Parasit Vectors. 2020;13:24.
272. Aliaga L, Cobo F, Mediavilla JD, Bravo J, Osuna A, Amador JM, et al. Localized mucosal leishmaniasis due to *Leishmania (Leishmania) infantum*: clinical and microbiologic findings in 31 patients. Medicine. 2003;82:147–58.
273. Alborzi A, Pouladfar GR, Ghadimi Moghadam A, Attar A, Drakhshan N, Khosravi Maharlooei M, et al. First molecular-based detection of mucocutaneous leishmaniasis caused by *Leishmania major* in Iran. J Infect Dev Ctries. 2013;7:413–6.
274. Dixit K, Singh R, Salotra P. Advancement in molecular diagnosis of post-kala-azar dermal leishmaniasis. Indian J Dermatol. 2020;65:465–72.
275. Mukhopadhyay D, Dalton JE, Kaye PM, Chatterjee M. Post-kala-azar dermal leishmaniasis: an unresolved mystery. Trends Parasitol. 2014;30:65–74.
276. Castilho TM, Shaw JJ, Lucile M, Floeter-Winter LM. New PCR assay using glucose-6-phosphate dehydrogenase for identification of *Leishmania* species. J Clin Microbiol. 2003;41:540–6.
277. Zelazny AM, Fedorko DP, Li L, Neva FA, Fischer SH. Evaluation of 7SL RNA gene sequences for the identification of *Leishmania* spp. Am J Trop Med Hyg. 2005;72:415–20.
278. McAvin JS, Swanson KI, Chan AST, Quintana M, Coleman RE. *Leishmania* detection in sand flies using a field-deployable real-time analytic system. Mil Med. 2012;177:460–6.
279. Fotouhi-Ardakani R, Ghafari SM, Ready PD, Parvizi P. Developing, modifying, and validating a TaqMan real-time PCR Technique for accurate identification of *Leishmania* parasites causing most leishmaniasis in Iran. Front Cell Infect Microbiol. 2021;11:731595.
280. Miranda A, Samudio F, Saldaña A, Castillo J, Brandão A, Calzada JE. The calmodulin intergenic spacer as molecular target for characterization of *Leishmania* species. Parasit Vectors. 2014;7:35.
281. Bhatia A, Sanyal R, Paramchuk W, Gedamu L. Isolation, characterization and disruption of the casein kinase II alpha subunit gene of *Leishmania chagasi*. Mol Biochem Parasitol. 1998;92:195–206.
282. Weirather JL, Jeronimo SMB, Gautam S, Sundar S, Kang M, Kurtz MA, et al. Serial quantitative PCR assay for detection, species discrimination, and quantification of *Leishmania* spp. in human samples. J Clin Microbiol. 2011;49:3892–904.
283. Jamjoom MB, Ashford RW, Bates PA, Chance ML, Kemp SJ, Watts PC, et al. *Leishmania donovani* is the only cause of visceral leishmaniasis in East Africa; previous descriptions of *L. infantum* and "*L. archibaldi*" from this region are a consequence of convergent evolution in the isoenzyme data. Parasitology. 2004;129:399–409.
284. da Silva RE, Sampaio BM, Tonhosolo R, da Costa APR, da Silva Costa LE, Nieri-Bastos FA, et al. Exploring *Leishmania infantum* cathepsin as a new molecular marker for phylogenetic relationships and visceral leishmaniasis diagnosis. BMC Infect Dis. 2019;19:895.
285. Chaouch M, Fathallah-Mili A, Driss M, Lahmadi R, Ayari C, Guizani I, et al. Identification of Tunisian *Leishmania* spp. by PCR amplification of cysteine proteinase B (cpb) genes and phylogenetic analysis. Acta Trop. 2013;125:357–65.
286. Rogers MB, Hilley JD, Dickens NJ, Wilkes J, Bates PA, Depledge DP, et al. Chromosome and gene copy number variation allow major structural change between species and strains of *Leishmania*. Genome Res. 2011;21:2129–42.
287. Wortmann G, Hochberg L, Houng HH, Sweeney C, Zapor M, Aronson N, et al. Rapid identification of *Leishmania* complexes by a real-time PCR assay. Am J Trop Med Hyg. 2005;73:999–1004.
288. Marcili A, Speranca MA, da Costa AP, Madeira Mde F, Soares HS, de Sanches O, et al. Phylogenetic relationships of *Leishmania* species based on trypanosomatid barcode (SSU rDNA) and gGAPDH genes: taxonomic revision of *Leishmania (L.) infantum* chagasi in South America. Infect Genet Evol. 2014;25:44–51.
289. Garcia L, Kindt A, Bermudez H, Llanos-Cuentas A, De Doncker S, Arevalo J, et al. Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. J Clin Microbiol. 2004;42:2294–7.
290. Baharia RK, Tandon R, Sahasrabuddhe AA, Sundar S, Dube A. Nucleosomal histone proteins of *L. donovani*: a combination of recombinant

H2A, H2B, H3 and H4 proteins were highly immunogenic and offered optimum prophylactic efficacy against *Leishmania* challenge in hamsters. PLoS ONE. 2014;9:e97911.
291. Zackay A, Nasereddin A, Takele Y, Tadesse D, Hailu W, Hurissa Z, et al. Polymorphism in the HASPB repeat region of East African *Leishmania donovani* strains. PLoS Negl Trop Dis. 2013;7:e2031.
292. de Almeida ME, Koru O, Steurer F, Herwaldt BL, da Silva AJ. Detection and differentiation of *Leishmania* spp. in clinical specimens by use of a SYBR green-based real-Time PCR assay. J Clin Microbiol. 2017;55:281–90.
293. De Almeida ME, Steurer FJ, Koru O, Herwaldt BL, Pieniazek NJ, Da Silva AJ. Identification of *Leishmania* spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. J Clin Microbiol. 2011;49:3143–9.
294. Zhang RG, Zhang J, Jing BQ. Virulence-associated gene profiling of different *Leishmania* spp. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 2009;27:307–11.
295. Aguiar BG, Coelho DL, Costa DL, Drumond BP, Coelho LF, Figueiredo LC, et al. Genes that encode NAGT, MIF1 and MIF2 are not virulence factors for kala-azar caused by *Leishmania infantum*. Rev Soc Bras Med Trop. 2014;47:593–8.
296. Tupperwar N, Vineeth V, Rath S, Vaidya T. Development of a real-time polymerase chain reaction assay for the quantification of *Leishmania* species and the monitoring of systemic distribution of the pathogen. Diagn Microbiol Infect Dis. 2008;61:23–30.
297. Zauli-Nascimento RC, Miguel DC, Yokoyama-Yasunaka JK, Pereira LI, Pelli de Oliveira MA, Ribeiro-Dias F, et al. In vitro sensitivity of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* Brazilian isolates to meglumine antimoniate and amphotericin B. Trop Med Int Health. 2010;15:68–76.
298. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I. Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. J Clin Microbiol. 2003;41:3147–53.
299. Waki K, Dutta S, Ray D, Kolli BK, Akman L, Kawazu S-I, et al. Transmembrane molecules for phylogenetic analyses of pathogenic protists: *Leishmania*-specific informative sites in hydrophilic loops of trans-endoplasmic reticulum *N*-acetylglucosamine-1-phosphate transferase. Eukaryot Cell. 2007;6:198–210.
300. Hadighi R, Mohebali M, Boucher P, Hajjaran H, Khamesipour A, Ouellette M. Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. PLoS Med. 2006;3:e162.
301. Hide M, Banuls AL. Species-specific PCR assay for *L. infantum/L. donovani* discrimination. Acta Trop. 2006;100:241–5.
302. Vallur AC, Duthie MS, Reinhart C, Tutterrow Y, Hamano S, Bhaskar KR, et al. Biomarkers for intracellular pathogens: establishing tools as vaccine and therapeutic endpoints for visceral leishmaniasis. Clin Microbiol Infect. 2014;20:O374–83.
303. Eslami G, Salehi R. Genetic variation in RPOIILS gene encoding RNA polymerase II largest subunit from *Leishmania major*. Mol Biol Rep. 2014;41:2585–9.
304. Giraud E, Martin O, Yakob L, Rogers M. Quantifying *Leishmania* metacyclic promastigotes from individual sandfly bites reveals the efficiency of vector transmission. Commun Biol. 2019;2:84.
305. Chiurillo MA, Sachdeva M, Dole VS, Yepes Y, Miliani E, Vazquez L, et al. Detection of *Leishmania* causing visceral leishmaniasis in the Old and New Worlds by a polymerase chain reaction assay based on telomeric sequences. Am J Trop Med Hyg. 2001;65:573–82.
306. Khosravi S, Hejazi S, Hashemzadeh M, Eslami G, Darani H. Molecular diagnosis of Old World leishmaniasis: real-time PCR based on tryparedoxin peroxidase gene for the detection and identification of *Leishmania* spp. J Vector Borne Dis. 2012;49:15–8.
307. Hitakarun A, Tan-ariya P, Siripattanapipong S, Mungthin M, Piyaraj P, Naaglor T, et al. Comparison of PCR methods for detection of *Leishmania siamensis* infection. Parasit Vectors. 2014;7:458.
308. Haouas N, Garrab S, Gorcii M, Khorchani H, Chargui N, Ravel C, et al. Development of a polymerase chain reaction-restriction fragment length polymorphism assay for *Leishmania major/Leishmania killicki/Leishmania infantum* discrimination from clinical samples, application in a Tunisian focus. Diagn Microbiol Infect Dis. 2010;68:152–8.
309. Romano A, Inbar E, Debrabant A, Charmoy M, Lawyer P, Ribeiro-Gomes F, et al. Cross-species genetic exchange between visceral and cutaneous strains of *Leishmania* in the sand fly vector. Proc Natl Acad Sci USA. 2014;111:16808–13.
310. Ibrahim ME, Barker DC. The origin and evolution of the *Leishmania donovani* complex as inferred from a mitochondrialcytochrome oxidase II gene sequence. Infect Genet Evol. 2001;1:61–8.
311. Yang BB, Chen DL, Chen JP, Liao L, Hu X, Xu JN. Analysis of kinetoplast cytochrome b gene of 16 *Leishmania* isolates from different foci of China: different species of *Leishmania* in China and their phylogenetic inference. Parasit Vectors. 2013;6:32.
312. Flegontov PN, Strelkova MV, Kolesnikov AA. The *Leishmania major* maxicircle divergent region is variable in different isolates and cell types. Mol Biochem Parasitol. 2006;146:173–9.
313. Zarean M, Maraghi SH, Hajjaran H, Mohebali M, Feiz-Haddad MH, Assarehzadegan MA. Correlation between clinical responses with the drug-susceptibility of parasites in Iranian cutaneous leishmaniasis caused by *Leishmania major*. Trop Biomed. 2016;34:338–45.

Publisher's Note

Chapter 2

Semi-Quantitative, Duplexed qPCR Assay for the Detection of *Leishmania* spp. Using Bisulphite Conversion Technology

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Publication:

Gow, I., Millar, D.S., Ellis, J., Melki, J., Stark, D. 2019., *Semi-Quantitative, Duplexed qPCR Assay for the Detection of Leishmania spp. Using Bisulphite Conversion Technology*. **Trop Med Infect Dis**, 4(4), 1-11.

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Date: March 2023

Tropical Medicine and Infectious Disease

MDPI

Article

Semi-Quantitative, Duplexed qPCR Assay for the Detection of *Leishmania* spp. Using Bisulphite Conversion Technology

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Received: 6 October 2019; Accepted: 28 October 2019; Published: 1 November 2019

Abstract: Leishmaniasis is caused by the flagellated protozoan *Leishmania*, and is a neglected tropical disease (NTD), as defined by the World Health Organisation (WHO). Bisulphite conversion technology converts all genomic material to a simplified form during the lysis step of the nucleic acid extraction process, and increases the efficiency of multiplex quantitative polymerase chain reaction (qPCR) reactions. Through utilization of qPCR real-time probes, in conjunction with bisulphite conversion, a new duplex assay targeting the 18S rDNA gene region was designed to detect all *Leishmania* species. The assay was validated against previously extracted DNA, from seven quantitated DNA and cell standards for pan-*Leishmania* analytical sensitivity data, and 67 cutaneous clinical samples for cutaneous clinical sensitivity data. Specificity was evaluated by testing 76 negative clinical samples and 43 bacterial, viral, protozoan and fungal species. The assay was also trialed in a side-by-side experiment against a conventional PCR (cPCR), based on the Internal transcribed spacer region 1 (ITS1 region). Ninety-seven percent of specimens from patients that previously tested positive for *Leishmania* were positive for *Leishmania spp.* with the bisulphite conversion assay, and a limit of detection (LOD) of 10 copies per PCR was achieved, while the LOD of the ITS1 methodology was 10 cells/1000 genomic copies per PCR. This method of rapid, accurate and simple detection of *Leishmania* can lead to improved diagnosis, treatment and public health outcomes.

Keywords: leishmaniasis; qPCR; bisulphite

1. Introduction

Leishmaniasis is an infection caused by some species of *Leishmania* parasites that affect the skin, organs and mucosal regions of the body, leading to serious morbidity and possibly death. It is classed as a Neglected Tropical Disease (NTD), affecting 12 million people worldwide, with a further 350 million people at risk of contracting the disease [1]. With the advent of increased human international travel, due to work, tourism or war, leishmaniasis is now an emerging infectious disease, with an increased impact on global mortality and morbidity [2]. It is becoming increasingly clear that there is a need for accurate and rapid detection of *Leishmania* in the form of a standardized and validated assay, to aid diagnosis, treatment and surveillance programs.

Many validated molecular *Leishmania* detection assays use conventional PCR (cPCR) for the detection of *Leishmania* infection [3–5]. Conventional PCR is a diagnostic method where DNA is amplified using a thermal cycler, amplicons are separated due to molecular weight by electrophoresis, and detected by stain (usually ethidium bromide or gel red) and UV light (via a transilluminator) [6].

This approach requires significantly more hands-on time, has a greater risk of contamination and makes multiplexing analysis more difficult if products are similar in size, compared to real-time PCR. Probe-based qPCR can overcome these issues. Additionally, specificity can be increased, and it allows for continuous monitoring of the PCR. The 18S rRNA gene (18S rDNA) is a highly conserved gene across all *Leishmania* species, despite having diverged from other similarly related species during the period Paleogene or Paleocene [7]. The gene exists in between 50–200 copies per *Leishmania* genome, making it an excellent choice for a pan-*Leishmania* detection assay [6]. To assess whether this target can be used in a novel diagnostic assay, based on bisulphite conversion and real-time PCR technologies, a series of experiments were performed to assess the limit of detection and sensitivity of the assay, and the new assay was compared to a cPCR, based on the ITS1 region, developed by Schönian et al. [8].

The development of this novel bisulphite-converted, qPCR assay methodology, based on genus-specific primer and probe designs for the 18S rDNA, and its validation, is described in this paper. Furthermore, the bisulphite conversion and purification of protozoan DNA are discussed. The assay's limit of detection was 10 cellular or genomic copies/PCR, with clinical sensitivity and specificity demonstrated to be 97.0% and 100%, respectively. The assay takes under 2.5 hours to complete, making the assay a potential diagnostic tool for both diagnostic and research laboratories worldwide.

2. Materials and Methods

2.1. Specimens Tested

DNA was purified from cell-cultured promastigotes of the following species: *L. donovani* (MHOM/IN/80/DD8 supplied at 2.3×10^7 cells/mL), *L. braziliensis* (MHOM/BR/75/M2903 supplied at 1.63×10^8 cells/mL), *L. tropica* (MHOM/SU/74/K27 supplied at 1.03×10^7 cells/mL), *L. amazonensis* (MHOM/BR/73/M2269 supplied at 9.9×10^6 cells/mL), *L. mexicana* (MHOM/BZ/82/BEL21 supplied at 1.51×10^8 cells/mL) and *L. major* (MHOM/SU/73/5-ASKH supplied at 7.1×10^6 cells/mL), obtained from the American type culture collection (ATCC, Manassas, USA). *Leishmania infantum* genomic DNA (supplied at 1.2×10^7 copies/mL) was obtained from Vircell (Vircell, Granada, Spain). The assay was initially evaluated by performing a 10-fold serial dilution series of the DNA from these strains to assess the limit of detection. In addition, DNA from 67 previously extracted cutaneous clinical samples (derived from 66 unique patients), that were previously identified by St. Vincent's Hospital, Sydney as positive for *Leishmania* by the cPCR method, during the period 2007–2016, were included in the study [8–10]. All DNA was initially extracted using the EZ1 tissue kit on the EZ1 biorobot (Qiagen, Hilden, Germany), in accordance with manufacturers' recommendations regarding direct sample or following culture. Specificity was assessed by extracting DNA using standard methods from 76 negative tissue samples, previously characterised at St. Vincent's Hospital, Sydney, and 43 potential cross-reacting organisms, and testing them in the assay (Table 1). The clinical specimens were tested in accordance with St Vincent's Hospital ethics approval, HREC number LNR/16/SVH/231.

Table 1. List of organisms used in this study for cross-reactivity testing for the novel bisulphite conversion assay.

Specimen Number	Organism
1	*Acinetobacter baumanni*
2	*Bacillus cereus*
3	*Bacillus subtilis*
4	*Clostridium perfringens*
5	*Clostridium sordelli*
6	*Escherichia coli*

Table 1. *Cont.*

Specimen Number	Organism
7	*Haemophilus influenzae*
8	*Klebsiella oxytoca*
9	*Klebsiella pneumoniae*
10	*Moraxella cattaharalis*
11	*Proteus mirabilis*
12	*Proteus vulgaris*
13	*Pseudomonas aeruginosa*
14	*Staphylococcus aureus*
15	*Staphylococcus hominis*
16	*Streptococcus pyogenes*
17	*Streptococcus sp. (mutans)*
18	*Yersinia sp.*
19	*Mycobacteria abscessus*
20	*Mycobacteria marinum*
21	*Mycobacteria sp.*
22	Herpes Simplex Virus Type I
23	Herpes Simplex Virus Type II
24	Varicella Zoster Virus
25	*Trichophyton tonsurans*
26	*Trichophyton mentagrophytes*
27	*Microsporum canis*
28	*Aspergillus fumigatus*
29	*Acromium pulluans*
30	*Acromium strictum*
31	*Aspergillus sp.*
32	*Bipolaris sp.*
33	*Fusarium sp.*
34	*Penicillium sp.*
35	*Scedosporium prolificans*
36	*Trichophyton rubrum*
37	Bovine
38	Human
39	*Trypanosoma cruzi*
40	*Crithidia lucilae*
41	*Trichomonas vaginalis*
42	*Giardia intestinalis*
43	*Entamoeba histolytica*

2.2. DNA Conversion and Quality Control

Genomic DNA was bisulphite converted by adding 2,880,000 or 28,800 copies of DNA/cellular standards, depending on available starting concentration, to a total volume of 150 µL with molecular grade H_2O, then adding 250 µL 3M sodium bisulphite. Alternatively, 5 µL of DNA, previously extracted from cutaneous clinical sample DNA, were added to 145 µL of molecular grade H_2O, then 250 µL 3M sodium bisulphite was added. One negative process control of 150 µL molecular grade H_2O was included in each run, to check for contamination. A total of 5×10^5copies/µL Lambda DNA (strain cI857 ind 1 Sam 7) (New England Biolabs, Ipswich, USA), an *Escherichia coli* bacteriophage, was added to each of these reactions, then the samples were mixed by vortexing, and incubated at 95 °C for 15 minutes. Subsequently 200 µL of this lysate was purified on the GS-mini (Genetic Signatures Ltd., Sydney, Australia) with the Sample Processing Pathogens A kit (Genetic Signatures Ltd., Sydney, Australia), according to the manufacturers' recommendations. The eluted DNA was then diluted in molecular grade H_2O in 10-fold dilution series, to 0.1 copy per PCR. The limit of detection (LOD) for this study was defined as the lowest concentration of DNA at which the assay detected 10 out of 10 replicates, in accordance with CLSI standards, which define the LOD as the lowest dilution where 95% of replicates are positive [11]. Cell and DNA concentrations were provided by the suppliers and copy number was calculated (https://www.thermofisher.com/au/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html). The GS-mini employs a closed cartridge-based system, whereby nucleic acid is bound to magnetic beads, with subsequent washing and, finally, elution steps, using heating and shaking to increase nucleic acid yield. Separate PCR areas were used for mastermix preparation, DNA seeding and PCR reactions, to prevent the possibility of PCR contamination. The addition of lambda bacteriophage DNA to the PCR reaction was used to monitor the efficiency of the bisulphite conversion, purification, and in assessing for possible false negatives due to PCR inhibition. A negative process control (molecular grade H_2O) controlled for possible PCR contamination.

An external positive control was developed by creating a geneblock—a synthetic double stranded 1000bp-long fragment of the 18S rDNA of *L. donovani*, (GenBank accession CP022642 positions 1047751 to 1048750), consisting of adenine, thymine, cytosine or guanine residues only. This was bisulphite converted and diluted to five copies/µL in molecular grade H_2O, using the previously described protocol.

2.3. PCR Primer and Probe Design

For the 18S rDNA assay forward, reverse primers and a probe were designed, based on multiple sequence alignments of the 18S rDNA in bisulphite converted form (that is, with all cytosines converted to thymines), of the species *Leishmania aethiopica, Leishmania amazonensis, Leishmania braziliensis, Leishmania colombiensis, Leishmania donovani, Leishmania guyanensis, Leishmania infantum, Leishmania lainsoni, Leishmania major, Leishmania mexicana, Leishmania naiffi, Leishmania panamensis, Leishmania shawi* and *Leishmania tropica*. This resulted in the identification of primers PL-18S-F2 (TTATTGTTTTGGTTTTTG) and PL-18S-R2 (AAACCAAAATTACAATAAAA) and probe PL-18S-P2 (GGAGATTATGGAGTTGTGTGATA), which amplify and detect DNA fragments of 82bp in length. The exogenous control was targeted by primers Lambda New F1 (AATATTGGTAGATTATGTTTGTG), Lambda New R1 (CTATCATCAAATCATACAATACC) and probe Lambda New P1 (TGATGTGATAGGAAGAATTTGTTGTTGTTGTTGTTG), which amplify a 100bp fragment of the Lambda bacteriophage DNA. The 18S rDNA and Lambda probes are intercalating, self-quenching probes, labeled with individual fluorophores (FAM and HEX, respectively) enabling the PCR to be performed as a duplex reaction.

2.4. PCR Preparation, Conditions, and Interpretation

The PCR mixture was prepared by using 10µL of 2x SensiFast (Bioline), 90ng of each primer PL-18S-F2/PL-18S-R2 and 8 pmol probe PL-18S-P2, 4ng primer Lambda New F1, 40ng primer Lambda New R1 and 3pmol probe Lambda New P1, 3.5µL of template, and molecular grade H_2O, to a final volume of 20µl. All DNA templates were tested in 10 PCR replicates. A negative template control reaction was included in each PCR run. PCRs were run on the MIC PCR thermal cycler (Bio Molecular Systems, Upper Coomera, Australia) using the following parameters: 95 °C for 3 min, and 50 cycles of 95 °C for 2 s and 50 °C for 10 s, 55 °C for 10 s (data acquisition step) and 60 °C for 10 s.

The new assay was tested against the Schönian method by processing the equivalent concentration of *Leishmania* cells or genomic DNA, diluting these in molecular grade H_2O, and heating at 70 °C for 15 minutes. Next, these lysates were processed on the GS-mini, using the MagPurix Viral/Pathogen Nucleic Acids Extraction Kit (Zinexts Life Science, Taipei, Taiwan) on the GS-mini, following the manufacturers' instructions. The eluates were diluted in the same fashion as the bisulphite-treated eluates and amplified in cPCR triplicates, according to the methodology developed by Schönian et al., with primers LITSR: CTGGATCATTTTCCGATG and L5.8S: TGATACCACTTATCGCACTT (targeting the ssu rRNA and 5.8S rRNA, respectively) [8].

3. Results

3.1. Specificity of the Real-Time PCR Assay Using Quantitated Cultured Cell or Purified DNA Standards

DNA converted from the panel of seven *Leishmania* quantitated standards (*L. donovani, L. braziliensis, L. tropica, L. amazonensis, L. major, L. mexicana* and *L. infantum)* were detected by the 18S rDNA assay (Table 2). As displayed in Table 2, there was a concordance between these results and the Schönian method, as all *Leishmania* species tested were detected [8].

Table 2. Detection limit of the conventional and novel PCR assays.

Species	Supplier	Schönian Method	Novel Method
L. donovani	ATCC	100 cells/PCR	10 cells/PCR
L. braziliensis	ATCC	100 cells/PCR	10 cells/PCR
L. tropica	ATCC	100 cells/PCR	10 cells/PCR
L. amazonensis	ATCC	100 cells/PCR	10 cells/PCR
L. mexicana	ATCC	100 cells/PCR	100 cells/PCR
L. major	ATCC	10 cells/PCR	10 cells/PCR
L. infantum	Vircell	1000 copies/PCR	10 copies/PCR

3.2. Specificity of the Real-Time PCR Assay Using Negative Control Samples

DNA, extracted from 76 negative clinical samples, did not produce any PCR products using the new assay, giving a specificity of 100% [8].

3.3. Specificity of the Real-Time PCR Assay Using Cross-Reactivity Specimens

To further investigate the specificity of the assay, a panel of DNA from 43 other phylogenetically related organisms, or those with a differential diagnosis related to leishmaniasis, was tested (Table 1). No PCR products were detected from any of these specimens, giving a specificity of 100%.

3.4. Limit of Detection of the Real-Time PCR Assay Using Quantitated Standards

The analytical sensitivity of the assay was evaluated using quantitated DNA and cell culture standards. Ten-fold serial dilutions were tested in the assay, and the LOD for *Leishmania* was shown to

be 10 cellular/genomic copies per PCR reaction, although this LOD differed between species, as outlined in Table 2. For *L. braziliensis*, for example, the LOD was 10 cellular copies, and an average of 38.7 cycle threshold (C_T) value was determined after testing the sample in 10 PCR replicates. When *L. braziliensis* was tested by the Schönian method, the LOD was 100 cellular copies/PCR when tested in triplicate (Figure 1). ATCC quantitation was given in cells/μL and Vircell quantitation was given in copies/μL, so this nomenclature has been upheld.

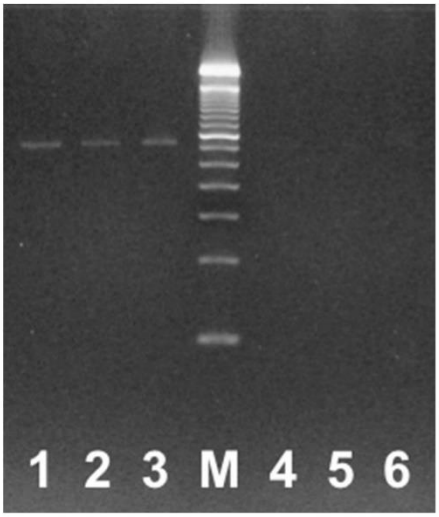


Figure 1. Sensitivity of the ITS1 cPCR assay for *L. braziliensis*, gel electrophoresis of conventional PCR result, using the Schönian method. Lanes 1, 2 and 3 are 100 copies/PCR; lane M is the 100bp ladder size standard, lanes 4, 5 and 6 are 10 copies/PCR.

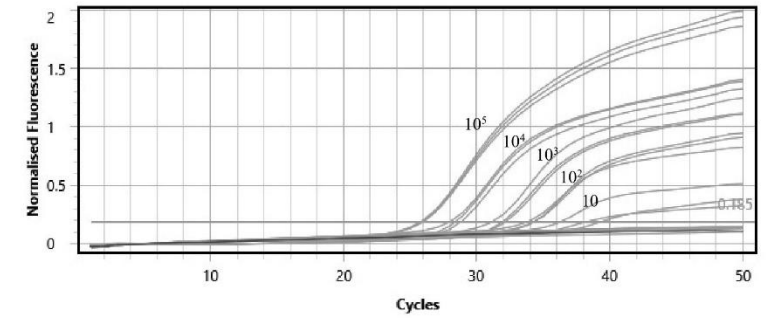
3.5. Sensitivity of the Real-Time PCR Assay Using Clinical Sample DNA

Previously extracted DNA from 67 clinical tissue samples was available from patients with confirmed diagnosis of cutaneous leishmaniasis. Although clinical data were not available for all specimens, of those samples with data available, 32 (72.7%) were male, and the age range was between one and 73 years. Forty-two patients had data available on previous travel; 21 (50.0%) of these patients had been to the middle east, 11 (26.2%) had been to South America, three (7.1%) had been to southern Europe, one (2.4%) to South Asia and five patients (11.9%) had been to multiple geographic regions. Reason for travel data were available for 39 patients; 26 (66.7%) were travellers, nine were immigrants (28.2%) and two (5.1%) were members of the army. Resulting cPCR (Schönian method) and restriction fragment length polymorphism analysis were used for detection and species differentiation, respectively [8]. Of the 67 clinical samples, the novel assay was detected in 65, thus 97.0 % concordance was achieved between the previous method and the 18S assay.

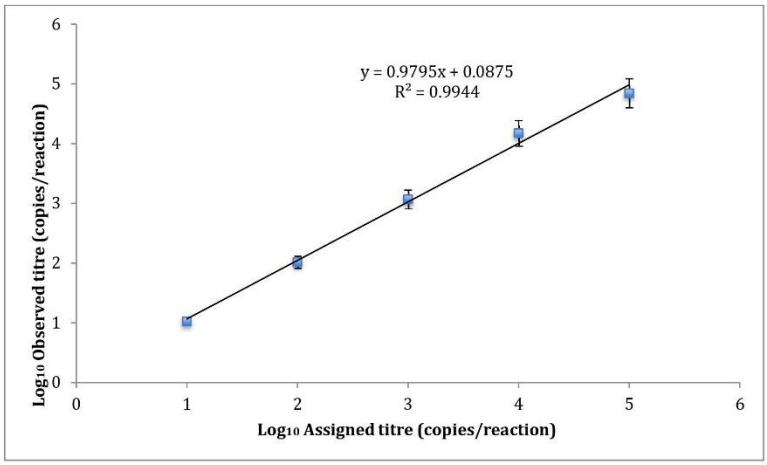
3.6. Precision of the Real-Time PCR Assay Using Quantitated Standards

Standard curves were produced for *L. braziliensis* and *L. tropica*, testing 10-fold serial dilutions in PCR triplicates, giving an R^2 value of 0.9945 for *L. braziliensis* (Figure 2a,b). This is a measure of the linearity of the generated curves and reflects efficiency and reproducibility. Error bars depict 95% confidence intervals, based upon two experimental replicates, comprising three PCR technical replicates

each. To measure the intra-experiment precision and agreement between experiments, five experiments were each performed over five consecutive days, with three replicates at two cellular concentrations for each species (Table 3). The results considered over 236 replicates, with four negative replicates for *L. tropica* excluded. Low coefficients of variation (CVs), related to intra-experiment variability, were observed, all <10%. These findings provide additional support that this novel, real-time PCR provides efficient and precise quantification of DNA within and between experiments.



(**a**)



(**b**)

Figure 2. Sensitivity of the novel qPCR assay for *L. braziliensis*. (**a**) FAM channel amplification curves, using 10-fold serial dilutions from 10^5 to 0.1 copies per PCR, tested in PCR triplicate. (**b**) Graphic depiction of the linear range of detection (10 to 10^5 copies per reaction). Error bars represent 95% CI.

Table 3. Summary of the Observed Precision Estimates for the novel assay.

Leishmania Species and Copy Number	Mean (Ct)	SD (Ct)	CV (%)
L. donovani 1000 c/PCR	30.26	0.88	2.92
L. donovani 100 c/PCR	33.28	0.99	2.98
L. braziliensis 100 c/PCR	30.78	0.87	2.84
L. braziliensis 10 c/PCR	33.71	0.67	1.99
L. tropica 100 c/PCR	34.07	0.95	2.80
L. tropica 10 c/PCR[1]	37.83	2.31	6.11
L. amazonensis 100 c/PCR	31.29	1.09	3.50
L. amazonensis 10 c/PCR	34.24	2.19	6.39
L. mexicana 1000 c/PCR	33.08	1.19	3.61
L. mexicana 100 c/PCR	36.22	1.52	4.19
L. major 100 c/PCR	31.96	0.84	2.62
L. major 10 c/PCR	35.09	0.74	2.10
L. infantum 1000 c/PCR	29.44	1.22	4.13
L. infantum 100 c/PCR	32.23	1.47	4.57

[1] For *L. tropica*, only 2/3 replicates were achieved on four of the five consecutive days tested. In order to assess mean, SD and CV, the negative data points were excluded from the data set.

3.7. Internal Control Reaction

No samples showed inhibition of the exogenous control.

4. Discussion

The development of a multiplexed, real-time PCR, targeting the 18S rDNA to detect all *Leishmania* species and the associated automated bisulphite conversion system, is described. The assay was validated on DNA and cell standards and the limit of detection, using seven individual strains of *Leishmania*. The LOD was compared with the method of Schönian et al. [8] and, as can be seen from Table 2, the LOD of all seven species improved upon using the real-time PCR method [8]. No cross-reactivity was observed using a panel of 43 possible cross-reacting organisms (Table 1) and 76 negative tissue samples, making the assay exclusive to *Leishmania* DNA detection. For clinical performance, the DNA of 67 previously described positive tissue samples were tested, alongside a conventional PCR method, as described by Schönian et al., and 65 samples tested positive in the 18S rDNA real-time PCR assay [8]. The Schönian method is based on the ITS1 region, a gene also located on the ribosomal DNA array, and thus present in the same number of copies as the 18S rRNA gene [12]. The novel assay includes an exogenous control, which controls for extraction and PCR performance, and an external positive control, controlling for PCR performance. The inclusion of quality controls, both internal and external, was highlighted as important in *Leishmania* detection assays [13]. The turn-around time is less than 2.5 hours from sample to result, and the system has a small laboratory footprint (the area required in the laboratory for instrumentation) of 75cm by 75cm.

The assay is based on the gene coding for the small subunit rRNA, a highly conserved region of the ribosomal DNA, located on chromosome 27. This gene was used for the detection of *Leishmania* in other assays, due to its excellent sensitivity, attributed to the fact that it is a multicopy gene, which is transcribed into abundant rRNA found in the cytoplasm, where it is predicted to be present at 10^4 copies [7,14–16].

The test utilises bisulphite conversion technology, whereby the genome is simplified to three nucleobases: A, T and G (Figure S1). This simplification of the genome enables easier design of primers and probes across subtypes and species variants, as single oligonucleotide sets can be designed to cover

a diverse population and reduce the need for multiplexing, in order to capture all species. Furthermore, the increase in homology allows for different primer and probe sets of differing targets to be designed with similar melting temperatures (Tm), reducing potential issues with specificity (Table S1). This was previously demonstrated in two clinical trials, where nearly 100% sensitivity and specificity were achieved by the increased homology and similar Tm of the primers and probes designed for the assays [17,18]. Bisulphite conversion technology is already in use in various diagnostic laboratories in the detection of clinical sample types, including gastro-intestinal infections [18,19]. The bisulphite conversion is included in the initial lysis step and therefore requires no extra steps by the end user. This is the first *Leishmania* detection assay exploiting bisulphite technology. The bisulphite conversion technology can also be used in an assay designed to differentiate *Leishmania* species. As there are over 20 *Leishmania* species pathogenic to humans, these will need to be multiplexed with up to four other targets into at least five panels [20]. A similar Tm greatly reduces the risk of non-specific amplification, as a lower melting temperature can be used across the PCR cycling protocol, but will accommodate all targets. In this way, a future assay may be designed to incorporate the novel pan-*Leishmania* assay to screen a given sample, then a reflex assay may be used to identify the causative *Leishmania* species. In intercalating self-quenching probes, such as those used in this assay, the fluorescent dye and quencher are at separate ends, that are in a hairpin conformation when not bound to target [21]. This gives less non-specific fluorescence, as the probe is in close proximity to the quencher, and, thus, is more effectively quenched.(See Supplementary Materials)

Leishmania DNA-based detection in the laboratory is dominated by cPCR, nested PCR or qPCR. Conventional PCR has sensitivities ranging from 56% to 100%, depending on clinical specimen and gene target [8,22]. In nested PCR, an inner and outer set of primers are designed and tested in two rounds to increase sensitivity and specificity [23]. In an Iranian study of cutaneous leishmaniasis patients, it gave a sensitivity of 100% [24]. Both these methods, however, are time-consuming and laborious, requiring gel electrophoresis and a transilluminator for imaging post-PCR. This may also leave the laboratory open to contamination risk during these post-PCR methods. Quantitative PCR is a closed-tube system, where one step is required between DNA addition and result, and results may be read in real-time [25]. It achieves sensitivities and specificities of up to 100% [26,27]. The novel qPCR achieved a lower LOD than cPCR, an outcome seen in other *Leishmania* assays utilising various targets [14,28,29]. Our future studies will determine the clinical sensitivity of samples previously tested positive for visceral leishmaniasis, to complement the clinical data obtained here.

Currently, there are very few commercial assays available on the market for the detection of all *Leishmania* species, particularly those based on the detection of *Leishmania* DNA, however, no formal evaluations are described in the scientific literature. Primer Design provide a primer and probe set with mastermix and controls, which claims to detect all *Leishmania* species, based on the cytochrome b gene. This is a qPCR test, providing lyophilized components, with a sensitivity of 100 copies (http://www.genesig.com/assets/files/leishmania_spp_std.pdf). Another assay detects *L. major* only (MyBioSource), through a qPCR assay containing the primers, probes, mastermix and controls. It claims a sensitivity of 100 copies of target template (https://www.mybiosource.com/images/tds/protocol_manuals/000000-799999/MBS486088_Easy.pdf). BioKits have a cPCR kit detection *Leishmania* spp., containing ready-to-use PCR mix and positive control, with a sensitivity of 20 copies/mL (http://www.biokits.com/productinfo/3587/Leishmania-sp.-PCR-Detection-Kit.html). The US army has an FDA-approved *Leishmania* qPCR detection kit called SMART Leish, developed in conjunction with Cepheid and the Walter Reed Army Institute of Research for the diagnosis of species associated with cutaneous leishmaniasis, with an LOD of four genome copies (http:/www.accessdata.fda.gov/cdrh_docs/pdf8/K081868.pdf). Its use is restricted to the Department of Defense laboratories, and thus not available commercially.

The novel pan-*Leishmania* assay provides a simple, economical solution for a high-tech molecular detection system, while retaining excellent sensitivity and specificity, that can be easily used in reference and satellite laboratories alike. Moreover, the automated nature of this system and its low cost means

its application is feasible in many countries where leishmaniasis is endemic, which may lack the finances and expertise to implement high-tech laboratory diagnostics, such as qPCR. Such an efficient workflow and quality performance assures that reliable patient results can be diagnosed quickly, treatment regimes can be administered, and prognosis can be assessed.

Supplementary Materials: The following are available online at http://www.mdpi.com/2414-6366/4/4/135/s1, Figure S1: Conventional and bisulphite converted alignments for the 18S rDNA gene, Table S1: Conventional and bisulphite converted primer and probe designs for the novel assay.

Author Contributions: Conceptualization, I.G., D.S., D.M. and J.E.; methodology, I.G.; validation, I.G.; formal analysis, I.G.; investigation, I.G.; resources, J.M.; data curation, I.G.; writing—original draft preparation, I.G.; writing—review and editing, D.S., D.M. and J.E; visualization, I.G.; supervision, D.S.; project administration, I.G.; funding acquisition, J.M.

Funding: This research received no external funding

Acknowledgments: We are grateful to Rogan Lee for the supply of *Leishmania*.

Conflicts of Interest: DM, IG and JM are employees of Genetic Signatures Ltd.

References

1. World Health Organisation. *Control of the Leishmaniases*; WHO Technical Report Series 949; World Health Organisation: Geneva, Switzerland, 2010.
2. Hotez, P.J. Human Parasitology and Parasitic Diseases: Heading Towards. *Adv. Parasitol.* **2018**, *100*, 29–38. [PubMed]
3. Gualda, K.P.; Marcussi, L.M.; Neitzke-Abreu, H.C.; Aristides, S.M.A.; Lonardoni, M.V.C.; Cardoso, R.F.; Silveira, T.G.V. New Primers for Detection of *Leishmania infantumusing* Polymerase Chain Reaction. *Rev. Inst. Med. Trop. Sao Paulo* **2015**, *57*, 377–383. [CrossRef] [PubMed]
4. Ranasinghe, S.; Wickremasinghe, R.; Hulangamuwa, S.; Sirimanna, G.; Opathella, N.; Maingon, R.D.; Chandrasekharan, V. Polymerase chain reaction detection of Leishmania DNA in skin biopsy samples in Sri Lanka where the causative agent of cutaneous leishmaniasis is Leishmania donovani. *Memórias Inst. Oswaldo. Cruz* **2015**, *110*, 1017–1023. [CrossRef] [PubMed]
5. De Cassia-Pires, R.; de Melo, M.F.; Barbosa, R.D.; Roque, A.L. Multiplex PCR as a tool for the diagnosis of Leishmania skDNA and the gapdh housekeeping gene of mammal hosts. *PLoS ONE* **2017**, *12*, e0173922. [CrossRef]
6. Srivastava, P.; Mehrotra, S.; Tiwary, P.; Chakravarty, J.; Sundar, S. Diagnosis of Indian Visceral Leishmaniasis by Nucleic Acid Detection Using PCR. *PLoS ONE* **2011**, *6*, e19304. [CrossRef]
7. Tuon, F.F.; Neto, V.A.; Amato, V.S. Leishmania: origin, evolution and future since the Precambrian. *FEMS Immunol. Med. Microbiol.* **2008**, *54*, 158–166. [CrossRef]
8. Schönian, G.; Nasereddin, A.; Dinse, N.; Schweynoch, C.; Schallig, H.D.F.H.; Presber, W.; Jaffe, C.L. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. *Diagn. Microbiol. Infect. Dis.* **2003**, *47*, 349–358. [CrossRef]
9. Roberts, T.; Barratt, J.; Sandaradura, I.; Lee, R.; Harkness, J.; Marriott, D.; Ellis, J.; Stark, D. Molecular Epidemiology of Imported Cases of Leishmaniasis in Australia from 2008 to 2014. *PLoS ONE* **2015**, *10*, e0119212. [CrossRef]
10. Lee, R.; Marriott, D.; Stark, D.; Van Hal, S.; Harkness, J. Leishmaniasis, an Emerging Imported Infection: Report of 20 Cases from Australia: Table. *J. Travel Med.* **2008**, *15*, 351–354.
11. Larrisey, M.P. *EP17-A2 Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures. Approved Guideline—Second Edition*; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2012; pp. 2–18.
12. Töz, S.; Özensoy; Çulha, G.; Zeyrek, F.Y.; Ertabaklar, H.; Alkan, M.Z.; Vardarlı, A.T.; Gunduz, C.; Özbel, Y. A Real-Time ITS1-PCR Based Method in the Diagnosis and Species Identification of Leishmania Parasite from Human and Dog Clinical Samples in Turkey. *PLoS Neglected Trop. Dis.* **2013**, *7*, e2205.
13. Gonçalves-de-Albuquerque, S.D.C.; Pessoa-e-Silva, R.; Trajano-Silva, L.A.M.; de Morais, R.C.S.; Brandao-Filho, S.P.; de Paiva-Cavalcanti, M. Inclusion of quality controls on leishmaniases molecular tests to increase diagnostic accuracy in research and reference laboratories. *Mol. Biotechnol.* **2015**, *57*, 318–324. [CrossRef] [PubMed]

14. León, C.M.; Muñoz, M.; Hernández, C.; Ayala, M.S.; Flórez, C.; Teherán, A.; Cubides, J.R.; Ramírez, J.D. Analytical Performance of Four Polymerase Chain Reaction (PCR) and Real Time PCR (qPCR) Assays for the Detection of Six Leishmania Species DNA in Colombia. *Front. Microbiol.* **2017**, *8*, 8. [CrossRef] [PubMed]
15. Vaish, M.; Mehrotra, S.; Chakravarty, J.; Sundar, S. Noninvasive Molecular Diagnosis of Human Visceral Leishmaniasis. *J. Clin. Microbiol.* **2011**, *49*, 2003–2005. [CrossRef] [PubMed]
16. Van Eys, G.J.J.M.; Schoone, G.J.; Kroon, N.C.; Ebeling, S.B. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of Leishmania parasites. *Mol. Biochem. Parasitol.* **1992**, *51*, 133–142.
17. Siah, S.P.; Merif, J.; Kaur, K.; Nair, J.; Huntington, P.G.; Karagiannis, T.; Stark, D.; Rawlinson, W.; Olma, T.; Thomas, L.; et al. Improved detection of gastrointestinal pathogens using generalised sample processing and amplification panels. *Pathology* **2014**, *46*, 53–59. [CrossRef]
18. Baleriola, C.; Millar, D.; Melki, J.; Coulston, N.; Altman, P.; Rismanto, N.; Rawlinson, W. Comparison of a novel HPV test with the Hybrid Capture II (hcII) and a reference PCR method shows high specificity and positive predictive value for 13 high-risk human papillomavirus infections. *J. Clin. Virol.* **2008**, *42*, 22–26. [CrossRef]
19. Stark, D.; Roberts, T.; Ellis, J.; Marriott, D.; Harkness, J. Evaluation of the EasyScreen™ Enteric Parasite Detection Kit for the detection of Blastocystis spp., Cryptosporidium spp., Dientamoeba fragilis, Entamoeba complex, and Giardia intestinalis from clinical stool samples. *Diagn. Microbiol. Infect. Dis.* **2014**, *78*, 149–152. [CrossRef]
20. Akhoundi, M.; Downing, T.; Votýpka, J.; Kuhls, K.; Lukeš, J.; Cannet, A.; Ravel, C.; Marty, P.; Delaunay, P.; Kasbari, M.; et al. Leishmania infections: Molecular targets and diagnosis. *Mol. Asp. Med.* **2017**, *57*, 1–29. [CrossRef]
21. Kjelland, V.; Stuen, S.; Skarpaas, T.; Slettan, A. Prevalence and genotypes of Borrelia burgdorferi sensu lato infection in Ixodes ricinus ticks in southern Norway. *Scand. J. Infect. Dis.* **2010**, *42*, 579–585. [CrossRef]
22. Saab, M.; El Hage, H.; Charafeddine, K.; Habib, R.H.; Khalifeh, I. Diagnosis of Cutaneous Leishmaniasis: Why Punch When You Can Scrape? *Am. J. Trop. Med. Hyg.* **2015**, *92*, 518–522. [CrossRef]
23. Haddad, M.H.F.; Ghasemi, E.; Maraghi, S.; Tavala, M. Identification of Leishmania Species Isolated from Human Cutaneous Leishmaniasis in Mehran, Western Iran Using Nested PCR. *Iran. J. Parasitol.* **2016**, *11*, 65–72.
24. Namazi, M.J.; Dehkordi, A.B.; Haghighi, F.; Mohammadzadeh, M.; Zarean, M.; Hasanabad, M.H. Molecular detection of Leishmania species in northeast of Iran. *Comp. Haematol. Int.* **2018**, *27*, 729–733. [CrossRef]
25. De Almeida, M.E.; Koru, O.; Steurer, F.; Herwaldt, B.L.; da Silva, A.J. Detection and Differentiation of Leishmania sin Clinical Specimens by Use of a SYBR Green-Based Real-Time PCR Assay. *J Clin. Microbiol.* **2017**, *55*, 281–290. [CrossRef] [PubMed]
26. Mohammadiha, A.; Mohebali, M.; Haghighi, A.; Mahdian, R.; Abadi, A.; Zarei, Z.; Yeganeh, F.; Kazemi, B.; Taghipour, N.; Akhoundi, B. Comparison of real-time PCR and conventional PCR with two DNA targets for detection of Leishmania (Leishmania) infantum infection in human and dog blood samples. *Exp. Parasitol.* **2013**, *133*, 89–94. [CrossRef] [PubMed]
27. Sudarshan, M.; Singh, T.; Chakravarty, J.; Sundar, S. A Correlative Study of Splenic Parasite Score and Peripheral Blood Parasite Load Estimation by Quantitative PCR in Visceral Leishmaniasis. *J. Clin. Microbiol.* **2015**, *53*, 3905–3907. [CrossRef] [PubMed]
28. Sterkers, Y.; Varlet-Marie, E.; Cassaing, S.; Brenier-Pinchart, M.-P.; Brun, S.; Dalle, F.; Delhaes, L.; Filisetti, D.; Pelloux, H.; Yera, H.; et al. Multicentric Comparative Analytical Performance Study for Molecular Detection of Low Amounts of Toxoplasma gondii from Simulated Specimens. *J. Clin. Microbiol.* **2010**, *48*, 3216–3222. [CrossRef]
29. Eroglu, F.; Koltas, I.S.; Uzun, S. Comparison of Clinical Samples and Methods in Chronic Cutaneous Leishmaniasis. *Am. J. Trop. Med. Hyg.* **2014**, *91*, 895–900. [CrossRef]

Chapter 3

Development of multiplexed real-time PCRs for the differentiation of *Leishmania* species targeting mini-exon DNA based on bisulphite technology

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Date: March				
2023				

Proprietary technology such as primer and probe sequences are not disclosed in this chapter for confidentiality reasons.

Development of multiplexed real-time PCRs for the differentiation of *Leishmania* species targeting mini-exon DNA based on bisulphite technology

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Abstract: The accurate and sensitive diagnosis of *Leishmania* species in clinical cases of leishmaniasis represents an important and essential step in the treatment and control of these deadly diseases. In this study, multiplexed real-time PCR panels for the detection of *Leishmania* species were created using the mini-exon gene as a PCR target, in conjunction with bisulphite DNA conversion technology. The assays differentiate amongst the main species, including *Leishmania mexicana, Leishmania amazonensis, Leishmania donovani/Leishmania infantum, Leishmania major, Leishmania tropica,* and *Leishmania braziliensis*. The sensitivity and specificity of the assays were tested using DNA sourced from clinical cases of leishmaniasis imported into Australia (from returned travelers, defense force personnel, and migrants from around the globe) as well as potentially cross-reacting organisms. The results were compared to those derived from a widely-used conventional ITS1-PCR RFLP method. This study demonstrated the multiplexed real-time PCR assays to be highly sensitive and specific, with sensitivity and specificity

of 100%. The extraction and amplification assays are performed on small, automated platforms with a turnaround time of less than 2 hours 30 minutes, making them suitable from national to provincial laboratories globally and easily deployable in outbreak situations. A limitation of this technology is that the *Leishmania Viannia* subgenera species were unable to be distinguished from each other; nevertheless, these assays now address the need for a standardized, differential test for the clinical market, of which none currently exists.

Importance: Leishmaniasis encompasses a spectrum of clinical syndromes, ranging from mild or asymptomatic infection to fatal systemic disease caused by *Leishmania* parasites. Rapid and accurate detection of the causative species provides important clinical and epidemiological information, leading to better patient outcomes and aiding in achieving disease elimination targets. Our assay is a novel system, differentiating clinically relevant *Leishmania* species using bisulphite technology with real-time PCR. Furthermore, accurate results for 12 samples at a time can be achieved in under 2.5 hours. The semi-portable and semi-automated nature of the system can be deployed in endemic and non-endemic regions, including in outbreak scenarios.

Keywords: leishmaniasis; diagnostics; real-time PCR, bisulphite; automation
Supplementary file: https://github.com/inekagow/Supplemental-data

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1. Introduction

Leishmania infections cause serious morbidity and mortality globally, often affecting the world's most disadvantaged people. Transmitted by the bite of a

female sandfly, if left untreated, the cutaneous (CL) or mucocutaneous (MCL) form can result in chronic skin ulceration or scarring, and the visceral form (VL) can result in death. Human *Leishmania* infections are caused by 21 different species, occurring in both sympatric and allopatric distributions and imported cases are significant. The increased movement of humans and expanding sandfly habitats are blurring the conventional lines of endemicity. Ten of these species are considered clinically important: *Leishmania donovani* and *Leishmania infantum* (within the *L. donovani* species complex*), Leishmania major* (within the *L. major* species complex*)*, *Leishmania tropica* and *Leishmania aethiopica* (within the *L. tropica* species complex*), Leishmania mexicana* and *Leishmania amazonensis* (within the *L. mexicana* species complex) and *Leishmania braziliensis* (within the *L. braziliensis* species complex) and *Leishmania guyanensis*, *Leishmania panamensis* (within the *L. guyanensis* species complex)[1]. Although the species are morphologically virtually indistinguishable, each *Leishmania* species is classically associated with a particular clinical manifestation(s); however, this notion is changing. Differences in terms of clinical presentation and disease progression are observed between species, and prognostic information such as time to healing and the risk of progression of MCL from CL disease can be estimated. Furthermore, treatment regimens have, more recently, been tailored to species-specific infections[1]. The planning and evaluation of intervention programs benefit greatly from the accurate assessment of diagnosis, distribution and prevalence of *Leishmania* species.

Currently, molecular methods are used to differentiate between *Leishmania* species, yet, it has been pointed out that no "gold standard" method for the differentiation of *Leishmania* species exists[2, 3]. Furthermore, in the clinical laboratory, assays targeting *Leishmania* are not standardized, and the inclusion of important controls, both internal and external, are limited in their

availability or use[4]. Several previous reports have described the differentiation of *Leishmania* species by PCR, either to the species complex or species level, from human or canine clinical specimens[5, 6]. However, many of these reports have not included all of the clinically relevant *Leishmania* species from around the globe. Furthermore, most of the *Leishmania* polymerase chain reaction (PCR) assays described in the literature have utilized conventional PCR (cPCR), which has limitations, including the risk of laboratory contamination and reduced ability to easily multiplex targets. The benefits of real-time PCR in *Leishmania* detection have been discussed extensively[7, 8, 9]. For *Leishmania* species differentiation, real-time PCR and its various forms (including high resolution melt) are increasingly being used to identify individual species[7, 10, 11]. It is a rapid detection method, and cross-contaminating is significantly reduced as it is a closed-tube system. In addition, parasitic load can be easily quantitated, which can aid in the monitoring of therapeutic response.

The need for differentiation between pathogenic *Leishmania* species is becoming increasingly acute in both endemic and non-endemic locations. In the study reported here, we set out to develop real-time PCR assays for *Leishmania* targeting the mini-exon gene, combined with bisulphite conversion technology for the rapid and reliable detection and differentiation of global *Leishmania* species. The mini-exon gene is less widely used for species typing than other gene targets, such as the hsp70 gene, but has nevertheless been described as suitable for both detection and typing purposes in human clinical samples (Table 1)[8, 12]. The gene is tandemly repeated, with 100 to 200 copies present per *Leishmania* genome. Each repeat contains a 39-nucleotide exon (highly conserved), an approximately 55-101 nucleotide intron (moderately conserved), and a non-transcribed intergenic spacer[13]. The intergenic spacer is of highly variable length and sequence (between 51 and 1,350 bp),

suggesting that this characteristic might be useful to discriminate between *Leishmania* species[14]. The mini-exon's tandem repeat nature makes it a good candidate for detection due to its high copy number providing increased sensitivity; however, it has been reported to have polymorphisms even within the same genome[15]. This is advantageous in cPCR as species may be separated by size difference but may affect efficiency of real-time PCR amplification and, therefore, sensitivity.

Bisulphite conversion, however, limits such polymorphisms by increasing the homology by simplifying the genome through chemical modification in order to design PCR assays with reduced complexity. By conversion of cytosines to uracils and ultimately into thymines during the first round of PCR, the targeted genome has increased sequence homology (Figure 1). This process makes closely related species more similar at the genomic level, which is especially advantageous in the variable mini-exon gene, meaning that primer and probe sets are designed to have fewer mismatches and can hybridize previously heterogeneous target regions more efficiently. For multiplexing, it allows for design of melting temperatures of the primers and probes to be made more similar. Bisulphite conversion technology has been utilized in detection assays for a range of infectious agents, including *Leishmania spp.* [16, 17, 18]. The authors' previous work to detect the *Leishmania* genus using bisulphite conversion technology was built upon in this study. The development of species-specific real-time PCR assays, designed to be utilized on the same DNA extraction and PCR platforms and alongside, or as a reflex test for, genus-wide assays. Multiplexed panels using bisulphite conversion technology, incorporating both endogenous and exogenous controls, were designed to differentiate *L. braziliensis*, *L. donovani*/*L. infantum*, *L. major*, *L. tropica*, *L. mexicana* and *L. amazonensis*. Performance was validated using previously purified clinical sample DNA arising from positive tissue

specimens, DNA standards, and fresh negative tissue samples, which were subsequently bisulphite converted, purified on an automated platform and amplified using the multiplex real-time PCR panels.

2. Materials and Methods

2.1. Samples and setting

A total of 98 cases of leishmaniasis diagnosed in Australia between 2008 and 2020 were included in this study[19]. These cases reflect a global distribution of *Leishmania* cases across five of the clinically relevant species complexes[20]. Bead-beating and extractions were performed at the time of receipt by hospital staff using 2mm beads at maximum speed for 30sec on the TissueLyser and the EZ1 tissue kit on the EZ1 biorobot, respectively (both Qiagen, Hilden, Germany) in accordance with the manufacturer's recommendations. All clinical DNA samples underwent conventional PCR targeting the ITS1 region followed by restriction fragment length polymorphism (RFLP) using the method developed by Schönian et al. (Schönian method), before storage at -20˚C[21].

The DNA of 45 potentially cross-reacting organisms were also included, including fungi, bacteria, mycobacteria, viruses, and human and bovine DNA (Table 2).

Commercially available whole genomic DNA and whole cells of the following species: *L. donovani (*MHOM/IN/80/DD8 supplied at 2.3 x 10^7 cells/mL), *L. braziliensis* (MHOM/BR/75/M2903 supplied at 1.63 x 10^8 cells/mL), *L. tropica (*MHOM/SU/74/K27 supplied at 1.03 x 10^7 cells/mL), *L. amazonensis* (MHOM/BR/73/M2269 supplied at 9.9 x 10^6 cells/mL), *L. mexicana* (MHOM/BZ/82/BEL21 supplied at 1.51 x 10^8 cells/mL), *L. infantum* (MHOM/TN/80/IPT-1 supplied at 2.2 x10^6 copies/mL) and *L. major* (MHOM/SU/73/5-ASKH supplied at 7.1 x 10^6 cells/mL) were also included

(obtained from the American Type Culture Collection [ATCC, Manassas, USA]). Synthetic constructs were used in the design phase of the assays, and for an external positive control (EPC), their details are available in the Supplementary file. Forty-seven tissue samples from unrelated clinical cases and previously characterized as negative for *Leishmania* at St. Vincent's Hospital, Sydney, Australia, by the above methods, were also included.

2.2. Limit of detection by ITS1-PCR

For the limit of detection by ITS1-PCR, *Leishmania* DNA was formulated by adding 2,880,000 or 28,800 DNA copies derived from ATCC standards to a total volume of 400µL of molecular grade H_2O, depending on the available starting concentration. These were heated at 70°C for 15 minutes, then processed on the DNA extraction platform, GS-mini (Genetic Signatures Ltd., Sydney, Australia), using the MagPurix Viral/Pathogen Nucleic Acids Extraction Kit (Zinexts Life Science, Taipei, Taiwan), following the manufacturers' instructions. The eluates were diluted in molecular grade H_2O in 10-fold dilution series to 0.1 copy per PCR and amplified in conventional PCR (cPCR) triplicates, according to the Schönian methodology, based on the primers LITSR: CTGGATCATTTTCCGATG and L5.8S: TGATACCACTTATCGCACTT (targeting the ribosomal internal transcribed spacer 1 [ITS1])[21]. The PCR was run on the BioRad T-100 thermocycler (BioRad, USA), and the PCR products were electrophorized on 4% agarose E-Gels (Invitrogen, LifeTechnologies, USA) run and visualized on the E-Gel Power Snap Electrophoresis System, using a 50 to 1,500bp ladder (ThermoFisher Scientific, USA).

2.3. Bisulphite conversion and quality control

Genomic DNA was bisulphite converted by adding 2,880,000 or 28,800 DNA copies derived from ATCC standards, depending on available starting concentration, to a total volume of 150 µL with molecular grade H_2O, and 250

μL 3M sodium bisulphite was added. Alternatively, 5 μL of DNA, extracted previously from a clinical sample DNA, was added to 145 μL of molecular grade H_2O, after which 250 μL 3M sodium bisulphite was added. For the negative tissue samples, 150 μL of tissue lysate, which had previously undergone a mechanical bead-beating process, was added to 250 μL 3M sodium bisulphite. A negative process control (NPC) of 150 μL molecular grade H_2O was included in each run. A total of 5×10^5copies/μL Lambda DNA (strain cI857 ind 1 Sam 7) (New England Biolabs, Ipswich, USA), an *Escherichia coli* bacteriophage, was added to each of these reactions as the exogenous control spike, then the samples were mixed by vortexing, and incubated at 95°C for 15 minutes. Subsequently, 200μL of this lysate was purified on the GS-mini (Genetic Signatures Ltd., Sydney, Australia) with the Sample Processing Pathogens A kit (Genetic Signatures Ltd., Sydney, Australia), according to the manufacturer's recommendations. The eluted DNA was then diluted in molecular grade H_2O in six 10-fold dilution series to 0.1 copy/PCR. A negative template control (NTC) of molecular grade H_2O and an external positive control (EPC) were included in each PCR. The EPC was developed by pooling synthetic constructs of the mini-exon regions of *L. donovani*, *L. braziliensis*, *L. tropica, L. amazonensis, L. mexicana,* and *L. major*, consisting of adenine, thymine, cytosine or guanine residues only (Sigma, Castle Hill, Australia). Further information regarding synthetic constructs are available in the Supplementary file. These were bisulphite converted, as described above, and diluted to five copies/μL in molecular grade H_2O. Separate PCR areas were used for mastermix preparation, DNA seeding and PCR reactions.

2.4. Bisulphite multiplex real-time PCR assay design

A novel multiplex real-time PCR was developed to differentiate the species *L. amazonensis*, *L. mexicana, L. donovani/L. infantum, L. major*, *L. tropica,* and *L. braziliensis* by comparing the mini-exon gene sequences. The Geneious

Prime tool (Biomatters, Auckland, NZ) was used to identify regions of difference between the genomes. Based on these comparisons, primer and probe sets, with individual probe fluorophores, were designed to amplify a partial segment of the mini-exon gene for each species. BLAST analyses confirmed the specificity of the designed primers and probes. Real-time multiplex PCR assays were formulated that contained three main components: (1) sets of mini-exon gene primer and probe set for *Leishmania* identification, (2) a human mitochondrial 12S rRNA gene primer and probe set as a host-specific endogenous control, or (3) a lambda bacteriophage primer and probe set targeting an exogenous spike control in the format as outlined in Table 3.

2.5. Limit of detection, sensitivity, and specificity studies

All bisulphite-converted DNA samples were analyzed using the described real-time PCR panels. The reactions were run on the BioRad CFX96 PCR platform (BioRad, USA) using 25µL volumes with a SensiFast Mastermix (Bioline, UK). The applied primer concentrations were 50ng, 25ng, and 6ng for target, 12S rRNA, and lambda bacteriophage primers, respectively, and the applied probe concentration were 5, 2.5, and 3.75 pmol, respectively. A 4µL volume of extracted DNA was used. Reaction conditions comprised 95°C for 3 min, followed by 50 cycles at 95°C for 2s, 55°C for 10s, and 60°C for 10s. A total of 98 clinical isolate DNA samples (derived from 89 unique patients) of imported *Leishmania* infections were collected by St. Vincent's Hospital, Sydney, Australia. The samples arose from returned travelers, defense force personnel and migrants from around the globe and were collected and stored at St. Vincent's Hospital, Sydney, Australia between 2008 and 2020. These were tested by the bisulphite real-time PCR assays and the results were compared to those derived from a widely-used conventional standard method (Schönian method) [21]. Clinical sample and potential cross-reacting organisms were tested in single replicates, synthetic constructs LOD were tested in three

replicates, and ATCC LOD were tested in ten replicates. In order to determine the LOD of the bisulphite multiplexed real-time PCR assays, the ATCC standards were used alongside diluted known concentrations of synthetic constructs, and LOD was defined as the lowest concentration of DNA at which the assay detected nine out of ten replicates. Real-time PCR analysis was performed on the BioRad CFX Maestro 1.1 program (version 4.1.2433.1219), where C_q method determination is by single threshold. Data points were excluded if they did not follow the one-in-ten series of approximately 3.3Ct values between points (due to inhibition by purification artifacts at high copy numbers or poor linearity at low copy numbers). The ATCC standard serial dilution limit of detection of 10^4 copies/PCR diluted down to 0.1 copy/PCR was run against a serial dilution of the previously-described synthetic constructs from 10^6 copies/µL diluted down to 0.1 copies/µL in 10-fold dilution series in order to generate a standard curve for each. An NTC reaction and EPC reaction were included in the PCR run.

2.6. Patient characteristics and ethics considerations

All patient records were anonymized and, as far as available, the following data were collected for molecular epidemiology purposes: gender, age, region of presumed infection, and biopsy sample site. The clinical specimens were tested in accordance with St Vincent's Hospital ethics approval, HREC number LNR/16/SVH/231.

3. Results

3.1. Limit of detection

The limit of detection of the ITS1-PCR was determined in our previous work (using the Schönian method) to be between 10 and 1000 copies of target DNA per PCR reaction, dependent upon the species tested[16, 21]. In this study reported here, the LOD for the bisulphite assays was found to be

between 10 and 100 copies per PCR reaction, dependent upon the species tested (Table 4). Figure 2 shows the LOD results obtained with the *Leishmania* differentiation assays using ATCC standard DNAs in the assay. The standard curve generated by a 10-fold serial dilution series of reference strain DNA had a linear dynamic range over 3 or 4 log-steps (dependent upon LOD) with a mean R^2 value of 0.9895.

3.2. Analytical sensitivity and species differentiation

Of the 98 clinical isolates determined to be *Leishmania* spp. by ITS1-PCR, 98 were positive with the bisulphite multiplex assays (100% sensitivity) (Table 5). In both cases, the external positive control was positive, and the exogenous and/or endogenous positive control was positive, indicating DNA extraction and/or correct sampling occurred. Table 6 lists the species differentiation results of the 98 clinical samples, where all but two samples were in agreement because they had no species assignment by the previous PCR-RFLP method.

3.3. Analytical specificity and cross-reactivity

To investigate the specificity of the assays, a panel of DNA from 47 negative clinical samples was tested and no PCR amplification was detected (Table 5). To further investigate the specificity of the assay, a panel of DNA from 45 phylogenetically organisms, or those with a differential diagnosis related to leishmaniasis, were tested. No PCR amplification was detected from any of these specimens, giving a specificity of 100%. In both cases, the external positive control was positive, indicating the lack of amplification was not due to failure of any component of the assay or the presence of PCR inhibitors.

3.4. Negative control reactions

No unexpected amplification was observed in NPC and NTC reactions.

4. Discussion

This is the first reported *Leishmania* differentiation system utilizing bisulphite conversion technology, which simplifies the genome for easier detection. The results generated in this report show the sensitive and specific detection and differentiation of clinically relevant *Leishmania* species using bisulphite conversion technology based upon synthetic construction, commercially available standards, and clinical DNA samples. Internal and external controls all gave the desired signals, confirming the positive and negative results. The internal controls consisted of two controls, an endogenous control (human mitochondrial 12S rRNA gene), indicating the validity of the sampling, bisulphite conversion, and extraction processes. The second, an exogenous control (bacteriophage lambda spike), isolated the validity of the bisulphite conversion and extraction processes. The EPC validated the PCR amplification reactions. The NPC and NTC assessed contamination of the bisulphite conversion, extraction, and PCR processes, respectively. Limit of detection cross-reactivity, sensitivity, specificity studies were performed. These studies determined the bisulphite real-time PCR assays to be highly sensitive and specific, with low LODs. The clinical sample results present excellent concordance with the previous PCR-RFLP method, where all analytical results were in agreement, apart from two previously-uncharacterized samples which were able to be assigned to a species with the real-time PCR method. This agreement with the previous method confirms the applicability of the bisulphite real-time PCR assays for use in the detection and differentiation of *Leishmania* species.

In this report, we have described the development of a DNA extraction and real-time PCR assay system (Figure 3) that can return patient results in under 2 hours 30 mins and uses platforms with a small footprint (60cm x 60cm x 60cm and 33cm x 46cm x 36cm, respectively). This means that patient results can be returned within half a day, and 12 individual patient samples

may be run at one time. Training for the system is simple, even for technicians with limited molecular experience, due to the cartridge-based extraction, pre-made multiplex panels, and inclusion of multiple controls for quality results analysis. This makes the system applicable for use from the national reference laboratory to provincial or mobile laboratories and could be easily deployed in outbreak scenarios.

It should be noted that no *L. amazonensis* positive clinical samples were available, and all positive clinical samples were derived from DNA, as no fresh, positive clinical samples were available during the study period. Further studies will need to be carried out to accurately determine the performance of the detection system as a clinical diagnostic test with fresh clinical samples, such as tissue samples, including *L. amazonensis* positive samples. Although not included in this study, due to limited availability, the inclusion of *Leptomonas seymouri* and *Leishmania tarentolae* DNA should be included in future cross-reactivity panels. There has been recent reports of *L. donovani*/*L. seymouri* co-infection in both CL and VL patients reported in the Indian subcontinent and of *L. tarentolae* and *L. infantum*/*L. tarentolae* co-infection in blood donors in Southern Italy[22, 23, 24].

Based on our findings in the design phase of the assays (data not shown), the bisulphite mini-exon real-time PCR has limitations differentiating between the South American *L. Viannia* subgenera species. Differentiation of the species is important in epidemiologic and clinical studies, as the clinical manifestations differ, particularly as *L. braziliensis, L. panamensis* and *L. guyanensis* are known to progress to MCL from CL[1, 25]. However, much debate has arisen as to whether *L. panamensis* and *L. peruviana* species are genetically distinct from other species in the *L. guyanensis* and *L. braziliensis* species complexes, respectively[26, 27, 28]. Further complicating the matter

of species designation in the *L. Viannia* subgenera, gene flow between these species is regarded as common, with around 10% of strains being genetically complex, including hybrids and mito-nuclear discordance [29, 30]. Hybrid species have been reported between *L. braziliensis* and *L. peruviana, L. panamensis* and *L. guyanensis,* and even between *L. braziliensis* and *L. guyanensis* (spanning the two *L. Viannia* species complexes)[30, 31, 32]. As well as the difficulties in the definition of species, as observed in *L. Viannia* species but also more widely, the quality of Genbank sequences for *Leishmania* has been queried on multiple occasions [33, 34, 35, 36]. Erroneous species classifications, attributed to contamination or laboratory error, complicates interpretation of sequence reads, presenting difficulties in isolate identification and subsequent assay design. Despite this, further work should be performed to differentiate between these species as current, and robust *L. Viannia* species data becomes available, in order to address clinical concerns.

Species-specific real-time PCRs have been deemed inappropriate for regions with sympatric *Leishmania* species, allopatric regions, or those regions concerned with returning global travelers and due to the presence of polymorphisms and hybrid species[7]. This assay covers all clinically relevant species from around the globe and is resistant to false negatives, which contribute to the sustained reservoir population. The sensitive and rapid nature of the assay ensures that responsive decision-making can be made to improve patient outcomes. This detection system may be paired with a genus-wide *Leishmania* spp. detecting system based upon the same extraction protocol as described, developed previously. Thus the assay addresses the need for a standardized, differential test for clinically relevant *Leishmania* species.

5. Conclusions

The use of a genus-wide and species-specific assay has been suggested as the optimal diagnostic method for leishmaniasis[7]. The *Leishmania* typing assay described here is highly sensitive and specific and, combined with the previously designed pan-*Leishmania* detection assay by the authors, represents a major step forward in meeting such requirements[16]. Furthermore, the system provides rapid results with a small footprint in an easy-to-use platform that may be used in both established laboratories globally or deployed in outbreak scenarios at a near point-of-care setting. We anticipate trialing the usefulness of this technology in a clinical setting in the near future.

References

1. Hodiamont CJ, Kager PA, Bart A, de Vries HJ, van Thiel PP, Leenstra T, de Vries PJ, van Vugt M, Grobusch MP, van Gool T. 2014. Species-directed therapy for leishmaniasis in returning travellers: a comprehensive guide. PLoS Negl Trop Dis 8:e2832.
2. Rodriguez-Cortes A, Ojeda A, Francino O, Lopez-Fuertes L, Timon M, Alberola J. 2010. *Leishmania* infection: laboratory diagnosing in the absence of a "gold standard". Am J Trop Med Hyg 82:251-6.
3. Gomes CM, Mazin SC, Raphael E, Cesetti MV, Albergaria G, Bächtold B, Henrique J, Cordeiro DF, Claudino F, Theodoro ET, Damasco S, Andrés S, Carranza V. 2015. Accuracy of mucocutaneous leishmaniasis diagnosis using polymerase chain reaction : systematic literature review and meta-analysis. 110:157-165.
4. Reithinger R, Dujardin JC. 2007. Molecular diagnosis of leishmaniasis: Current status and future applications. J Clin Microbiol 45:21-25.
5. Weirather JL, Jeronimo SMB, Gautam S, Sundar S, Kang M, Kurtz MA, Haque R, Schriefer A, Talhari Ss, Carvalho EM, Donelson JE, Wilson ME. 2011. Serial quantitative PCR assay for detection, species discrimination,

and quantification of *Leishmania* spp. in human samples. J Clin Microbiol 49:3892-3904.

6. Harris E, Kropp G, Belli A, Rodriguez B, Agabian N. 1998. Single-step multiplex PCR assay for characterization of New World Leishmania complexes. J Clin Microbiol 36:1989-95.
7. Galluzzi L, Ceccarelli M, Diotallevi A, Menotta M, Magnani M. 2018. Real-time PCR applications for diagnosis of leishmaniasis. Parasit Vectors 11:273.
8. Conter CC, Mota CA, dos Santos BA, de Souza Braga L, de Souza Terron M, Navasconi TR, Fernandes ACBS, Demarchi IG, de Castro KRR, Aristides SMA, Lonardoni MVC, Teixeira JJV, Silveira TGV. 2019. PCR primers designed for new world Leishmania: A systematic review. Exp Parasitol 207:107773.
9. Moreira OC, Yadon ZE, Cupolillo E. 2018. The applicability of real-time PCR in the diagnostic of cutaneous leishmaniasis and parasite quantification for clinical management: Current status and perspectives. Acta Trop 184:29-37.
10. Leon CM, Munoz M, Hernandez C, Ayala MS, Florez C, Teheran A, Cubides JR, Ramirez JD. 2017. Analytical Performance of Four Polymerase Chain Reaction (PCR) and Real Time PCR (qPCR) Assays for the Detection of Six *Leishmania* Species DNA in Colombia. Front Microbiol 8:1907.
11. Diotallevi A, Buffi G, Ceccarelli M, Neitzke-Abreu HC, Gnutzmann LV, da Costa Lima MS, Jr., Di Domenico A, De Santi M, Magnani M, Galluzzi L. 2020. Real-time PCR to differentiate among Leishmania (Viannia) subgenus, Leishmania (Leishmania) infantum and Leishmania (Leishmania) amazonensis: Application on Brazilian clinical samples. Acta Trop 201:105178.

12. Van der Auwera G, Dujardin JC. 2015. Species typing in dermal leishmaniasis. Clin Microbiol Rev 28:265-94.
13. Cook G, Donelson J. 1987. Mini-exon gene repeats of Trypanosoma (Nannomonas) congolense have internal repeats of 190 base pairs. . Mol Biochem Parasitol 25:113-22.
14. Marfurt J, Niederwieser I, Makia N, Beck H, Felger I. 2003. Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP. Diagn Microbiol Infect Dis 46:115-124.
15. Van der Auwera G, Ravel C, Verweij J, Bart A, Schönian G, Felger I. 2014. Evaluation of four single-locus markers for *Leishmania* species discrimination by sequencing. J Clin Microbiol 52:1098-104.
16. Gow I, Millar D, Ellis J, Melki J, Stark D. 2019. Semi-Quantitative, Duplexed qPCR Assay for the Detection of *Leishmania* spp. Using Bisulphite Conversion Technology. Trop Med Infect Dis 4.
17. Garae C, Kalo K, Pakoa GJ, Baker R, Isaacs P, Millar DS. 2020. Validation of the easyscreen flavivirus dengue alphavirus detection kit based on 3base amplification technology and its application to the 2016/17 Vanuatu dengue outbreak. PLoS One 15:e0227550.
18. Siah SP, Merif J, Kaur K, Nair J, Huntington PG, Karagiannis T, Stark D, Rawlinson W, Olma T, Thomas L, Melki JR, Millar DS. 2014. Improved detection of gastrointestinal pathogens using generalised sample processing and amplification panels. Pathology 46:53-59.
19. Roberts T, Barratt J, Sandaradura I, Lee R, Harkness J, Marriott D, Ellis J, Stark D. 2015. Molecular epidemiology of imported cases of leishmaniasis in Australia from 2008 to 2014. PLoS One 10:1-11.
20. World Health Organization. 2020. Global leishmaniasis surveillance, 2017–2018, and first report on 5 additional indicators. World Health Organization, Organization WH, Geneva, Switzerland.

21. Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig H, Presber W, Jaffe C. 2003. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis 47:349-58.
22. Iatta R, Mendoza-Roldan JA, Latrofa MS, Cascio A, Brianti E, Pombi M, Gabrielli S, Otranto D. 2021. Leishmania tarentolae and Leishmania infantum in humans, dogs and cats in the Pelagie archipelago, southern Italy. PLoS Negl Trop Dis 15:e0009817.
23. Thakur L, Kushwaha HR, Negi A, Jain A, Jain M. 2020. Leptomonas seymouri Co-infection in Cutaneous Leishmaniasis Cases Caused by Leishmania donovani From Himachal Pradesh, India. Front Cell Infect Microbiol 10:345.
24. Ahuja K, Vats A, Beg MA, Kariyawasam K, Chaudhury A, Chatterjee M, Karunaweera ND, Selvapandiyan A. 2020. High resolution melting based method for rapid discriminatory diagnosis of co-infecting Leptomonas seymouri in Leishmania donovani-induced leishmaniasis. Parasitol Int 75:102047.
25. Esteves S, Costa I, Amorim C, Santarem N, Cordeiro-da-Silva A. 2018. Biomarkers in Leishmaniasis: From Basic Research to Clinical Application. doi:10.5772/intechopen.75315.
26. Coughlan S, Taylor AS, Feane E, Sanders M, Schonian G, Cotton JA, Downing T. 2018. Leishmania naiffi and Leishmania guyanensis reference genomes highlight genome structure and gene evolution in the Viannia subgenus. R Soc Open Sci 5:172212.
27. Bañuls A, Dujardin J, Guerrini F, Doncker S, Jacquet D, Arevalo J, Noël S, Ray D, Tibayrenc M. 2000. Is Leishmania (Viannia) peruviana a Distinct Species? A MLEE/RAPD Evolutionary Genetics Answer. The Journal of Eukaryotic Microbiology 47:197–207.

28. Boite MC, Mauricio IL, Miles MA, Cupolillo E. 2012. New insights on taxonomy, phylogeny and population genetics of Leishmania (Viannia) parasites based on multilocus sequence analysis. PLoS Negl Trop Dis 6:e1888.
29. Noyes H, Arana B, Chance M, Wingon R. 1997. The Leishmania hertigi (Kinetoplastida; Trypanosomatidae) Complex and the Lizard Leishmania: Their Classification and Evidence for a Neotropical Origin of the Leishmania-Endotrypanum Clade.
30. Kato H, Caceres AG, Gomez EA, Tabbabi A, Mizushima D, Yamamoto DS, Hashiguchi Y. 2021. Prevalence of Genetically Complex Leishmania Strains With Hybrid and Mito-Nuclear Discordance. Front Cell Infect Microbiol 11:625001.
31. Banuls A, Guerrini F, Pont F, Barrera C, Espinel I, Guderian R, Echeverria R, Tibayrenc M. 1997. Evidence for Hybridization by Multilocus Enzyme Electrophoresis and Random Amplified Polymorphic DNA Between Leishmania braziliensis and Leishmania panamensis/guyanensis in Ecuador. The Journal of Eukaryotic Microbiology 44:408–411.
32. Dujardin J, Bañuls A, Llanos-Cuentas A, Alvarez E, DeDoncker S, Jacquet D, Le Ray D, Arevalo J, Tibayrenc M. 1995. Putative Leishmania hybrids in the Eastern Andean valley of Huanuco, Peru. Acta Trop 59:293-307.
33. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, Sereno D. 2016. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. PLoS Negl Trop Dis 10:e0004349-e0004349.
34. Van der Auwera G, Bart A, Chicharro C, Cortes S, Davidsson L, Di Muccio T, Dujardin JC, Felger I, Paglia MG, Grimm F, Harms G, Jaffe CL,

Manser M, Ravel C, Robert-Gangneux F, Roelfsema J, Toz S, Verweij JJ, Chiodini PL. 2016. Comparison of Leishmania typing results obtained from 16 European clinical laboratories in 2014. Euro Surveill 21.

35. Foulet F, Botterel F, Buffet P, Morizot G, Rivollet D, Deniau M, Pratlong F, Costa J-M, Bretagne S. 2007. Detection and identification of Leishmania species from clinical specimens by using a real-time PCR assay and sequencing of the cytochrome B gene. J Clin Microbiol 45:2110-5.
36. Van der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Van Esbroeck M, Van Gompel A, Clerinx J, Dujardin J. 2013. Heat-shock protein 70 gene sequencing for Leishmania species typing in European tropical infectious disease clinics. Euro Surveill 18:20543.
37. Hassan M, Ghosh A, Ghosh S, Gupta M, Basu D, Mallik K, Adhya S. 1993. Enzymatic amplification of mini-exon-derived RNA gene spacers of Leishmania donovani: primers and probes for DNA diagnosis. . Parasitology 107:509-17.
38. Fernandes O, Murthy VK, U, Degrave W, Campbell D. 1994. Mini-exon gene variation in human pathogenic Leishmania species. Mol Biochem Parasitol 66:261-71.
39. Piarroux R, Fontes M, Perasso R, Gambarelli F, Joblet C, Dumon H, Quilici M. 1995. Phylogenetic relationships between Old World Leishmania strains revealed by analysis of a repetitive DNA sequence. Mol Biochem Parasitol 73:249-52.
40. Ramos A, Maslov DA, Fernandes O, Campbell DA, L. S. 1996. Detection and Identification of Human Pathogenic Leishmania and Trypanosoma Species by Hybridization of PCR-Amplified Mini-exon Repeats. Exp Parasitol 82:242-50.

41. Kebede A, De Doncker S, Arevaloc J, Le Raya D, Dujardin JC. 1999. Size-polymorphism of mini-exon gene-bearing chromosomes among natural populations of Leishmania, subgenus Viannia. Int J Parasitol 29:549-57.
42. Vernal J, Cazzulo J, Nowicki C. 2003. Cloning and heterologous expression of a broad specificity aminotransferase of *Leishmania* mexicanapromastigotes. FEMS Microbiol Lett 229:217-222.
43. Paiva B.R., Passos L.N., Falqueto A., Malafronte R.S., H.F. A. 2004. Single step polymerase chain reaction (PCR) for the diagnosis of the Leishmania (Viannia) subgenus. Rev Inst Med Trop Sao Paulo 46:335-8.
44. van der Snoek EM, Lammers AM, Kortbeek LM, Roelfsema JH, Bart A, Jaspers CA. 2009. Spontaneous cure of American cutaneous leishmaniasis due to *Leishmania naiffi* in two Dutch infantry soldiers. Clin Exp Dermatol 34:e889-91.
45. Osman A. 2011. Evaluation of molecular genotyping in clinical and epidemiological study of leishmaniasis in Sudan. . Sci Parasitol 12:131-137.

Figures and Tables

Sequence	Before	After
Seq 1	G A T G G C G A T A T G G T T G A C A C	G A T G G T G A T A T G G T T G A T A T
Seq 2	G A T G G T G A C A T G G T A G A T A C	G A T G G T G A T A T G G T A G A T A T
Seq 3	G A T G G T G A T A T G G T G G A C A C	G A T G G T G A T A T G G T G G A T A T
Seq 4	G A T G G T G A T A T G G T A G A T A T	G A T G G T G A T A T G G T A G A T A T
Seq 5	G A T G G T G A T A T G G T G G A C A C	G A T G G T G A T A T G G T G G A T A T
Seq 6	G A T G G C G A C A T G G T T G A T A T	G A T G G T G A T A T G G T T G A T A T
Seq 7	G A T G G T G A T A T G G T G G A C A C	G A T G G T G A T A T G G T G G A T A T
Seq 8	G A T G G T G A C A T G G T A G A T A C	G A T G G T G A T A T G G T A G A T A T
Seq 9	G A T G G T G A T A T G G T A G A T A C	G A T G G T G A T A T G G T A G A T A T
Seq 10	G A T G G T G A T A T G G T G G A T A C	G A T G G T G A T A T G G T G G A T A T

Consensus

Sequence	G A T G G Y G A Y A T G G T D G A Y A Y	G A T G G T G A T A T G G T D G A T A T
	• 75% sequence similarity over 20 bases • **48** possible primer combinations	• 95% sequence similarity over 20 bases • **3** possible primer combinations

Figure 1. Example of the bisulphite conversion mechanism. When Cs are detected as Ts, in this example, homology is increased from 75% to 95% from the "Before" to "After" consensus regions.

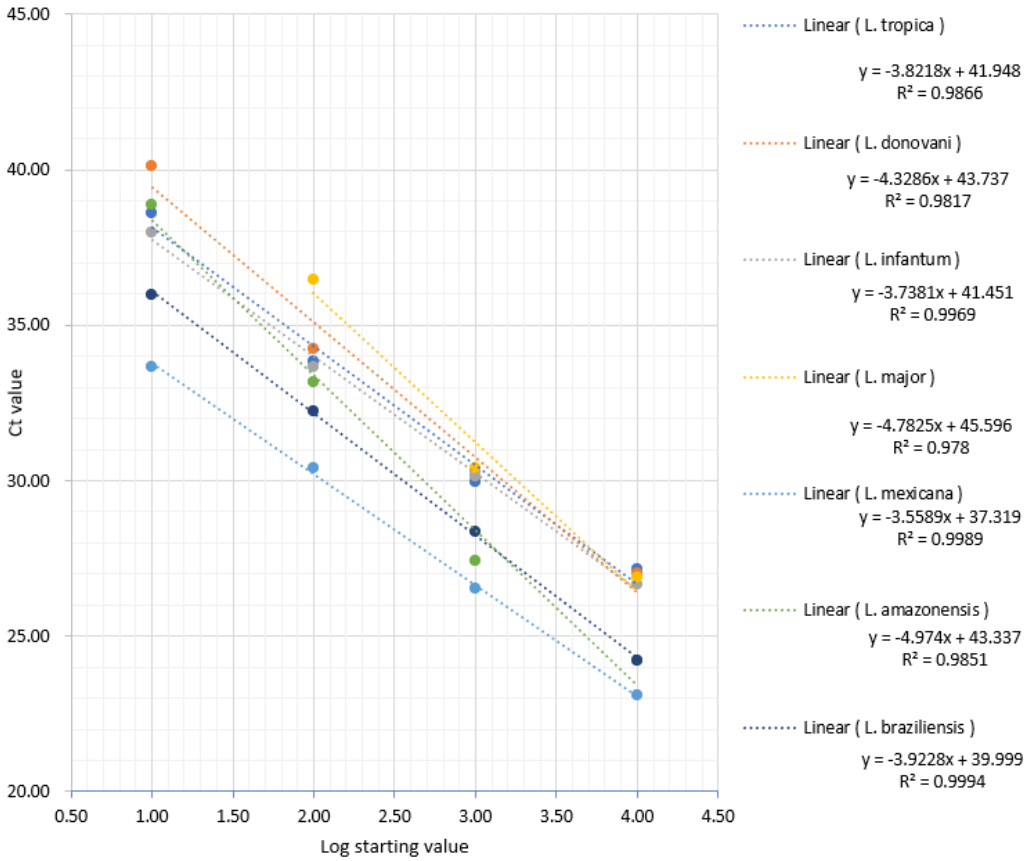

Figure 2. Results obtained using the differentiation assays on ATCC standards (Cycle threshold (Ct) averaged from 10x PCR replicates). The standard curve was plotted using log10 concentration versus Ct value.

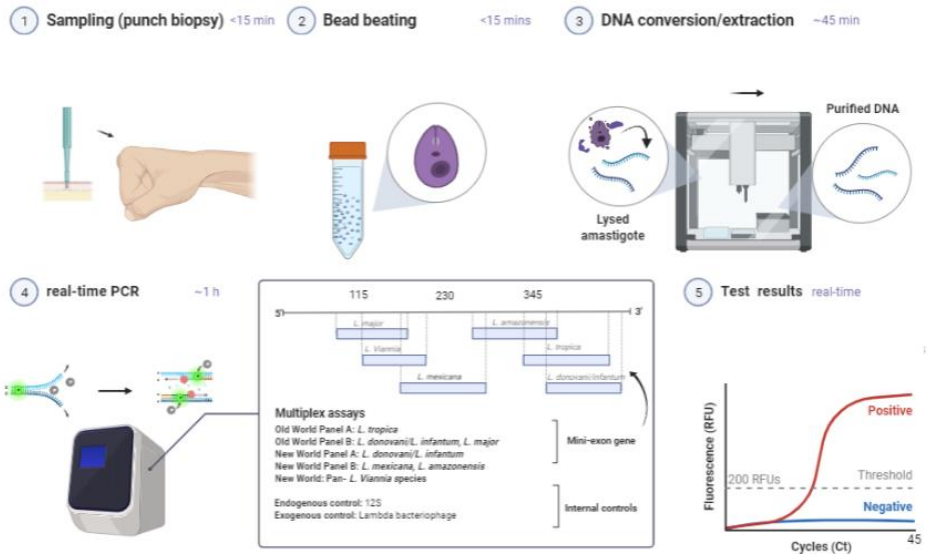

Figure 3. Overview of the complete *Leishmania* differentiation system when performed on fresh tissue samples. 1. Circular blade removes ulcerated site from dermal layer. 2. Rapid agitation with grinding media (metallic beads) in a bead beater releases amastigotes. 3. DNA is bisulphite converted and purified from lysed amastigotes. 4. Purified DNA is amplified by real-time PCR. 5. Positive *Leishmania* patients cross the threshold line (over 200 Relative fluorescence units) within 45 cycles (<45.00 Ct).

Table 1. Previously published *Leishmania* detection assays in human samples, utilizing original mini-exon gene designs (cPCR- conventional PCR, qPCR- real-time PCR).

Application	**Primers and probes 5' – 3' (name: sequence)**	**Typing**	**Geographical region**	**Reference**
cPCR/dot hybridization	0-22: TTCCGGAAGGTTTCGCATAC 0-23: GTCTTCCGGCAAGATTTTGG	Yes	Old World/ New World	[37]
cPCR/sequencing	Forward: GGGAATTCAATATAGTACAGAAACTG Reverse: GGGAAGCTTCTGTACTTTATTGGTA	Yes	Old World/ New World	[38]
cPCR	T2: CGGCTTCGCACCATGCGGTG B4: ACATCCCTGCCCACATACGC	Yes	Old World	[39]
cPCR with hybridization probes	S-1629: GGGAATTCAATA(A/T)AGTACAGAAACTG S-1630: GGGAAGCTTCTGTACT(A/T)TATTGGTA (conserved region) S-1593:A(A/G)(C/T)GGCACCCCCCTCACA(G/A)CGA CCTGGGCA (*L. braziliensis, L. guyanensis, L. panamensis*)	Yes	Old World/ New World	[40]

	S-1698: CGGCCATGGTGGTGAC(G/A)CGCGGGCCCGTGC (*L. chagasi, L. donovani, L. infantum*) S-1595: GGGCG(C/A)CGGC(A/G)GCCGTGAC(A/G)CGTGG(C/T)CCGG (*L. amazonensis, L. mexicana*) S-1932: GCGTGCGCGGAGAACATCCA (*L. aethiopica*) S-1933: GCGTGCGCGGGGAACGGCCA (*L. major*) S-1981: GCGTGCGCGGAGAACATCCATCAA (*L. tropica*)			
cPCR	LU-5A: TTTATTGGTATGCGAAACTTC LB-3C: CG(C/G)CCGAACCCCGTGTC LM-3A: GCACCGCACCGG(A/G)CCAC LC-3L: GCCCGCG(C/T)GTCACCACCAT	Yes	New World	[6]
Probe hybridization	Probe: AACTAACGCTATATAAGTATCAGTTTCTGTACTTTATTG	Yes	New World	[41]

Hemi-nested PCR	Mini-Exon fw: AACTAACGCTATATAAGTATCAGTTTCTGTAC N-Ter-region-2 rev: GTCCAGAAGAAGCTGCTCAGCC N-Term-region-3 rev: AAGGTAGATGGGCGTCTTCTCAGCG	No	New World	[42]
cPCR	LV1: CGTCTTCCGGCAACATTT LV2: CGTTAGTTGGAAGCCCAAGG	No	New World	[43]
cPCR/sequencing	F(RIVM): ACTTCCGGAACCTGTCTTCC R(RIVM): CAGAAACTGATACTTATATAGCGTTA F(AMC): ACTTTATTGGTATGCGAAACTTCCGG R(AMC): ACAGAAACTGATACTTATATAGCGTTAG	Yes	New World	[44]
cPCR	ME/Unit primers ME1: CAATATAGTACAGAAACT G ME2: TTCTGTACTTTATTGGTA ME/Other primers ME1: CAATATAGTACAGAAACTG ME3: ACTTTATTGGTATGCGAA	Yes	Old World/ New World	[45]

qPCR	Miniexon 1 F: CGAAACTTCCGGAACCTGTCTT R: CACCACACGCACGCACAC P:CGGCAAGATTTTGGAAGCGCGCA Miniexon 2 F: GTGTGGTGGCGGGTGTATGT R: GCCCAGGTCGCTGTGAGG	No	Old World/ New World	[5]

Table 2. Cross-reactivity panel tested with the *Leishmania* differentiation assays

Bacteria	Fungi	Protozoa	Viral	Mammalian
Acinetobacter baumannii	*Acremonium strictum*	*Crithidia fasciculata*	Herpes Simplex Virus Type I	*Bos taurus*
Bacillus cereus	*Aspergillus fumigatus*	*Giardia intestinalis*	Herpes Simplex Virus Type II	*Homo sapiens*
Bacillus subtilis	*Aspergillus sp.*	*Entamoeba histolytica*	Varicella Zoster Virus	
Clostridium perfringens	*Aureobasidium pulluans*	*Trypanosoma cruzi*		
Clostridium sordelli	*Bipolaris sp.*	*Trichomonas vaginalis*		
Escherichia coli	*Fusarium sp.*			
Enterococcus faecalis	*Microsporum canis*			
Haemophilus influenzae	*Penicillium sp.*			
Klebsiella oxytoca	*Scedosporium prolificans*			
Klebsiella pneumoniae	*Trichophyton mentagrophytes*			
Moraxella cattaharalis	*Trichophyton rubrum*			
Proteus mirabilis	*Trichophyton tonsurans*			
Proteus vulgaris				
Providencia stuartii				

Pseudomonas aeruginosa
Staphylococcus aureus
Staphylococcus hominis
Stenotrophomonas maltophilia
Streptococcus pyogenes
Streptococcus mutans
Yersinia sp.
Mycobacteria abscessus
Mycobacteria sp.

Table 3. Real-time PCR Panels for the differentiation of *Leishmania* species (amplicon length in base pairs).

Target description	**Old World A**	**Old World B**	**New World A**	**New World B**	**New World C**
Species complex/ species/subgenera	*L. tropica* (109bp)	*L. donovani*/*L. infantum* (93bp) *L. major* (97bp)	*L. donovani*/*L. infantum* (93bp)	*L. mexicana* (110 bp) *L. amazonensis* (109bp)	*L. braziliensis* (79bp)
Internal control	*12S* rRNA gene (105bp)	*12S* rRNA gene (105bp)	Lambda bacteriophage (101bp)	Lambda bacteriophage (101bp)	Lambda bacteriophage (101bp)

Table 4. Limit of detection of ATCC standards.

Target	Detection limit (copies/PCR)
L. tropica	10 copies
L. donovani	10 copies
L. infantum	10 copies
L. major	100 copies
L. mexicana	10 copies
L. amazonensis	10 copies
L. braziliensis	10 copies

Table 5. Comparison of the bisulphite real-time PCR assays and ITS1-PCR for the detection of *Leishmania spp*.

Method		ITS1-PCR result		
		Positive	**Negative**	**Total**
Bisulphite real-time PCR result	**Positive**	98	0	98
	Negative	0	47	47
	Total	98 (100% sensitivity)	47 (100% specificity)	145

Table 6. Side-by-side analysis of the bisulphite real-time PCR assays and ITS1-PCR for the differentiation of *Leishmania* species in clinical sample DNA.

Species	ITS1-PCR result	Bisulphite real-time PCR result
L. tropica	40	41
L. donovani/L. infantum	27	27
L. major	7	7
L. mexicana	2	2
L. braziliensis	20	21
No species designation	2	0
Total	98	98

Chapter 4

Less-invasive sampling and 'test of cure' for visceral leishmaniasis and post-kala-azar dermal leishmaniasis: addressing diagnostic priorities

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Certificate:

I certify the following chapter is largely my own work although the contributions of other authors are duly recognised. The contributions of other authors are detailed as follows:

- By providing suggestions on topics to be reviewed
- By proof reading draft manuscripts
- By correcting spelling and grammatical errors in drafts
- By providing suggestion to improve writing style and language
- By providing suggestions to improve layout

Otherwise, the core composition of this work is credited to me.

I hereby certify that the above statements are true and correct:

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Signatures removed prior to publication.

Ineka Gow	Dr. Damien Stark	Prof. Nicholas Smith	Em. Prof. John Ellis

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Date: March 2023

1. Abstract

Visceral leishmaniasis and a post-treatment complication, post-kala-azar dermal leishmaniasis, cause significant mortality and morbidity, respectively, to those living in endemic regions. The diagnosis of these two diseases, caused by the parasite *Leishmania*, is plagued with diagnostic pitfalls and challenges. Thus, the World Health Organisation has prioritised actions to aid in reaching elimination targets for these diseases, through less invasive methods of detection and a test-of-cure. The study presented here aims to address these diagnostic challenges, incorporating several innovative technologies and platforms. Whole peripheral blood was utilised as a less-invasive sampling method, integrating bisulphite modification and target capture technology, with an observed detection limit of 100 cells/mL. A potential test-of-cure was also developed, also incorporating bisulphite technology, detecting RNA presence in clinical sample nucleic acid, an indicator of parasite viability. Although the study would greatly benefit from further work employing fresh clinical samples, including blood samples, pertinent diagnostic solutions to help reach these elimination targets were achieved.

2. Introduction

The visceral form of leishmaniasis, known as kala-azar, is a chronic disease affecting primarily the spleen, liver, and bone marrow. The disease is caused by *Leishmania* parasites, which exist in two forms during their life cycles, amastigotes (in the mammalian host stage) and promastigotes (in the sand fly vector stage). While in the human host, the amastigotes preferentially reside in macrophages. Unlike the cutaneous form of the disease, visceral leishmaniasis (VL) is fatal in 95% of cases if treatment is not administered [46]. A complication of VL, post-kala-azar dermal leishmaniasis (PKDL), characterised by papular or nodular rashes, occurs months to years after successful VL treatment in 5-15% of cases. Both forms of this disease are caused by *Leishmania donovani* or *Leishmania infantum* species, although the forms are prominent in differing regions. It is estimated that 50,000 to 90,00 new cases of the VL disease occur each year, with the Indian subcontinent making up the majority of the cases, as well as a significant number of cases occurring in East Africa and Brazil [47].

Diagnostic pitfalls are associated with both VL and PKDL. Aside from assessment of clinical signs and simple serological tests, visceral leishmaniasis is in some settings detected by the microscopic assessment of lymph node, bone marrow, or splenic biopsies, all procedures that present significant risks to the patient. Splenic biopsies are of major concern, as they can result in death if not performed correctly. Rapid antigen and antibody tests, although cost effective and simple to use, can return poor sensitivity[48]. Post-kala-azar dermal leishmaniasis is currently detected by slit skin smears, with poor detection rates of between 4-58% [49]. According to the World Health Organisation (WHO), strengthening diagnostics is a top priority for some neglected tropical diseases, one specific goal being to "Develop less invasive test of cure of post-kala-azar dermal and visceral leishmaniasis" [50]. In addition, the WHO identified that current detection for both VL and PKDL could be enhanced through a "test of

cure" (TOC) diagnostic test. This will allow monitoring of the active status of VL cases and help predict which VL cases are likely to develop into PKDL. This data is important for case management and epidemiological monitoring and elimination programs such as the Kala-Azar Elimination Programme (KAEP) [47].

In response to these priorities, two real-time PCR assays were developed: one aimed to validate the use of whole peripheral blood as a less invasive sample type for VL and PKDL diagnosis (incorporating nucleic acid extraction optimisation); and the other to accurately assess TOC through parasite viability. These two assays were designed incorporating the already embedded bisulphite technology developed in the pan-*Leishmania* and *Leishmania* species differentiation assays developed previously (Chapters 2 and 3) [16]. Bisulphite conversion is a method of chemically simplifying the genome by converting cytosines to thymines. This can be done within the lysis step of clinical sample preparation, resulting in increased genomic homology. Assay design targeting this more homologous genome can, therefore, cover more subtypes and polymorphisms, which is particularly useful in analysing samples that may include multiple *Leishmania* species.

As part of the study design, a target product profile (TPP) was constructed to outline the efficacy, safety, and user needs to define desired attributes of a test (Table 1). A TPP is a strategic document that outlines all relevant technical, medical, and scientific information related to the product, in this instance, a diagnostic test. The TPP was targeted to address the WHO diagnostic priorities by defining “Optimal” and “Minimal” desired attributes of the test [50]. Some of these desired attributes are embedded into the test by integrating equipment from previous studies, whereas others were able to be designed *de novo* to meet specific requirements. For instance, the use of whole peripheral blood was prioritised over blood components (*e.g.,* buffy coat) to remove the need for a

centrifuge in the extraction process. The use of a centrifuge in the extraction process requires a piece of large and expensive laboratory equipment, is time-consuming, and creates an additional risk of contamination. Peripheral blood is known to be inhibitory to downstream PCR processes and poses challenges to bisulphite conversion studies, due to its heterogenous nature [51].

Table 1. Target Product Profile for an automated real-time PCR based near point-of-care diagnostic test for visceral leishmaniasis and post-kala-azar dermal leishmaniasis (adapted from [52]).

SCOPE	**Optimal**	**Minimal**	**Rationale**
Intended use	Detection of VL or PKDL with the purpose of initiating treatment the same day	Detection of VL or PKDL	VL is the most deadly form of leishmaniasis, 95% fatality rate if not treated
Target population	Individuals with clinical signs suggestive of VL, and PKDL	Individuals with clinical signs suggestive of VL	Test ordered after clinical sign assessment
Target operator of test	Trained laboratory staff- technicians	Trained laboratory staff- scientists	Endemic region/mobile laboratories may have limited expertise
Target use setting	Health care facilities with minimal laboratory	Conventional laboratory	This test could replace gold standard (microscopy)

	infrastructure, or mobile team		
Target analyte to be detected	*Leishmania* DNA and internal control	*Leishmania* DNA	PCR-based technique
PERFORMANCE CHARACTERISTICS	**Optimal**	**Minimal**	**Rationale**
Limit of detection	5-10 cells/mL	100-200 cells/mL	Measured against a reference method
Clinical specificity	100%	>95%	Tested on negative blood samples
Leishmania species-specificity	*Leishmania* species-specific	*Leishmania* genus-specific	VL/PKDL known to be caused by *L. donovani* complex species
Type of analysis	Quantitative	Qualitative	Parasite load is valuable for treatment monitoring
TEST PROCEDURE	**Optimal**	**Minimal**	**Rationale**
Training requirements	One day for any level health care worker	One week for any level health care worker	PCR experience is likely limited with staff

Sample type	Peripheral (whole) blood	Splenic, bone marrow, lymph node biopsy or split skin smear	Minimally invasive sampling procedures and not requiring centrifugation preferred
Number of steps to be performed by operator	<3	<10	Risk of contamination/user error
Sample transfer requirements	Disposable transfer device provided	Pipettes required	Sample will need to be added from collection tube manually
Time to result	<1hr	<3hrs	For clinical decision-making
Internal control	Endogenous (included)	Exogenous (needs to be added)	Internal control to confirm validity of the test
Result interpretation	Automated result to LIS	Interpretation of amplification results	Results generally need to be validated
Auxiliary equipment	Semi-portable extraction and PCR equipment	Large, fixed extraction and PCR equipment	Equipment is required for PCR-based testing
Power Requirements	Intermittent (required for time of experiment)	Constant	Electricity supply often cannot be guaranteed

Maintenance/spare parts	Provided by supplier	Performed by/sourced by testing laboratory	Laboratory equipment requires maintenance
OPERATIONAL CHARACTERISTICS	**Optimal**	**Minimal**	**Rationale**
Operating conditions	5–50 °C, up to 90% relative humidity (RH), 0–4000 m above sea level	5–40 °C, up to 80% RH, 0–2000 m above sea level	High environmental temperatures and high humidity are often a problem in endemic countries
Reagent kit transport	No cold chain required; tolerance of transport stress for a minimum of 72h at −15 °C to 50 °C	Transport on ice packs required; tolerance of transport stress for a minimum of 72h at −15 °C to 20 °C	Refrigerated transport is costly and often cannot be guaranteed during the entire transportation process. Frequent delays in transport are common
Reagent kit storage/stability	No cold chain required. Up to 24 months at 50 °C, up to 90% humidity	4 °C storage required up to 24 months.	Refrigerated and freezer storage is costly and electricity supply often cannot be guaranteed

Reagents reconstitution/preparation	All reagents ready to use	Minimal transfer (no equipment needed)	Reduced interaction with sample/reagent reduces risk of contamination/user error
In use stability	<3h for single use test after reagents reconstituted	<1h for single use test after reagents reconstituted	High environmental temperatures and high humidity are often a problem in endemic countries
Biosafety requirement	No need for biosafety cabinet	Need for biosafety cabinet	Standard biosafety precautions when handling potentially infectious materials
PRICING	**Optimal**	**Minimal**	**Rationale**
Maximum price for individual test	<10 USD per test	<20 USD per test	Endemic regions often have constrained budgets
Maximum price for instrumentation.	<2000 USD	<20,000 USD	DNA extraction and PCR equipment may be used for other assays
Expected scale of manufacture	500,000 tests per year	100,000 tests per year	Based on 100,000-400,000 estimated VL cases (including follow up testing); and provided the test replaces reference method (microscopy)

Therefore, initial experimental work was performed to investigate pre-treatments to whole blood both before and after the bisulphite conversion step, followed by conventional extraction with a commercially available kit.

The next phase of whole blood extraction experimentation explored target capture technology to remove target DNA directly from the clinical sample and then carried through purification and conversion processes (Figure 1). This method was also chosen as it has the option to be adapted to an automated platform. The kinetoplast DNA (kDNA) minicircle was selected as the gene target of choice as it is present in extremely high copy numbers (~10,000 copies/genome), which mitigates potential inhibitory effects of residual peripheral blood [7]. kDNA minicircles encode guide RNA and are highly heterogeneous and have been reported to undergo rapid evolution (although this has been contested) [53, 54]. By selecting the conserved region, combined with bisulphite modification, this heterogeneity can be moderated, and a pan-*Leishmania* target region was exploited in the target capture method. Indirect sequence-specific target capture is the process by which DNA is hybridised with a target region-specific biotinylated capture probe and then subsequently captured with streptavidin-coated magnetic beads. This allows for the removal of undesirable DNA and inhibitors from complex clinical samples such as whole blood and for concentrating this target DNA for downstream applications. Additionally, 12S mitochondrial DNA was used as an endogenous control to measure background tissue presence.

A TOC for VL and PKDL was prioritised by the WHO, largely because drug resistance (particularly for pentavalent antimonials) can result in relapse of

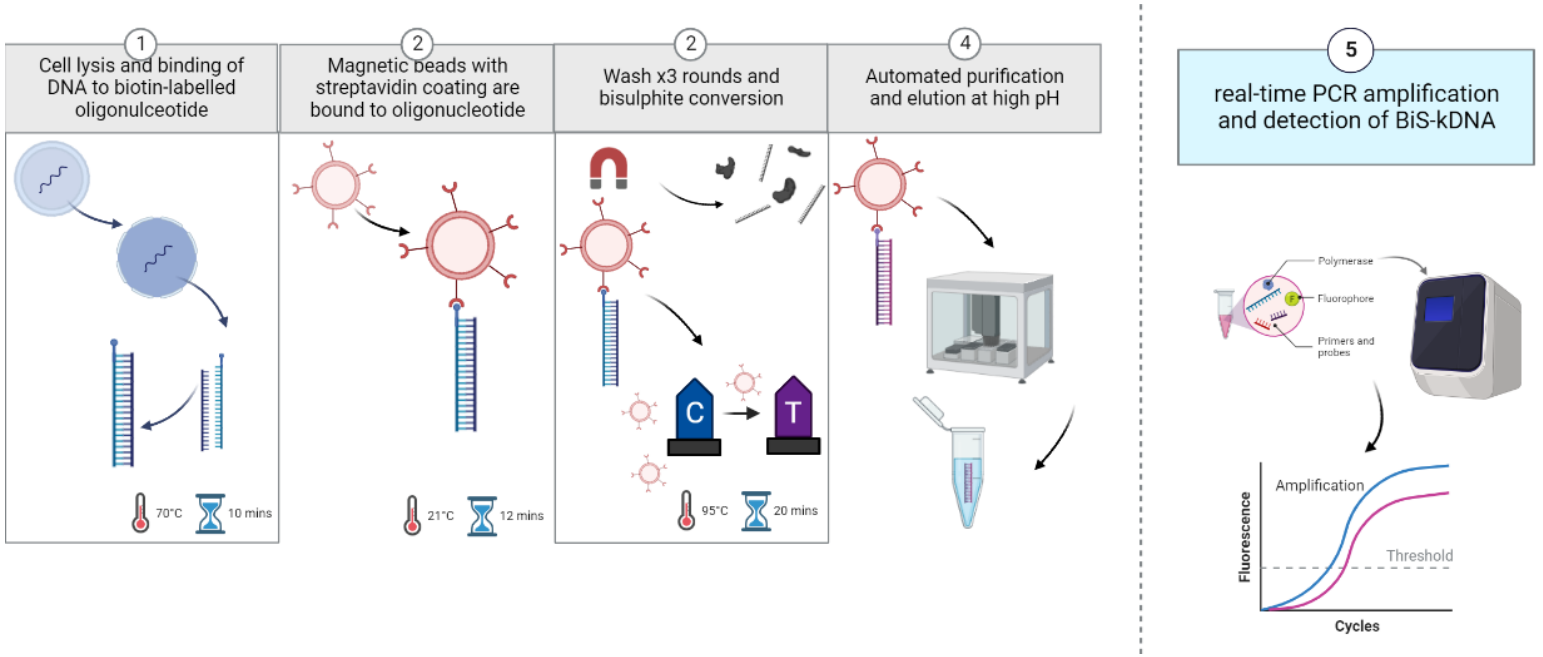

Figure 1. Target capture assay workflow, demonstrating the pathway for DNA binding to the capture oligonucleotide, through bisulphite conversion, washing and elution steps and the final real-time PCR amplification for detection. BiS- bisulphite converted.

disease [55]. Additionally, confirming viability can give information on carriers of the disease that act as reservoirs of the parasite. PCR is the most sensitive test of parasitological cure, performed one month and six months post-treatment [55]. However, due to its stability, non-viable *Leishmania* DNA remains in clinical samples and is detected for around 24 hours after parasite death. Thus, it has been suggested that RNA-based amplification is preferred [4]. The spliced leader RNA (SL-RNA) has been proposed as a measure of viability as it quickly degrades after the death of the pathogen, so can be measured against the presence of a DNA target [56, 57]. The SL-RNA sequences are highly conserved among all *Leishmania* species, are present in high copy numbers (~150 copies/ genome), are responsible for RNA processing and have roles in transcription and translation [58]. For viability status, an assay based on the SL-RNA was developed and tested, both with and without the reverse transcriptase enzyme, to measure how much amplified product originated from RNA as opposed to DNA. Bisulphite technology was incorporated into this assay design to ensure integration into the previously developed extraction system and to be used alongside, or as a reflex to, the previously developed *Leishmania* detection assays (Chapters 2 and 3).

3. Materials and methods

3.1 kDNA minicircle assay design for the detection of target *Leishmania* DNA

Literature searches were performed to identify assays designed to conserved regions of the kDNA minicircle gene. Designs from three papers were identified [59, 60, 61], and BLAST analyses were performed on each region. The assay designed by Francino et al. [61]was selected and multiple sequence alignments (MSAs) were performed for this design using the

MUSCLE algorithmic approach implemented in the Geneious Prime 2020.2.3 software package (https://www.geneious.com). The regions corresponding to a 188bp amplicon was identified (Figure 2a). A forward and reverse primer, and a probe were identified manually. Two unavoidable potential mismatches were observed at position 885 and 889, but designed so as to not occur near the 3' end of the reverse primer. Primers and probe were then converted to the bisulphite converted form, that is, with thymines replacing cytosines (and reverse complemented for the reverse primer)(Table 2). Primers and a probe (EasyBeacon probe technology) were ordered from suppliers IDT (Coralville, USA) and Pentabase (Odense, Denmark), respectively.

3.2 12S mitochondrial DNA assay design for the detection of an endogenous control

GenBank nucleotide searches were performed to identify conserved regions of the 12S mitochondrial DNA gene (GenBank accession number MK617223). BLAST analyses were performed on these regions, and MSAs were performed and a conserved region corresponding to a 74bp amplicon was identified for the wild type (four base) region and to a 99bp amplicon for the bisulphite converted region (Figure 2b). Forward and reverse primers, and a probe were identified for each design, then transformed to the bisulphite converted form for the bisulphite converted design (and reverse complemented for the reverse primer), (Table 2). Primers and probes (EasyBeacon probe technology) were ordered in both the bisulphite converted form and wild type form from suppliers, IDT and Pentabase, respectively.

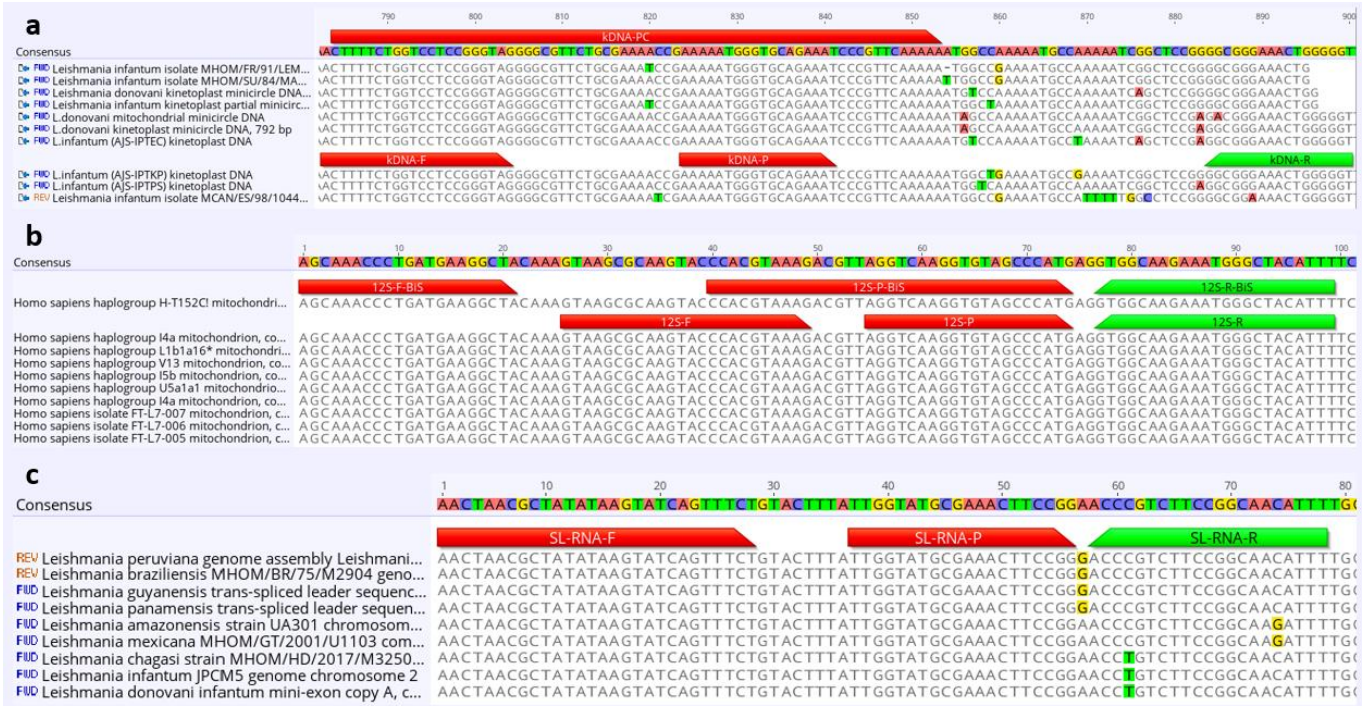

Figure 2. Multiple sequence alignments for wild type (four base) kinetoplast DNA (a), 12S mitochondrial DNA (b), and Spliced-leader RNA (c) used for assay design. The primer and probe names relate to those described in Table 2. Designed assays were converted to bisulphite form (three base) manually. Accession numbers are as follows, (a) AF190475, AF190476, AJ010081, AJ275331, X84844, Y11401, Z35271, Z35272, Z35273, EU437403 (reversed); (b) ON688208, ON457162, ON409192, ON682947, ON640629, ON682948, ON602072, ON156780, ON156779, ON156778; (c) LN609266 (reversed), LS997601 (reversed), MG010485, MG010486, CP040130, FR799555, CP048158.1, FR796434, AF097653, respectively.

Table 2. Primer and probe details for the described assays. Primer melting temperatures were calculated for 50mM NaCl, probe melting temperatures were assessed through melt curve.

Assay (target gene)	Sequence name	Sequence (5′-3′)	Melting temperature
Blood detection (kDNA)	kDNA-F	AATTTTTTTGGTTTTTTGGGTAG	48.9˚C
	kDNA-P	FAM-AAAAATGGGTGTAGAAAT-BHQ1	61.3 ˚C
	kDNA-R	ACCCCCAATTTCCCACC	55.1 ˚C
Blood detection/bisulphite conversion (12S mitochondrial DNA)	12S-F-BiS	AGTAAATTTTGATGAAGGTTA	45.0 ˚C
	12S-P-BiS	Penta Yellow-TTATGTAAAGATGTTAGGTTAAGGTGTAGTTTATG-BHQ1	68.6 ˚C
	12S-R-BiS	AAATATAACCCATTTCTTACCAC	48.7 ˚C
Bisulphite conversion (12S	12S-F	GTAAGCGCAAGTACCCACGTAAAG	59.7 ˚C
	12S-P	Penta Yellow-AGGTCAAGGTGTAGCCCATG-BHQ1	68.9 ˚C
	12S-R	AAATGTAGCCCATTTCTTGCCAC	56.2 ˚C

mitochondrial DNA)			
Viability (SL-RNA)	SL-RNA-F	AATTAATGTTATATAAGTATTAGTTTTT	44.8 ˚C
	SL-RNA-P	FAM-TTGGTATGTGAAATTTTTGG-BHQ1	63.3 ˚C
	SL-RNA-R	AAATATTACCAAAAAACAAAT	42.0 ˚C
Target capture (kDNA)	kDNA-PC	BioTinTEG/CTTTTCTGGTCCTCCGGGTAGGGGCGTTCTGCGAAAACCGAAAAATGGGTGCAGAAATCCCGTTCAAAAA	72.3 ˚C

3.3 Whole peripheral blood extraction pre-treatments

In order to investigate the extraction and bisulphite conversion of *Leishmania* DNA from whole peripheral blood, early exploratory work was performed on a range of clinical sample pre-treatments both directly before and after bisulphite conversion step. This was performed on contrived clinical samples of spiked *L. donovani* cells to a concentration of 1000 cells/mL in four pooled whole blood samples, derived from a pool of three clinical blood samples each. All patient samples were de-identified and tested in accordance with St Vincent's Hospital ethics approval, HREC number LNR/16/SVH/231. Pre-treatment conditions were performed according to Table 3. The workflow of pre-treatment, bisulphite conversion, and DNA extraction was according to Figure 3, vortexing briefly between each step. A control sample was tested, with no pre-treatment applied, according to Figure 3. The total pre-treatment lysate was extracted on the GS-mini platform (Genetic Signatures Ltd., Sydney, Australia), using the Zinexts Blood 1200 Kit (Taipei, Taiwan), with 100µL elution volume (with the 1000µL elution buffer position replaced with 1000µL of Genetic Signatures Ltd. elution solution (pH 12.2), (Sydney, Australia)).

Table 3. Volumes of the pre-treatment added to the reaction and incubation conditions (temperature and time). The pre-treatments were added to 150µl sample and 400uL sodium bisulphite. 95˚ for 20 mins. SDS- sodium dodecyl sulfate, ProK- Proteinase K.

Pre-treatment	Supplier	Volume added	Incubation step
Triton X-100	Sigma-Aldrich, UK	15µL	*95˚C for 5 min
SDS 10%w/v	Sigma-Aldrich, UK	40µL	*37˚C for 10min
ProK	Versant, Switzerland	15µL	*37˚C for 10min
SDS + ProK	Sigma-Aldrich, UK, Versant, Switzerland	40µL SDS, 15µL ProK	*37˚C for 10min
*Dithiothreitol (*Sputasol)	Thermofisher, USA	150µL	*37˚C for 10min
tris(2-carboxyethyl) phosphine) (TCEP)	Sigma-Aldrich, UK	5µL	*37˚C for 10min
Control (no additive)	N/A	N/A	N/A

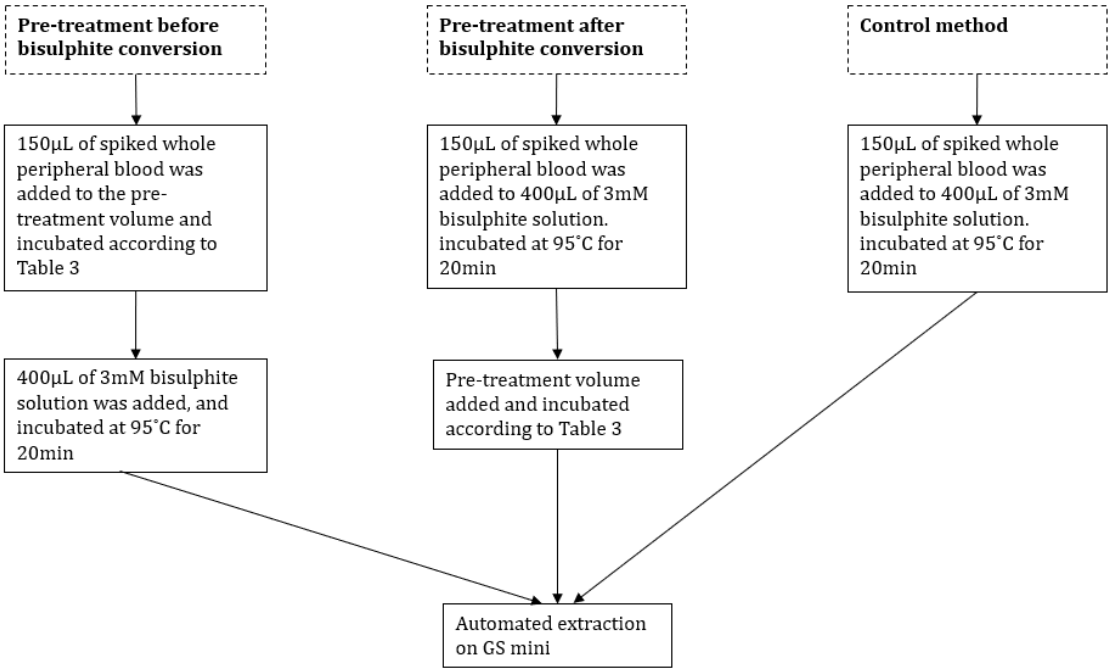

Figure 3. Work flow of pre-treatment application, before or after bisulphite conversion.

The kDNA/12S mitochondrial DNA duplex assay was tested in duplicate, and PCR reactions contained 10µL Bioline SensiFast mastermix (London, UK), 0.9µM of each kDNA primer and 0.2µM kDNA probe, 0.18µM of each endogenous control primer and 0.13µM control probe, 2µL contrived samples and molecular grade water up to a reaction volume of 20µL. PCR cycling conditions were as follows: 95˚C for 3 mins; 40 cycles of 95˚C for 2sec; 55˚C for 10 sec (data collection point); and 60˚C for 10 sec.

A 12S mitochondrial DNA singleplex assay was tested against a wild type (four-base) version of the 12S DNA mitochondrial assay design, that is, with no cytosines converted to thymines. These assays were amplified in singleton and PCR reactions contained 10µL SensiFast mastermix, 0.18µM of each primer and 0.13µM probe, 2µL contrived samples, and molecular grade water up to a reaction volume of 20µL. PCR cycling conditions were as follows: 95˚C for 3 mins; 40 cycles of 95˚C for 2sec; 55˚C for 10 sec (data collection point); and 60˚C for 10 sec.

The relative expression of bisulphite conversion was calculated by the ΔC_T method [62], using the following equation:

$$=2^{(\text{Ct bisulphite converted-Ct non-bisulphite converted})}$$

(assuming that each PCR cycle doubles the number of amplicons).

3.4 Target capture kDNA biotinylated capture probe design

A 70bp region of the conserved kDNA region was identified from the previously described kDNA MSAs (Figure 2a), and labelled with a biotin label at the 5' end of the oligonucleotide using a C6 spacer by IDT.

3.5 Target capture and blood extraction validation

2x "Binding Wash Buffer" (BWB) was prepared as the following solution: 10mM TrisHCl; 1mM EDTA; 2M NaCl. ThermoFisher Streptavidin beads (Massachusetts, USA) were diluted 1:1 in 2x BWB. 5mL of peripheral blood was collected in a Copan EDTA vacutainer (Murrieta, USA) and stored at 4°C.

Leishmania donovani promastigotes, obtained from the American Type Culture Collection (ATCC, Manassas, USA) were diluted to 5.6x10^5 cells/mL in molecular grade water, and diluted in a further 1 in 10 dilution series in molecular grade H_2O to 5.6x10^0 cells/mL. 17.8µL of each dilution was added to 100µL whole blood (to create contrived *L. donovani* positive whole peripheral blood samples) or molecular grade H_2O background to a total concentration of 10^5cells/mL down to 0.1 cells/mL. A negative process control and negative clinical samples, (where no cells were added to molecular grade H_2O background or blood background, respectively), were also included.

These diluted cells and contrived samples were then incubated at 70°C for 10 minutes with 5µL 10µM biotin-labelled oligonucleotide, 850µL molecular grade H_2O, 850µL 2x BWB and, 200µL of the diluted streptavidin beads, which was incubated at room temperature for 12 minutes, with slow inversion. The beads were magnetised, and the supernatant discarded.

The DNA/oligonucleotide/bead complex was washed with 1mL 1x BWB solution for three rounds, then eluted in 200µL molecular grade H_2O at 72°C for 10 mins and subsequently magnetised. 150µL of this supernatant was added to 250µL 3mM bisulphite and incubated at 95°C for 20 mins. The contrived samples were eluted directly into 400µL of 3mM bisulphite solution, and 200µL of this lysate was extracted on the GS-mini platform using the Pathogens A kit with an elution volume of 100µL.

The diluted cells and contrived samples were tested against a commercial kit reference method (Blood 1200 extraction kit, Zinexts), using contrived blood

samples prepared as described above. A total concentration of 10^5cells/mL down to 1 cells/mL were added to 900µL 3M bisulphite solution, incubated at 95˚C for 20 minutes. The total 1000µL lysate was extracted on the GS-mini using the Blood 1200 kit (with the 1000µL elution buffer position replaced with 1000µL of Genetic Signatures Ltd. elution solution [pH 12.2]) with an elution volume of 100µL.

The kDNA/12S mitochondrial DNA duplex assay was tested in duplicate, and PCR reactions contained 10µL SensiFast mastermix, 0.9µM of each kDNA primer and 0.2µM kDNA probe, 0.18µM of each endogenous control primer, 0.13µM control probe and 2µL eluate (of contrived sample DNA, diluted promastigote *L. donovani* DNA from 100 cells/mL to 0.1 cells/mL, control samples) up to a reaction volume of 20µL. PCR cycling conditions were as follows: 95˚C for 3 mins; 40 cycles of 95˚C for 2sec; 55˚C for 10 sec (data collection point); and 60˚C for 10 sec.

3.6 Viability assay design

Literature searches were performed to identify conserved regions of the SL-RNA region. Designs from two papers were identified [56, 63], and BLAST analyses were performed on each region. MSAs were performed for these designs using the MUSCLE algorithmic approach implemented in the Geneious Prime 2020.2.3 software package (https://www.geneious.com), and a conserved region corresponding to a 78bp amplicon was identified (Figure 2c). The regions corresponding to the forward and reverse primers, and a probe were identified manually. One unavoidable potential mismatch was observed at position 74, but designed so as to not occur near the 3' end of the reverse probe. Primers and probes were then converted to the bisulphite converted form, that is, with thymines replacing cytosines (and reverse complemented

for the reverse primer) Table 2). Primers and probes (EasyBeacon probe technology) were ordered from suppliers, IDT and Pentabase, respectively.

3.7 Viability assay validation

Leishmania nucleic acid was extracted, and bisulphite converted from 81 previously described *Leishmania* positive DNA samples [16]. These were previously analysed by ITS1 PCR-RFLP method, and the DNA samples were stored at -20˚C. Although validated previously for DNA extraction, the Pathogens A kit is also developed for RNA extraction. This kit is a closed cartridge-based system whereby nucleic acid is bound to magnetic beads, with subsequent washing and finally elution steps, using heating and shaking to increase nucleic acid yield. Downstream bisulphite conversion, post-conversion extraction (with the Pathogens A kit), and storage (at -80˚C) were all appropriate for RNA. The SL-RNA assay was tested for separate DNA and combined DNA/RNA detection in clinical samples.

The *Leishmania* nucleic acid clinical samples were assayed in duplicate by the SL-RNA assay, with and without the use of a reverse transcriptase (RT) enzyme and the reverse transcriptase step, to assess how much of the PCR signal originated from RNA as opposed to DNA. The percentage of DNA detected by the SL RNA assay was calculated by the ΔC_T method [62], using the following equation:

$$= \frac{100\%}{2(Cq\ with\ RT - Cq\ without\ RT)}$$

(assuming that each PCR cycle doubles the number of amplicons).

For LOD studies, commercially available whole genomic DNA and whole cells of the following species: *L. donovani (*MHOM/IN/80/DD8 supplied at 2.3 x 10^7 cells/mL), *L. braziliensis* (MHOM/BR/75/M2903

supplied at 1.63 x 10^8 cells/mL), *L. tropica* (MHOM/SU/74/K27 supplied at 1.03 x 10^7 cells/mL), *L. amazonensis* (MHOM/BR/73/M2269 supplied at 9.9 x 10^6 cells/mL), *L. mexicana* (MHOM/BZ/82/BEL21 supplied at 1.51 x 10^8 cells/mL), *L. infantum* (MHOM/TN/80/IPT-1 supplied at 2.2 x10^6 cells/mL) and *L. major* (MHOM/SU/73/5-ASKH supplied at 7.1 x 10^6 cells/mL) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Genomic DNA was bisulphite converted by adding 2,880,000 or 28,800 DNA copies (quantified by manufacturer and using an online calculator, https://www.thermofisher.com/au/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html), depending on available starting concentration, to a total volume of 150 µL with molecular grade H_2O, and 250 µL 3M sodium bisulphite was added. A negative process control (NPC) of 150 µL molecular grade H_2O was included in each run. The samples were mixed by vortexing and incubated at 95°C for 15 minutes. Subsequently, 200µL of this lysate was purified on the GS-mini with the Sample Processing Pathogens A kit (Genetic Signatures Ltd., Sydney, Australia), according to the manufacturer's recommendations, eluted in a volume of 50µL. The eluted DNA was then diluted in molecular grade H_2O in six 10-fold dilution series to 0.1 copy/PCR. The SL-RNA assay was tested in singletons for combined DNA/RNA detection in the LOD studies.

The DNA-based PCR reaction was conducted with 10µL SensiFast mastermix, 1.3mM MgCl2, 0.3µM of each primer and 0.25µM probe, 2µL *Leishmania* nucleic acid extracted previously according to the methods described in the pan-*Leishmania* assay [16]. Molecular grade water was added to a reaction volume of 20µL. PCR cycling conditions were as follows: 95°C for 3 mins; 40 cycles of 55°C for 10 sec (data collection point);

and 60°C for 10 sec. The combined DNA/RNA-based PCR reactions were conducted with 10μL SensiFast mastermix, 1.3mM $MgCl_2$, 0.263μL BioRad iScript reverse transcriptase (Hercules, USA), 0.3μM of each primer, and 0.25μM probe, 2μL *Leishmania* nucleic acid extracted previously according to the methods described in the pan-*Leishmania* assay [16]. Molecular grade water was added to a reaction volume of 20μL. PCR cycling conditions were as follows: 42°C for 10 mins; 95°C for 3 mins; 40 cycles of 95°C for 2sec; 55°C for 10 sec (data collection point); and 60°C for 10 sec. A negative template control (NTC) of molecular grade H_2O was included in each PCR.

4. Results

4.1 Peripheral blood assay pre-treatments

The following conditions were excluded due to mechanical failure as a result of pellet formation, restricting the pipette head of the instrument from aspirating and transferring lysate from the sample tube: Triton X-100 (before bisulphite conversion); Triton X-100 (after bisulphite conversion); SDS (before bisulphite conversion); Proteinase K (after bisulphite conversion); TCEP (before bisulphite conversion); and the 'no additive' control condition. Even if the mechanical failure occurred in fewer than the total four contrived clinical samples, the entire condition was excluded due to the future potential for platform error.

Of the remaining conditions, the endogenous control was detected for all samples across all conditions, except for one of the two replicates in one condition – SDS (after bisulphite conversion), thus denoted a 'fail'. Averages of the duplicate Ct values were taken, ranging from 26.3Ct to 38.5 (12.2Ct range). For the kDNA target amplification, a positive result 'pass' was accepted if both

duplicate PCR reactions were positive. Across the four contrived clinical samples the pass rate, and Ct range was as per Table 4.

Table 4. 'Pass' rate of the four contrived clinical samples and associated Ct value range (no range available if one sample passed)

Pre-treatment	**'Pass' rate**	**Ct range**
SDS post- bisulphite conversion	2/4	38.7-39.3
ProK pre- bisulphite conversion	1/4	38.9
SDS + ProK pre- bisulphite conversion	2/4	38.6-39.3
SDS + ProK post- bisulphite conversion	1/4	39.3
Sputasol pre- bisulphite conversion	2/4	38.3-38.7
Sputasol post- bisulphite conversion	2/4	39.4-39.6
TCEP post- bisulphite conversion	1/4	39.1

As a pass rate of at most 2/4 contrived samples occurred across all remaining conditions and delayed Cts of between 38.3-39.6 occurred, the conversion efficiency was queried. The condition resulting in the lowest averaged Ct value, across the four contrived clinical samples was 38.7Cts. For 1000 cells/mL, lower Ct values within the 32Ct range would be expected, as defined in early experimental work (data not shown). Thus, side-by-side amplification of bisulphite and non-bisulphite converted assays were run to assess bisulphite conversion ratio. The samples that underwent the pre-treatments that were excluded due to mechanical failure in the previous sample set were again excluded from conversion analysis. Of these samples, two samples had no detectable bisulphite or non-bisulphite 12S mitochondrial DNA, and three samples detected only non-bisulphite converted material (Ct range 26.7-47.7), thus were excluded from the conversion ratio analysis. Of the remaining samples, three had predominantly bisulphite converted material, and 26 samples had predominantly non-bisulphite-converted material (Table 5). Figure 4 demonstrates the average difference in Ct value of each pre-treatment condition across the four clinical samples and the calculated relative abundance on non-bisulphite converted material.

No amplification was detected in the NPC or the NTC.

Table 5. Ct values for each pre-treatment condition for bisulphite-converted and non-bisulphite material using the 12S mitochondrial DNA assay, and resultant relative expression

Sample and pre-treatment condition	Bisulphite-converted material	Non-bisulphite converted material	Ct difference	Relative expression
Sample 1 SDS after bisulphite conversion	33.19	28.76	4.43	21.6
Sample 1 ProK before bisulphite conversion	26.9	25.37	1.53	2.9
Sample 1 SDS + ProK before bisulphite conversion	25.68	21.24	4.44	21.7
Sample 1 SDS + ProK after bisulphite conversion	27.64	26.61	1.03	2.0
Sample 1 Sputasol before bisulphite conversion	28.99	25.03	3.96	15.6
Sample 1 Sputasol after bisulphite conversion	27.1	26.87	0.23	1.2
Sample 1 TCEP after bisulphite conversion	29.88	27.03	2.85	7.2
Sample 2 SDS after bisulphite conversion	39.11	40.89	-1.78	0.3
Sample 2 ProK before bisulphite conversion	27.3	28.01	-0.71	0.6
Sample 2 SDS + ProK before bisulphite conversion	25.5	25.23	0.27	1.2
Sample 2 SDS + ProK after bisulphite conversion	26.92	27.71	-0.79	0.6
Sample 2 Sputasol before bisulphite conversion	27.03	24.43	2.6	6.1

Sample 2 Sputasol after bisulphite conversion	32.18	25.35	6.83	113.8
Sample 2 TCEP after bisulphite conversion	37.69	31.07	6.62	98.4
Sample 3 SDS after bisulphite conversion	37.57	30.71	6.86	116.2
Sample 3 ProK before bisulphite conversion	26.91	23.11	3.8	13.9
Sample 3 SDS + ProK before bisulphite conversion	42.47	25.59	16.88	120610.8
Sample 3 SDS + ProK after bisulphite conversion	28.53	22.86	5.67	50.9
Sample 3 Sputasol before bisulphite conversion	35.36	24.9	10.46	1408.6
Sample 3 Sputasol after bisulphite conversion	29.96	24.51	5.45	43.7
Sample 3 TCEP after bisulphite conversion	32.09	23.55	8.54	372.2
Sample 4 SDS after bisulphite conversion	29.72	24.63	5.09	34.1
Sample 4 ProK before bisulphite conversion	25.58	19.23	6.35	81.6
Sample 4 SDS + ProK before bisulphite conversion	27.53	22.43	5.1	34.3
Sample 4 SDS + ProK after bisulphite conversion	27.87	21.16	6.71	104.7
Sample 4 Sputasol before bisulphite conversion	26.03	23.17	2.86	7.3
Sample 4 Sputasol after bisulphite conversion	30.81	24.94	5.87	58.5
Sample 4 TCEP after bisulphite conversion	26.44	24.03	2.41	5.3

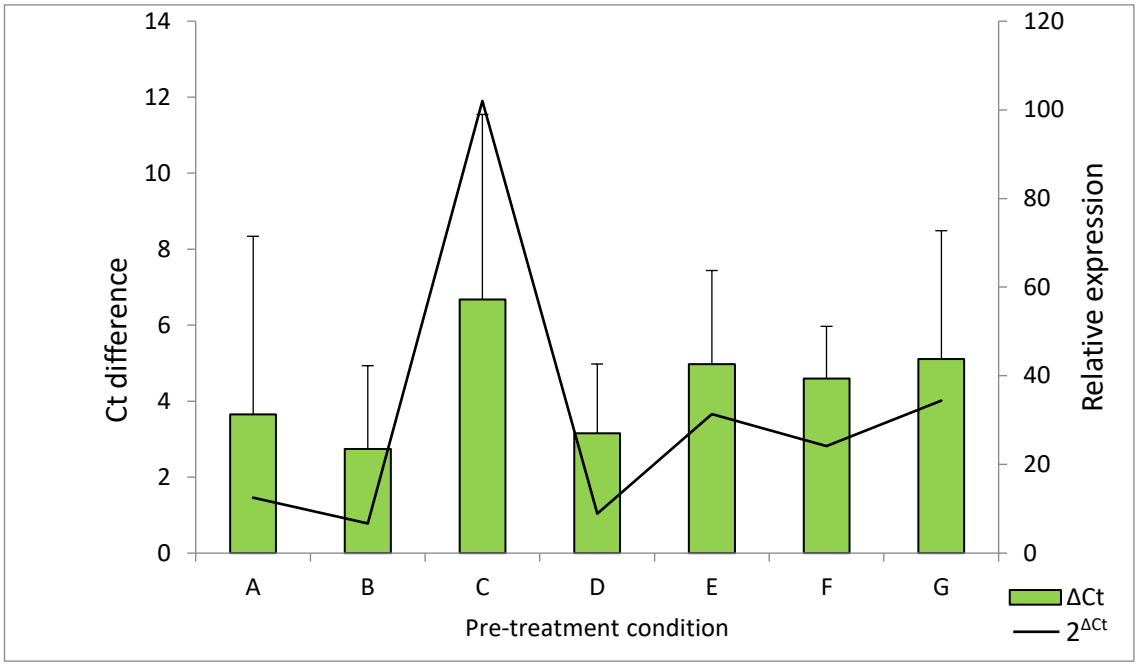

Figure 4. Average difference in Ct values (ΔCt) for each pre-treatment condition and calculated relative expression of non-bisulphite material ($2^{\Delta Ct}$). A- SDS after bisulphite conversion, B- ProK before bisulphite conversion, C- SDS + ProK before bisulphite conversion, D- SDS + ProK after bisulphite conversion, E- Sputasol before bisulphite conversion, F- Sputasol after bisulphite conversion, G- TCEP after bisulphite conversion. Error bars represent standard error of the difference.

When calculating the ratio of bisulphite conversion for each pre-treatment condition and each sample, an average of 4410-fold more non-bisulphite converted material was observed (Table 5). Across all samples, only three conditions returned predominantly more bisulphite converted material; however, these were all observed in one contrived clinical sample (Sample 2). This may indicate that the sample itself was less inhibitory. Of the conditions, Proteinase K pre-treatment before bisulphite conversion gave the highest conversion rates when averaged across the four samples; however, this method still retained 6.7-fold more non-bisulphite converted material (Figure 4). Given the incomplete conversion ratios and delayed Ct target results, the target capture method was pursued.

4.2 Peripheral blood target capture

The commercial method for the extraction of *L. donovani* promastigotes spiked into whole blood did not produce any meaningful results for the kDNA target gene. The target capture extracted cells were detected to a limit of detection (LOD) of 10 cells/mL, whereas the target capture-extracted contrived samples were detected to a LOD of 100 cells/mL. No amplification was detected in the negative process control or the negative clinical sample.

The endogenous 12S mitochondrial DNA was not detected in the H_2O diluted cells but was detected with an average Ct value of 23.1 for the target capture-extracted contrived samples and 25.6 for the commercial kit-extracted contrived samples.

4.3 Viability assay

RNA was detected in all but two clinical nucleic acid samples and DNA in all but one. Sample 63 showed no amplification, in line with the results from our previous study [16], thus presumed a true negative and omitted from the results. Sample 5 showed no amplification in the "without RT" condition, so it was considered erroneous and omitted from the results. The amplification curves for Sample 49 are shown, with a lower Ct value observed for the “with RT” condition (Figure 5). The Ct values of the 79 samples screened by the SL-RNA assay, with and without reverse transcriptase added, are shown in Table 6 and summarised in Figure 6. The ratio between the two Ct values for the positive samples was calculated from the "with RT" PCR and the above equation, reflecting the presence and amount of surviving DNA. The calculated mean difference in Ct values (excluding RNA negative samples) was -0.26Ct values (standard deviation ±0.34), and the mean DNA presence was 122.8% (±28.1). DNA presence over 100% indicates no RNA was present, occurring in 64 of the 79 samples (Table 6).

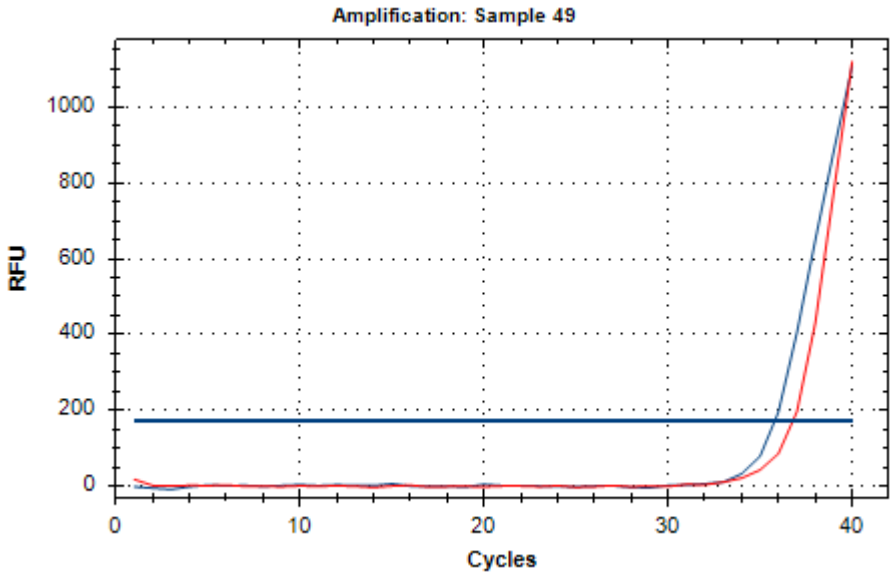

Figure 5. Amplification curves for Sample 49, “with Reverse Transcriptase” (blue) and “without Reverse Transcriptase” (red).

Table 6. Ct values of the SL-RNA assay for each clinical sample, with and without reverse transcriptase enzyme and calculation of DNA percentage. N/A- not detected.

Sample ID	Ct with RT	Ct without RT	Ct difference	% DNA	Sample ID	Ct with RT	Ct without RT	Ct difference	% DNA
1	27.24	26.91	-0.33	125.70	**41**	22	21.58	-0.42	133.79
2	26.2	26.14	-0.06	104.25	**42**	23.45	23.19	-0.26	119.75
3	33.05	32.32	-0.73	165.86	**43**	25.06	24.47	-0.59	150.52
4	39.3	38.76	-0.54	145.40	**44**	31.26	30.7	-0.56	147.43
6	31.62	31.16	-0.46	137.55	**45**	23.4	22.93	-0.47	138.51
7	25.52	25.46	-0.06	104.25	**46**	27.4	27.22	-0.18	113.29
8	31.23	31.3	0.07	95.26	**47**	35.22	35.31	0.09	93.95
9	26.49	26.13	-0.36	128.34	**48**	26.82	26.42	-0.4	131.95
10	31.05	30.97	-0.08	105.70	**49**	35.78	36.76	0.98	50.70
11	30.58	29.27	-1.31	247.94	**50**	23.65	23.67	0.02	98.62
12	31.34	31	-0.34	126.58	**51**	32.5	32.06	-0.44	135.66
13	25.95	25.79	-0.16	111.73	**52**	36.37	36.42	0.05	96.59

14	30.63	29.89	-0.74	167.02	**53**	31.29	30.84	-0.45	136.60
15	28.81	28.41	-0.4	131.95	**54**	28.06	27.65	-0.41	132.87
16	25.18	25.36	0.18	88.27	**55**	22.31	21.89	-0.42	133.79
17	36.36	36.74	0.38	76.84	**56**	30.49	30.1	-0.39	131.04
18	28.48	28.3	-0.18	113.29	**57**	23.02	23.04	0.02	98.62
19	26.29	26.17	-0.12	108.67	**58**	25.18	25.18	0	100.00
20	24.71	24.49	-0.22	116.47	**59**	28.86	28.39	-0.47	138.51
21	29.21	28.6	-0.61	152.63	**60**	28.5	28.43	-0.07	104.97
22	32.07	32.77	0.7	61.56	**61**	31.87	31.46	-0.41	132.87
23	30.55	30.59	0.04	97.27	**62**	29.78	29.42	-0.36	128.34
24	33.59	33.03	-0.56	147.43	**64**	23.85	23.38	-0.47	138.51
25	24.19	24.05	-0.14	110.19	**65**	30.1	29.78	-0.32	124.83
26	27.54	26.96	-0.58	149.48	**66**	31.02	30.78	-0.24	118.10
27	24.7	24.24	-0.46	137.55	**67**	27.68	27.38	-0.3	123.11
28	33.4	32.9	-0.5	141.42	**68**	33.99	33.82	-0.17	112.51
29	22.44	21.86	-0.58	149.48	**69**	32.86	33.1	0.24	84.67

30	26.43	25.95	-0.48	139.47	**70**	34.11	34.13	0.02	98.62
31	32.59	32.27	-0.32	124.83	**71**	33.47	33.18	-0.29	122.26
32	30.75	30.84	0.09	93.95	**72**	35.86	35.89	0.03	97.94
33	33.27	33.22	-0.05	103.53	**73**	32.47	32.23	-0.24	118.10
34	26.07	25.29	-0.78	171.71	**74**	29.75	29.53	-0.22	116.47
35	31.59	31.23	-0.36	128.34	**75**	21.08	20.43	-0.65	156.92
36	32.73	32.53	-0.2	114.87	**76**	26.1	26	-0.1	107.18
37	32.55	32.6	0.05	96.59	**77**	25.66	25.34	-0.32	124.83
38	31.75	31.34	-0.41	132.87	**78**	27.94	28.62	0.68	62.42
39	29.22	28.75	-0.47	138.51	**79**	27.87	27.49	-0.38	130.13
40	35.1	34.25	-0.85	180.25	**80**	24.5	24.06	-0.44	135.66
					81	24.45	24.34	-0.11	107.92

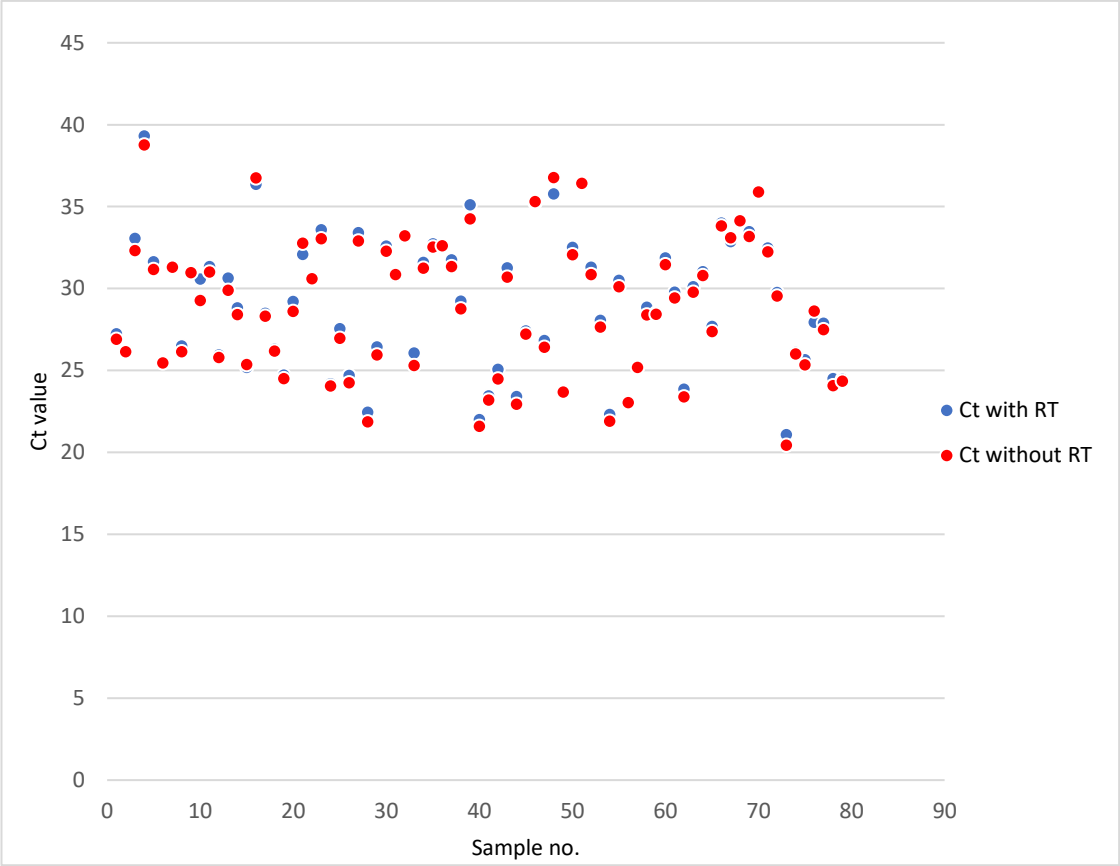

Figure 6. Ct values associated with each sample, of the "With RT" and "Without RT" assay results. The "paired" nature of Ct values returned for each sample indicates the small Ct difference between the two conditions, despite the wide Ct value range observed.

In order to determine the LOD, promastigotes from seven *Leishmania* species were used (Table 7). LOD was defined as the lowest dilution in which DNA/RNA was detected. LOD differed among *Leishmania* species, the lowest LOD reaching 1 genomic copy/PCR for *L. amazonensis, L. donovani,* and *L. tropica. L. major* and *L. mexicana* were detected at 10 genomic copies/PCR and *L. braziliensis* and *L. infantum* at 100 genomic copies/PCR.

No amplification was detected in the negative process control or the negative clinical sample.

Table 7. Ct values of the SL-RNA assay as detected in the LOD study. N/D- not detected

copies/PCR	***L. amazonensis***	***L. braziliensis***	***L. donovani***	***L. infantum***	***L. major***	***L. mexicana***	***L. tropica***
10,000	25.33	25.54	22.03	26.33	27.36	27.59	24.34
1,000	28.13	28.66	24.87	29.07	30.02	30.15	26.36
100	29.03	33.87	28.82	31.33	31.85	35.95	28.35
10	33.08	N/D	33.09	N/D	38.09	38.66	31.64
1	38.76	N/D	35.13	N/D	N/D	N/D	35.28
0.1	N/D	N/D	N/D	N/D	N/D	N/D	N/D

5. Discussion

Attempts to develop validation of methods for extraction and detection of *Leishmania* promastigote nucleic acid using target capture technology targeting the kDNA minicircle gene are described here. This approach used whole peripheral blood to simulate clinical samples or molecular grade water background, and was compared against a commercial blood extraction kit. Each of these methods was coupled with downstream bisulphite conversion and real-time PCR detection in the bisulphite-converted form. Target capture is an appropriate technology to pursue in challenging samples such as whole blood with bisulphite conversion. The method was validated following early optimisation work, exploring various pre-treatments applied to peripheral whole blood samples. The pre-treatments were applied directly before and after the bisulphite conversion step in order to improve detection of the kDNA target gene. For many of these pre-treatments, *Leishmania* cells could not be processed in blood due to mechanical failure of the automated platform employed. Moreover, where successful pre-treatments were applied, the best-performing condition, adding of *Dithiothreitol (*Sputasol) reagent prior to the bisulphite conversion reaction, still resulted in a delayed Ct range of 38.3-38.7Cts when detecting 1000 cells/mL. Not only were delayed target Ct values observed, poor bisulphite-conversion rates occurred, thus, target capture was explored as an alternative nucleic acid extraction method.

A selection of previous studies utilising peripheral blood for *Leishmania* detection with real-time PCR are listed in Table 8, although these vary whether detected from whole blood or separated blood components. Only studies targeting human peripheral blood were included. These studies explored the extraction of whole peripheral blood, with downstream detection by real-time PCR. The studies utilised commercial kits or standard DNA extraction methods directly from sample. This study is the first to explore an enrichment

Table 8. Selection of clinical studies using various real-time PCR methods to detect *Leishmania* from peripheral blood in humans.

Geographical region	Target gene	Type of sample	Assay type	Assay chemistry	Typing	Reference
Old World	rDNA	Blood (whole)	Quantitative	Fluorescent probe	No	[64]
Old World/ New World	rDNA	Blood (PBMC)/ bone marrow/ skin biopsy	Quantitative	Fluorescent probe	Yes	[65]
New World	kDNA	Blood (whole)/ bone marrow/ skin biopsy/ sand flies	Qualitative	Intercalating dye	Yes	[66]
Old World	kDNA	Blood(PBMC)/ bone marrow	Quantitative	Fluorescent probe	No	[67]
Old World	rDNA	Blood (whole)/ bone marrow/ lymph node/cutaneous lesion aspirates	Qualitative	Fluorescent probe	Yes	[68]
New World	kDNA	Blood (whole)	Quantitative	Intercalating dye	No	[69]

Old World Blood	REPL repeats (L42486.1)	Blood (buffy coat)	Quantitative	Fluorescent probe	Specific for *L. infantum* and *L. donovani*	[70]
New World	kDNA	Blood (whole)/ urine	Quantitative	Intercalating dye	No	[71]
Old World/ New World	rDNA	Blood (whole)/ skin biopsy/ bone marrow	Qualitative	Intercalating dye	Yes	[72]
Old World	REPL repeats (L42486.1)	Blood (buffy coat)/ skin	Quantitative	Fluorescent probe	Specific for *L. infantum* and *L. donovani*	[73]
Old World	G3PD	Blood (whole)	Quantitative	Intercalating dye	Specific for *L. infantum*	[74]
Old World	ITS1	Blood (buffy coat)	Quantitative	Intercalating dye	Yes	[75]

PBMC- Peripheral blood mononuclear cells, rDNA- ribosomal DNA, kDNA- kinetoplast DNA, ITS1- internal transcribed spacer 1, SLACS- splice leader-associated retrotransposons, MAG- MSP-associated gene

method prior to DNA extraction- in this case, target capture technology. The LOD observed in the contrived clinical samples of 100 cells/mL is clinically relevant, although other studies report lower LODs with this marker [65, 69, 76, 77]. When the assay was run with *Leishmania* cells in a molecular grade water background, the detection limit observed was 10 cells/mL, evidencing that the inhibitory nature of blood retains a negative effect on the performance. Our study aimed to avoid PCR inhibitors found in heparin or citrate-based peripheral blood collection tubes by using EDTA collection tubes, nevertheless, innate blood components such as haemoglobin and immunoglobulin G have been identified as PCR inhibitors[51, 78]. Although the contrived clinical samples do not precisely mimic human clinical infection (*Leishmania* cells reside within macrophages), macrophages are osmotically lysed with the addition of water, occurring in the initial lysis step of our extraction process. For these reasons, further work is required with positive whole peripheral blood samples from both symptomatic and asymptomatic patients to assess clinical sensitivity. However, this work was not possible during the study period due to restrictions from the SARS-CoV-2 pandemic. All clinical sample DNA was derived from *Leishmania* cases imported into Australia from sources such as migrants, travellers, and defence force personnel. As international travel and migration were restricted and suspended, no new *Leishmania*-positive cases were received during the study period. Nevertheless, this assay indicates detection of *Leishmania* in peripheral blood to an acceptable detection limit, a classically challenging sample type for PCR detection. Furthermore, the detection system, inclusive of the DNA extraction method utilising target capture technology, can fulfill at least the minimal attributes to render the test applicable for the end user as identified in the TPP generated.

For the viability assay, RNA from *Leishmania* positive samples was considered a better marker than DNA which remains intact for a longer time after cell death. RNA is produced by metabolically active cells, and after cell death occurs, becomes rapidly unstable and degrades. In this way, its presence, when compared directly to DNA presence (which remains intact in the cell after cell death), can be utilised as a proxy measure of cell viability. Furthermore, as real-time PCR technology is used, quantitation of parasite load can be performed and monitored readily over a treatment course [79]. Bisulphite technology was incorporated into the assay so as to seamlessly integrate with extraction methods utilised in the detection and differentiation assays developed previously, appropriate for both DNA and RNA extraction [16]. The SL-RNA is highly conserved among all *Leishmania* species so the increase in primer homology after bisulphite conversion will not have a resultant effect on species coverage in the assay (Figure 2c). One mismatch was observed in two *Leishmania* species, *L. amazonensis* and *L. mexicana*, at position 74. The mismatch, however, was able to be designed away from the 3' end of the reverse primer, a position known to be detrimental to PCR priming [80]. These samples were, however, originally extracted with reagents and methods developed for DNA extraction and stored at a temperature inappropriate for RNA (-20˚C). DNA and RNA presence was observed in the stored *Leishmania* nucleic acid samples, albeit in higher proportions than expected, with average DNA presence of 122.8% (±28.1). DNA presence over 100% indicates no RNA presence and in 15 of the 79 samples tested. In a recent study, DNA content from freshly extracted *L. major*-positive sandfly extracts was observed at 3.8% (±2.7%), also targeting the SL-RNA [56]. This difference in results may be explained by the sub-optimal extraction and storage conditions described. Nevertheless, here a potentially reliable and useful tool for the detection of viable *Leishmania* parasites is described, as opposed to residual parasite DNA. Furthermore, the extraction

process has the potential to be integrated onto cartridge-based automated platforms with minimal handling steps, as all reagents can be stored at ambient temperatures or 4˚C.

To perform the role of a TOC a diagnostic test would optimally be performed one month and six months post-treatment for VL or PKDL. Obviously, the limitation of this study described here is the need for examination of freshly extracted *Leishmania* positive clinical samples or concurrently cultured promastigotes for full validation. This work was not possible during the study period due to restrictions occurring as a result of the SARS-CoV-2 pandemic. In terms of the TPP generated, the necessity of the reverse transcriptase enzyme for this assay will fall short of achieving desired operational characteristics, as this regent requires -20˚C storage, a practical challenge in many endemic regions.

Overall, this study presents two real-time PCR assays aimed at addressing recently identified diagnostic priorities for the detection of *Leishmania* parasites by the WHO. The detection of *Leishmania* cells in whole peripheral blood was achieved, using the novel combination of target capture and bisulphite conversion technologies. Next, a TOC assay, detecting RNA as a measure of cell viability was able to present DNA/RNA ratios present in a given sample. Due to the need for naturally infected blood samples, and recently obtained clinical samples, stored correctly, further work is required for clinical validation of these assays.

Bibliography

1. Hodiamont CJ, Kager PA, Bart A, de Vries HJ, van Thiel PP, Leenstra T, et al. Species-directed therapy for leishmaniasis in returning travellers: a comprehensive guide. PLoS Negl Trop Dis. 2014;8 5:e2832.
2. Rodriguez-Cortes A, Ojeda A, Francino O, Lopez-Fuertes L, Timon M, Alberola J. *Leishmania* infection: laboratory diagnosing in the absence of a "gold standard". Am J Trop Med Hyg. 2010;82 2:251-6.
3. Gomes CM, Mazin SC, Raphael E, Cesetti MV, Albergaria G, Bächtold B, et al. Accuracy of mucocutaneous leishmaniasis diagnosis using polymerase chain reaction : systematic literature review and meta-analysis. Mem Inst Oswaldo Cruz. 2015;110 2:157-65.
4. Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: Current status and future applications. J Clin Microbiol. 2007;45 1:21-5.
5. Weirather JL, Jeronimo SMB, Gautam S, Sundar S, Kang M, Kurtz MA, et al. Serial quantitative PCR assay for detection, species discrimination, and quantification of *Leishmania* spp. in human samples. J Clin Microbiol. 2011;49 11:3892-904.
6. Harris E, Kropp G, Belli A, Rodriguez B, Agabian N. Single-step multiplex PCR assay for characterization of New World Leishmania complexes. J Clin Microbiol. 1998;36 7:1989-95.
7. Galluzzi L, Ceccarelli M, Diotallevi A, Menotta M, Magnani M. Real-time PCR applications for diagnosis of leishmaniasis. Parasit Vectors. 2018;11 1:273.
8. Conter CC, Mota CA, dos Santos BA, de Souza Braga L, de Souza Terron M, Navasconi TR, et al. PCR primers designed for new world Leishmania: A systematic review. Exp Parasitol. 2019;207:107773.
9. Moreira OC, Yadon ZE, Cupolillo E. The applicability of real-time PCR in the diagnostic of cutaneous leishmaniasis and parasite quantification for clinical management: Current status and perspectives. Acta Trop. 2018;184:29-37.
10. Leon CM, Munoz M, Hernandez C, Ayala MS, Florez C, Teheran A, et al. Analytical Performance of Four Polymerase Chain Reaction (PCR) and Real Time PCR (qPCR) Assays for the Detection of Six *Leishmania* Species DNA in Colombia. Front Microbiol. 2017;8:1907.
11. Diotallevi A, Buffi G, Ceccarelli M, Neitzke-Abreu HC, Gnutzmann LV, da Costa Lima MS, Jr., et al. Real-time PCR to differentiate among Leishmania (Viannia) subgenus, Leishmania (Leishmania) infantum and Leishmania (Leishmania) amazonensis: Application on Brazilian clinical samples. Acta Trop. 2020;201:105178.
12. Van der Auwera G, Dujardin JC. Species typing in dermal leishmaniasis. Clin Microbiol Rev. 2015;28 2:265-94.

13. Cook G, Donelson J. Mini-exon gene repeats of Trypanosoma (Nannomonas) congolense have internal repeats of 190 base pairs. . Mol Biochem Parasitol. 1987;25 1:113-22.
14. Marfurt J, Niederwieser I, Makia N, Beck H, Felger I. Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP. Diagn Microbiol Infect Dis. 2003;46 2:115-24.
15. Van der Auwera G, Ravel C, Verweij J, Bart A, Schönian G, Felger I. Evaluation of four single-locus markers for *Leishmania* species discrimination by sequencing. J Clin Microbiol. 2014;52 4:1098-104.
16. Gow I, Millar D, Ellis J, Melki J, Stark D. Semi-Quantitative, Duplexed qPCR Assay for the Detection of *Leishmania* spp. Using Bisulphite Conversion Technology. Trop Med Infect Dis. 2019;4 4.
17. Garae C, Kalo K, Pakoa GJ, Baker R, Isaacs P, Millar DS. Validation of the easyscreen flavivirus dengue alphavirus detection kit based on 3base amplification technology and its application to the 2016/17 Vanuatu dengue outbreak. PLoS One. 2020;15 1:e0227550.
18. Siah SP, Merif J, Kaur K, Nair J, Huntington PG, Karagiannis T, et al. Improved detection of gastrointestinal pathogens using generalised sample processing and amplification panels. Pathology. 2014;46 1:53-9.
19. Roberts T, Barratt J, Sandaradura I, Lee R, Harkness J, Marriott D, et al. Molecular epidemiology of imported cases of leishmaniasis in Australia from 2008 to 2014. PLoS One. 2015;10 3:1-11.
20. World Health Organization. Global leishmaniasis surveillance, 2017–2018, and first report on 5 additional indicators. In Secondary Global leishmaniasis surveillance, 2017–2018, and first report on 5 additional indicators. World Health Organization. 2020.
21. Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig H, Presber W, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003;47 1:349-58.
22. Iatta R, Mendoza-Roldan JA, Latrofa MS, Cascio A, Brianti E, Pombi M, et al. Leishmania tarentolae and Leishmania infantum in humans, dogs and cats in the Pelagie archipelago, southern Italy. PLoS Negl Trop Dis. 2021;15 9:e0009817.
23. Thakur L, Kushwaha HR, Negi A, Jain A, Jain M. Leptomonas seymouri Co-infection in Cutaneous Leishmaniasis Cases Caused by Leishmania donovani From Himachal Pradesh, India. Front Cell Infect Microbiol. 2020;10:345.
24. Ahuja K, Vats A, Beg MA, Kariyawasam K, Chaudhury A, Chatterjee M, et al. High resolution melting based method for rapid discriminatory diagnosis of co-infecting Leptomonas seymouri in Leishmania donovani-induced leishmaniasis. Parasitol Int. 2020;75:102047.

25. Esteves S, Costa I, Amorim C, Santarem N, Cordeiro-da-Silva A. Biomarkers in Leishmaniasis: From Basic Research to Clinical Application. 2018.
26. Coughlan S, Taylor AS, Feane E, Sanders M, Schonian G, Cotton JA, et al. Leishmania naiffi and Leishmania guyanensis reference genomes highlight genome structure and gene evolution in the Viannia subgenus. R Soc Open Sci 2018;5 4:172212.
27. Bañuls A, Dujardin J, Guerrini F, Doncker S, Jacquet D, Arevalo J, et al. Is Leishmania (Viannia) peruviana a Distinct Species? A MLEE/RAPD Evolutionary Genetics Answer. The Journal of Eukaryotic Microbiology. 2000;47 3:197–207.
28. Boite MC, Mauricio IL, Miles MA, Cupolillo E. New insights on taxonomy, phylogeny and population genetics of Leishmania (Viannia) parasites based on multilocus sequence analysis. PLoS Negl Trop Dis. 2012;6 11:e1888.
29. Noyes H, Arana B, Chance M, Wingon R. The Leishmania hertigi (Kinetoplastida; Trypanosomatidae) Complex and the Lizard Leishmania: Their Classification and Evidence for a Neotropical Origin of the Leishmania-Endotrypanum Clade. 1997.
30. Kato H, Caceres AG, Gomez EA, Tabbabi A, Mizushima D, Yamamoto DS, et al. Prevalence of Genetically Complex Leishmania Strains With Hybrid and Mito-Nuclear Discordance. Front Cell Infect Microbiol. 2021;11:625001.
31. Banuls A, Guerrini F, Pont F, Barrera C, Espinel I, Guderian R, et al. Evidence for Hybridization by Multilocus Enzyme Electrophoresis and Random Amplified Polymorphic DNA Between Leishmania braziliensis and Leishmania panamensis/guyanensis in Ecuador. The Journal of Eukaryotic Microbiology. 1997;44 5:408–11.
32. Dujardin J, Bañuls A, Llanos-Cuentas A, Alvarez E, DeDoncker S, Jacquet D, et al. Putative Leishmania hybrids in the Eastern Andean valley of Huanuco, Peru. Acta Trop. 1995;59 4:293-307.
33. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. PLoS Negl Trop Dis. 2016;10 3:e0004349-e.
34. Van der Auwera G, Bart A, Chicharro C, Cortes S, Davidsson L, Di Muccio T, et al. Comparison of Leishmania typing results obtained from 16 European clinical laboratories in 2014. Euro Surveill. 2016;21 49.
35. Foulet F, Botterel F, Buffet P, Morizot G, Rivollet D, Deniau M, et al. Detection and identification of Leishmania species from clinical specimens by using a real-time PCR assay and sequencing of the cytochrome B gene. J Clin Microbiol. 2007;45 7:2110-5.

36. Van der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Van Esbroeck M, et al. Heat-shock protein 70 gene sequencing for Leishmania species typing in European tropical infectious disease clinics. Euro Surveill. 2013;18 30:20543.
37. Hassan M, Ghosh A, Ghosh S, Gupta M, Basu D, Mallik K, et al. Enzymatic amplification of mini-exon-derived RNA gene spacers of Leishmania donovani: primers and probes for DNA diagnosis. . Parasitology. 1993;107:509-17.
38. Fernandes O, Murthy VK, U, Degrave W, Campbell D. Mini-exon gene variation in human pathogenic Leishmania species. Mol Biochem Parasitol. 1994;66 2:261-71.
39. Piarroux R, Fontes M, Perasso R, Gambarelli F, Joblet C, Dumon H, et al. Phylogenetic relationships between Old World Leishmania strains revealed by analysis of a repetitive DNA sequence. Mol Biochem Parasitol 1995;73 1-2:249-52.
40. Ramos A, Maslov DA, Fernandes O, Campbell DA, L. S. Detection and Identification of Human Pathogenic Leishmania and Trypanosoma Species by Hybridization of PCR-Amplified Mini-exon Repeats. Exp Parasitol. 1996;82 3:242-50.
41. Kebede A, De Doncker S, Arevaloc J, Le Raya D, Dujardin JC. Size-polymorphism of mini-exon gene-bearing chromosomes among natural populations of Leishmania, subgenus Viannia. Int J Parasitol. 1999;29 4:549-57.
42. Vernal J, Cazzulo J, Nowicki C. Cloning and heterologous expression of a broad specificity aminotransferase of *Leishmania* mexicanapromastigotes. FEMS Microbiol Lett. 2003;229 2:217-22.
43. Paiva B.R., Passos L.N., Falqueto A., Malafronte R.S., H.F. A. Single step polymerase chain reaction (PCR) for the diagnosis of the Leishmania (Viannia) subgenus. Rev Inst Med Trop Sao Paulo. 2004;46 6:335-8.
44. van der Snoek EM, Lammers AM, Kortbeek LM, Roelfsema JH, Bart A, Jaspers CA. Spontaneous cure of American cutaneous leishmaniasis due to *Leishmania naiffi* in two Dutch infantry soldiers. Clin Exp Dermatol. 2009;34 8:e889-91.
45. Osman A. Evaluation of molecular genotyping in clinical and epidemiological study of leishmaniasis in Sudan. . Sci Parasitol 2011;12 3:131-7.
46. World Health Organization. Leishmaniasis [Fact Sheet]. In Secondary Leishmaniasis [Fact Sheet]. World Health Organization. 2021. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis. Accessed 20/5/21.
47. Moulik S, Sengupta S, Chatterjee M. Molecular Tracking of the Leishmania Parasite. Front Cell Infect Microbiol. 2021;11:623437.

48. Mahajan R, Owen SI, Kumar S, Pandey K, Kazmi S, Kumar V, et al. Prevalence and determinants of asymptomatic Leishmania infection in HIV-infected individuals living within visceral leishmaniasis endemic areas of Bihar, India. PLoS Negl Trop Dis. 2022;16 8:e0010718.
49. Sengupta R, Chaudhuri SJ, Moulik S, Ghosh MK, Saha B, Das NK, et al. Active surveillance identified a neglected burden of macular cases of Post Kala-azar Dermal Leishmaniasis in West Bengal. PLoS Negl Trop Dis. 2019;13 3:e0007249.
50. World Health Organization. Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021−2030. In Secondary Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021−2030. World Health Organization. 2020.
51. Sidstedt M, Hedman J, Romsos EL, Waitara L, Wadso L, Steffen CR, et al. Inhibition mechanisms of hemoglobin, immunoglobulin G, and whole blood in digital and real-time PCR. Anal Bioanal Chem. 2018;410 10:2569-83.
52. World Health Organization. Target product profile for a point-of-care diagnostic test for dermal leishmaniases. In Secondary Target product profile for a point-of-care diagnostic test for dermal leishmaniases. World Health Organization. 2022.
53. Sanchez DO, Frasch AC, Carrasco AE, Gonzalez-Cappa SM, de Isola ED, Stoppani AO. Rapid evolution of kinetoplast DNA mini-circle subpopulations in Trypanosoma cruzi. Mol Biochem Parasitol. 1984;11:169-78. .
54. Brewster S, Barker DC. Analysis of minicircle classes in Leishmania (Viannia) species. Trans R Soc Trop Med Hyg 2002;96 1.
55. Gradoni L, Lopez-Velez R, Mokni M. Manual on case management and surveillance of the leishmaniases in the WHO European Union. In Secondary Manual on case management and surveillance of the leishmaniases in the WHO European Union. World Health Organization Regional Office for Europe. 2017.
56. Pareyn M, Hendrickx R, Girma N, Hendrickx S, Van Bockstal L, Van Houtte N, et al. Evaluation of a pan-Leishmania SL RNA qPCR assay for parasite detection in laboratory-reared and field-collected sand flies and reservoir hosts. Parasit Vectors. 2020;13 1:276.
57. Zelazny AM, Fedorko DP, Li L, Neva FA, Fischer SH. Evaluation of 7SL RNA gene sequences for the identification of *Leishmania* spp. Am J Trop Med Hyg. 2005;72 4:415-20.
58. Miller S, Landfear S, Wirth D. Cloning and characterization of a Leishmania gene encoding a RNA spliced leader sequence. Nucleic Acids Res. 1986;14 18:7341–60.

59. Losada-Barragan M, Cavalcanti A, Umana-Perez A, Porrozzi R, Cuervo-Escobar S, Vallejo AF, et al. Detection and quantification of Leishmania infantum in naturally and experimentally infected animal samples. Vet Parasitol. 2016;226:57-64.
60. Mary C, Faraut F, Lascombe L, Dumon H. Quantification of Leishmania infantum DNA by a real-time PCR assay with high sensitivity. J Clin Microbiol. 2004;42 11:5249-55.
61. Francino O, Altet L, Sanchez-Robert E, Rodriguez A, Solano-Gallego L, Alberola J, et al. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Vet Parasitol. 2006;137 3-4:214-21.
62. Silver N, Best S, Jiang J, Thein SL. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol. 2006;7:33.
63. Eberhardt E, Van den Kerkhof M, Bulte D, Mabille D, Van Bockstal L, Monnerat S, et al. Evaluation of a Pan-Leishmania Spliced-Leader RNA Detection Method in Human Blood and Experimentally Infected Syrian Golden Hamsters. J Mol Diagn. 2018;20 2:253-63.
64. Bossolasco S, Gaiera G, Olchini D. Real-time PCR assay for clinical management of human immunodeficiency virus-infected patients with visceral leishmaniasis. Journal of clinical. 2003;41 11:5080-4.
65. Schulz A, Mellenthin K, Schonian G, Fleischer B, Drosten C. Detection, Differentiation, and Quantitation of Pathogenic *Leishmania* Organisms by a Fluorescence Resonance Energy Transfer-Based Real-Time PCR Assay. J Clin Microbiol. 2003;41 4:1529-35.
66. Pita-Pereira D, Lins R, Oliveira MC, Lima RB, Pereira BAS, Moreira OC, et al. SYBR Green-based Real-Time PCR targeting kinetoplast DNA can be used to discriminate between the main etiologic agents of Brazilian cutaneous and visceral leishmaniases. 2012.
67. Molina I, Fisa R, Riera C, Falco V, Elizalde A, Salvador F, et al. Ultrasensitive real-time PCR for the clinical management of visceral leishmaniasis in HIV-Infected patients. Am J Trop Med Hyg. 2013;89 1:105-10.
68. Toz SO, Culha G, Zeyrek FY, Ertabaklar H, Alkan MZ, Vardarli AT, et al. A real-time ITS1-PCR based method in the diagnosis and species identification of Leishmania parasite from human and dog clinical samples in Turkey. PLoS Negl Trop Dis. 2013;7 5:e2205.
69. da Costa Lima MS, Zorzenon DCR, Dorval MEC, Pontes ERJC, Oshiro ET, Cunha R, et al. Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood. Asian Pacific Journal of Tropical Disease. 2013;3 1:10-5.
70. Vallur AC, Duthie MS, Reinhart C, Tutterrow Y, Hamano S, Bhaskar KR, et al. Biomarkers for intracellular pathogens: establishing tools as vaccine

and therapeutic endpoints for visceral leishmaniasis. Clin Microbiol Infect. 2014;20 6:O374-83.
71. Pessoa ESR, Mendonca Trajano-Silva LA, Lopes da Silva MA, da Cunha Goncalves-de-Albuquerque S, de Goes TC, Silva de Morais RC, et al. Evaluation of urine for Leishmania infantum DNA detection by real-time quantitative PCR. J Microbiol Methods. 2016;131:34-41.
72. de Almeida ME, Koru O, Steurer F, Herwaldt BL, da Silva AJ. Detection and Differentiation of Leishmania spp. in Clinical Specimens by Use of a SYBR Green-Based Real-Time PCR Assay. J Clin Microbiol. 2017;55 1:281-90.
73. Hossain F, Ghosh P, Khan MAA, Duthie MS, Vallur AC, Picone A, et al. Real-time PCR in detection and quantitation of *Leishmania donovani* for the diagnosis of Visceral leishmaniasis patients and the monitoring of their response to treatment. PLoS One. 2017;12 9:e0185606.
74. Trajano-Silva LAM, Pessoa ESR, Goncalves-de-Albuquerque SDC, Morais RCS, Costa-Oliveira CND, Goes TC, et al. Standardization and evaluation of a duplex real-time quantitative PCR for the detection of *Leishmania infantum* DNA: a sample quality control approach. Rev Soc Bras Med Trop. 2017;50 3:350-7.
75. Asfaram S, Fakhar M, Mohebali M, Ziaei Hezarjaribi H, Mardani A, Ghezelbash B, et al. A Convenient and Sensitive kDNA-PCR for Screening of Leishmania infantum Latent Infection Among Blood Donors in a Highly Endemic Focus, Northwestern Iran. Acta Parasitol. 2022;67 2:842-50.
76. Mary C, Faraut F, Lascombe L, Dumon H. Quantification of *Leishmania infantum* DNA by a Real-Time PCR Assay with High Sensitivity. J Clin Microbiol. 2004;42 11:5249-55.
77. Nicolas LM, Genevieve; Prina, Eric Rapid differentiation of Old World Leishmania species by LightCycler polymerase chain reaction and melting curve analysis. 2002.
78. Cai D, Behrmann O, Hufert F, Dame G, Urban G. Direct DNA and RNA detection from large volumes of whole human blood. Sci Rep. 2018;8 1:3410.
79. Verrest L, Kip AE, Musa AM, Schoone GJ, Schallig H, Mbui J, et al. Blood Parasite Load as an Early Marker to Predict Treatment Response in Visceral Leishmaniasis in Eastern Africa. Clin Infect Dis. 2021;73 5:775-82.
80. Wu JH, Hong PY, Liu WT. Quantitative effects of position and type of single mismatch on single base primer extension. J Microbiol Methods. 2009;77 3:267-75.

Chapter 5

Molecular detection of neglected tropical diseases: the case for automated near point-of-care diagnosis of leishmaniasis

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Publication:

Gow, I., Smith, N., Stark, D. & Ellis, J., 2023., *Molecular Detection of Neglected Tropical Diseases: The Case for Automated Near Point-of-Care Diagnosis of Leishmaniasis.* **Am. J. Trop. Med. Hyg**. 108(1), 2-6.

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Date: March 2023

Am. J. Trop. Med. Hyg., 108(1), 2023, pp. 2–6
doi:10.4269/ajtmh.22-0373

Perspective Piece

Molecular Detection of Neglected Tropical Diseases: The Case for Automated Near–Point-of-Care Diagnosis of Leishmaniasis

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Abstract. Neglected tropical diseases affect those in poorer nations disproportionately across the globe. One example of these, leishmaniasis, is a debilitating and potentially fatal parasitic infection. Molecular detection of this disease can provide accurate and fast diagnosis, and with near point-of-care technologies, detection can be provided in many health-care settings. Traditionally, the perceived limitations to such detection methods have hindered their provision to resource-limited nations, but new technologies and techniques are helping to overcome these perceptions. The current pandemic offers an opportunity to maintain and develop further advances, ensuring molecular diagnostics are accessible to all.

The 20 neglected tropical diseases (NTDs) place huge health, social, and economic burdens on 1 billion people globally. The availability of effective, standardized, and affordable diagnostics may help ameliorate morbidity, and lower intervention program costs and achieve WHO elimination targets.[1] Of the 19 infectious NTDs, leishmaniasis—both the visceral and cutaneous forms—are associated with an estimated 50,000 to 90,000 new cases of visceral leishmaniasis (VL) and 600,000 to 1 million new cases of cutaneous leishmaniasis each year.[2] Early diagnosis was attributed to the success of the 2005 intervention program, targeted to eliminate VL in India, Nepal, and Bangladesh. This program reduced VL cases in these regions from 2,220 per 10,000 inhabitants per year to 254 per 10,000 inhabitants per year between 2003 and 2017.[3] The development and approval of nucleic acid-based tests to overcome the limitations of the current antigen-based testing has been encouraged to achieve elimination targets in these regions.

Concurrently, major advances in the detection of leishmaniasis and other infectious NTDs have occurred during the past few decades. However, these sensitive and specific molecular methods have often been deemed inappropriate for the geographical regions that need them most.[4–7] Real-time polymerase chain reaction (PCR), and the nucleic acid extraction preceding it, is being performed increasingly by automated platforms in the laboratory in many parts of the globe. Such platforms aim to free researchers and technicians from manual processes and increase accuracy, reproducibility, and throughput of results. Here, we discuss—with a focus on leishmaniasis—that the oft-disregarded molecular detection assays and the automated platforms that can perform them have become more relevant in resource-limited settings. The automation of manual molecular techniques has increased in reach and performance in the form of near–point-of-care (NPOC) testing. These are device-based or low–equipment-based technologies enabling onsite, decentralized testing. Now, more than ever before, in the setting of the SARS-CoV-2 global pandemic, is an opportune time for automation to be applied to the detection of these NTDs using NPOC testing.[8]

In recent years, molecular diagnostic technologies have improved in terms of accuracy and meeting user needs on a global scale. This includes the automation of nucleic acid extraction or the PCR master mix setup to sample-to-result function (including onboard nucleic acid extraction, amplification, and analysis). Automation can increase reproducibility, and reduce the risk of laboratory contamination and human error, such as sample mix-ups and laboratory-acquired infection. It is acknowledged that such processing could improve throughput, speed, and sensitivity of *Leishmania* detection. For the detection of a related species, such as *Trypanosoma cruzi*, automated methods achieved the same performance as an in-house, manual method.[9] Furthermore, that study concluded that the broader use of real-time PCR methods could help to standardize methods across different laboratories.[9,10] Table 1 lists common automated nucleic acid extraction liquid-handling systems, highlighting the range of throughput, speed, and processing capabilities and the area (or "footprint") the instrument requires in a laboratory.[11]

The implementation of diagnostic tests differs at varying levels of national health-care systems, depending on their affordability, accessibility, and accuracy (Figure 1).[12–14] This tiered system relates to the provision of services (tests, staffing, communication infrastructure, equipment, turnaround times, and surveillance networks) at each level. Tier 0 is characterized by community health centers or outreach programs serving outpatients performing point-of-care (POC) tests and refers further tests to tiers 2 or 3. Tier 1 includes primary care/health center laboratories serving mostly outpatients and performing POC/single-use tests, and refers tests to tiers 2 or 3. Tier 2 laboratories are within district hospitals, serving inpatients, and receives referrals from tiers 0 and 1, performing a limited number of routine tests. Tier 3 laboratories are within regional hospitals, serving inpatients; receives referrals from tiers 0, 1, and 2; and performs multidisciplinary routine testing. Tier 4 laboratories in national or teaching hospitals serve inpatients and receive referrals from tiers 0, 1, 2, and 3. They perform routine tests and highly specialized tests, and provide education/training for all tiers. Although staffing may be relatively fixed within each tier, the diagnostic technologies and their increasing accessibility are being adapted to suit the lower, less-resourced tiers. It is in

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TABLE 1
Automated liquid handling platforms

Instrument	Manufacturer	No. of samples	Runtime, min	Sample input volume, µL	Elution volume, µL	Dimensions, cm; width × height × depth
m2000sp	Abbott	24–96	90–250	400–4000	15–190	145 × 217.5 × 79.4
EasyMag*	Biomerieux	1–24	40–60	10–1,000	25–110	100 × 53 × 65
GS-mini*	Genetic Signatures Ltd.	1–12	40–75	100–2,000	50–400	56 × 59 × 51
chemagic Prime 8 Instrument	PerkinElmer	1–192	55–75	10–10,000	Various	86.6 × 194 × 228.5
Maxwell RSC 48*	Promega	1–48	30–70	100–300	30–100	53.3 × 35.6 × 53.3
EZ1 Advanced XL*	Qiagen	1–14	20–50	200–400	50–100	51 × 57 × 51
QIAsymphonySP	Qiagen	1–96	90–290	> 200	30–500	128 × 103 × 73
Magnapure 96	Roche	1–96	50–170	50–4,000	50–200	136 × 100 × 81.5

*Suitable for near-point-of-care testing.

tier 2, tier 3, and tiers 0 and 1 (when serviced by mobile laboratories) where the implementation of automated molecular detection could have the most impact in low- and middle-income countries (LMICs).[15,16] The challenge is to bring these technologies down the tiers; however, this requires changing the perspectives and assumptions of key stakeholders.[17] This implementation is important as part of routine testing schedules and in outbreak scenarios, when the ability to upscale is imperative—when the demand on the health-care system increases.

In a 2002 report,[18] scientific experts identified "Modified molecular technologies for affordable, simple diagnosis of infectious diseases" as the major biotechnology that could improve health in developing countries. The authors found that many assumptions made regarding the lack of usefulness and cost of molecular diagnostics in controlling infectious diseases in poorer nations were not supported by evidence. Since then, the continued view that infectious diseases diagnostics are not accessible to these developing countries has led to the ASSURED/REASSURED criteria. These criteria emphasize the ideal characteristics of a diagnostic test across all health-care levels, encompassing affordability, accessibility, and accuracy (Figure 2).[19,20] POC tests, generally accepted as those tests performed and analyzed at the place of patient care, have broad and fluid definitions, with many derivatives still requiring a laboratory infrastructure.[21,22] Although promising and fulfilling many of the ASSURED/REASSURED criteria, POC tests can also be limited in sensitivity and specificity in *Leishmania* diagnostics, and molecular-based POC tests can be prohibitively expensive.[23,24] It is important to view the move from large-scale centralized laboratory testing to "true" POC tests as a continuum where varying testing modes overlap in technology and usefulness in situations in which they are used.[25] NPOC tests (Table 2) can be placed along this continuum, decentralizing testing by eliminating or reducing the need for sample transport and reducing turnaround time. Furthermore, NPOC tests have the flexibility of interchangeable assays and can retain the greater throughput that is lost in true POC tests. This becomes increasingly critical during times of outbreak, which occur frequently for both forms of leishmaniasis.[26–28] Tiers 0 and 1 settings with no or minimal infrastructure, including locations with no or intermittent electricity or no assigned laboratory space, may continue to be a challenge for the molecular diagnosis of *Leishmania* and other NTDs.[29] However, well-designed NPOC tests—including their automation—could find a place in most regions of the world in tier 2 and tier 3 laboratories (and mobile laboratories).

The major challenge in providing molecular testing and automation to resource-limited settings is that they have traditionally been considered an expensive diagnostic method. However, automation reduces direct and indirect staffing costs, and the miniaturization of PCR platforms and reagent

FIGURE 1. The different levels of healthcare and staffing requirements associated with these, adapted from ref.[16]

FIGURE 2. The ASSURED/REASSURED criteria for the ideal characteristics of a diagnostic test, adapted from ref.[20,24]

production methods has been accompanied by a further reduction in cost for these technologies.[10,30] It is predicted in LMICs that, as staffing costs increase over time, there will be a greater need to drive down the cost per test through automation.[17] Furthermore, the broad and interchangeable diagnostic panels and their capability for assay multiplexing (the detection of multiple organisms simultaneously) can offset the initial equipment costs, increasing impact and cost-effectiveness in the appropriate contexts.

Automation decreases the level of interaction between the user and the test; therefore, the risks of human error, laboratory-based accidents, and cross-contamination of samples are minimized. This is particularly apparent in settings where staff lack sufficient specialist training and educational background in manual molecular techniques.[31] In molecular diagnostics, real-time PCR with lyophilized reagents has also greatly reduced the risk of contamination that may be introduced to a laboratory.[32] For instance, sample-to-result systems eliminate manual preanalytical sample processing and postamplification analysis steps through premeasured, cartridge-based, and lyophilized reagents.[31] Large, complex, and high-throughput automated equipment share drawbacks in terms of instrument errors and breakdowns that require complex troubleshooting performed by specialist technicians.[33] The simplified nature of NPOC devices and platforms (e.g., cartridge-contained reagents) are less prone to such errors.

Endemic areas serviced by laboratories that currently lack the required physical infrastructure, including access to refrigerated transport or storage, sterile workspaces, or permanent laboratories, are considered unsuitable to perform molecular testing.[34] Freeze-dried PCR reagents were developed in the late 1990s and were found to be stable for up to 12 months at ambient temperatures, allowing reagents to be cold-chain independent.[35] DNA-free areas are less critical when reactions can be fully enclosed in an instrument, and DNA-free water can be provided with testing kits.[36] The advent of small, automated systems now allows for flexibility in laboratory location and may be incorporated into mobile laboratories or even a mobile suitcase laboratory (developed for pathogen detection such as *Leishmania* in the field).[15,37,38] Automation in other NTD detection is being seen in diverse technologies such as microscopy, loop-mediated isothermal amplification, and DNA extraction.[39–41]

The preanalytical phase of diagnostic testing can present challenges to retain sample quality when decentralized. Staff training and expertise, sample collection methods, containers, and handling all affect specimen quality. Although cutaneous leishmaniasis specimens are collected increasingly by relatively simple methods such as tape strips, skin scrapings, or exudate, VL specimen collection often requires invasive sampling methods, including spleen, lymph, and bone marrow biopsies.[42–45] These sampling methods may have to be performed using ultrasound guidance, and in the case of splenic aspirates, face the risk of patient death if performed improperly. Recently, the WHO has prioritized less-invasive, highly specific tests to measure parasite levels for VL to reach elimination targets for the disease.[1] Less-invasive sampling methods for PCR detection of *Leishmania* in visceral cases, such as peripheral blood collection, although not yet recommended, are being investigated with increasingly improving detection limits.[46,47] When applied to real time-PCR, the potential for quantification of parasitic load in the blood is possible. Monitoring parasitic load during and after treatment can give an indication of relapse, as validated in blood samples.[48] However, collection of blood specimens is not yet designed for POC, in terms of sample collection and prevention of diagnostic errors.[49] Clinical sample referral and transport needs to be avoided to keep the testing near to the patient. Thus, the challenge remains that the simplicity of specimen collection must be in line with the resources and limitations of the laboratory tier in which they are collected. For VL, sensitive detection of *Leishmania* DNA in the urine was possible, and its depletion correlated with treatment.[45] Adapting novel sample types for molecular detection of VL and monitoring of parasite load to NPOC testing could present a solution for specific WHO elimination priorities.

The global pandemic experience exemplifies that NPOC testing can and has been implemented across most healthcare levels. This challenges the assumptions of the lack of appropriateness of molecular technologies in LMICs and resource-limited settings. Many LMICs have had increased opportunities to develop infrastructure, logistic, administrative, and workforce systems skilled in testing procedures suitable for mass diagnosis and screening programs. Concurrently, manufacturers of the assays and their associated diagnostic platforms have scaled up capacity for product production and

TABLE 2
Characteristics of the "true" POC test vs. the near-POC test

Characteristic	True POC test	Near-POC test
Turnaround time	Minutes	Hours
Throughput	Single test, predetermined target	12 Samples per run, flexible target selection
Infrastructure	No need for electricity or air conditioning	Constant electricity, computer interface, and some degree of temperature control
Staff expertise	Nonlaboratory training required	Basic laboratory training required
Cost	More than conventional	Can be reduced to conventional depending on the type of device used, the type of test run, and where the device is placed
Quality	Decentralized quality control, equipment maintenance, supply chain and waste management	Centralized quality control, equipment maintenance, supply chain and waste management
Example test	CL Detect Rapid Test™ (Inbios International Inc., Seattle, WA)	GeneXpert (Cepheid, Sunnyvale, CA), GS-mini (Genetic Signatures Ltd., New South Wales, Australia)

POC: point-of-care

provision of expertise to these settings. If the momentum we are seeing in the diagnostic development and delivery capabilities for SARS-CoV-2 is not sustained and applied further to NTDs such as leishmaniasis in a postpandemic environment, it could be a missed opportunity to achieve important global public health gains in the fight against NTDs.

Received June 2, 2022. Accepted for publication August 13, 2022.

Published online November 30, 2022.

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REFERENCES

1. World Health Organization, 2020. *Ending the Neglect to Attain the Sustainable Development Goals: A Road Map for Neglected Tropical Diseases 2021–2030.* Geneva, Switzerland: WHO.
2. World Health Organization, 2021. *Leishmaniasis.* Geneva, Switzerland: WHO.
3. Selvapandiyan A, Croft SL, Rijal S, Nakhasi HL, Ganguly NK, 2019. Innovations for the elimination and control of visceral leishmaniasis. *PLoS Negl Trop Dis 13:* 1–5.
4. Castellanos-Gonzalez A, White AC, Melby P, Travi B, 2018. Molecular diagnosis of protozoan parasites by recombinase polymerase amplification. *Acta Trop 182:* 4–11.
5. Gunaratna G et al., 2018. Evaluation of rapid extraction and isothermal amplification techniques for the detection of *Leishmania donovani* DNA from skin lesions of suspected cases at the point of need in Sri Lanka. *Parasit Vectors 11:* 1–7.
6. Burza S, Croft SL, Boelaert M, 2018. Leishmaniasis. *Lancet 392:* 951–970.
7. Sundar S, Singh OP, 2018. Molecular diagnosis of visceral leishmaniasis. *Mol Diagn Ther 22:* 443–457.
8. Al-Hall H, Mirza F, Al Hashemi A, Ahmad MN, Iqbal M, Tang P, Hasan MR, 2021. Evaluation of automated molecular tests for the detection of SARS-CoV-2 in pooled nasopharyngeal and saliva specimens. *J Clin Lab Anal 35:* 1–6.
9. Abras A et al., 2018. Introducing automation to the molecular diagnosis of *Trypanosoma cruzi* infection: a comparative study of sample treatments, DNA extraction methods and real-time PCR assays. *PLoS One 13:* 1–14.
10. Galluzzi L, Ceccarelli M, Diotallevi A, Menotta M, Magnani M, 2018. Real-time PCR applications for diagnosis of leishmaniasis. *Parasit Vectors 11:* 1–13.
11. Felder RA, Jackson KD, Walter AM, 2014. Process evaluation of an open architecture real-time molecular laboratory platform. *J Lab Autom 19:* 468–473.
12. World Health Organization, 2019. *Second WHO Model List of Essential In Vitro Diagnostics.* WHO Technical Report Series, No. 1017. Geneva, Switzerland: WHO.
13. World Health Organization, 2018. *World Health Organization Model List of Essential In Vitro Diagnostics.* WHO Technical Report Series, No. 1017. Geneva, Switzerland: WHO.
14. World Health Organization, 2021. *The Selection and Use of Essential In Vitro Diagnostics.* WHO Technical Report Series, No. 1031. Geneva, Switzerland: WHO.
15. Wolfel R et al., 2015. Mobile diagnostics in outbreak response, not only for Ebola: a blueprint for a modular and robust field laboratory. *Euro Surveill 20:* 1–9.
16. World Health Organization, 2019. *Consolidated Guidelines on HIV Testing Services.* Geneva, Switzerland: WHO.
17. Fleming KA et al., 2017. An essential pathology package for low- and middle-income countries. *Am J Clin Pathol 147:* 15–32.
18. Daar A, Thorsteinsdóttir H, Martin D, Smith A, Nast S, Singer P, 2002. Top ten biotechnologies for improving health in developing countries. *Nat Genet 32:* 229–232.
19. Mabey D, Peeling RW, Ustianowski A, Perkins MD, 2004. Diagnostics for the developing world. *Nat Rev Microbiol 2:* 231–240.
20. Land KJ, Boeras DI, Chen XS, Ramsay AR, Peeling RW, 2019. REASSURED diagnostics to inform disease control strategies, strengthen health systems and improve patient outcomes. *Nat Microbiol 4:* 46–54.
21. Hänscheid T, Rebelo M, Grobusch MP, 2014. Point-of-care tests: where is the point? *Lancet Infect Dis 14:* 922.
22. Drain PK, Hyle EP, Noubary F, Freedberg KA, Wilson D, Bishai WR, Rodriguez W, Bassett IV, 2014. Diagnostic point-of-care tests in resource-limited settings. *Lancet Infect Dis 14:* 239–249.
23. Schallig H, Hu RVP, Kent AD, van Loenen M, Menting S, Picado A, Oosterling Z, Cruz I, 2019. Evaluation of point of care tests for the diagnosis of cutaneous leishmaniasis in Suriname. *BMC Infect Dis 19:* 1–6.
24. Shah K et al., 2017. Field-deployable, quantitative, rapid identification of active Ebola virus infection in unprocessed blood. *Chem Sci (Camb) 8:* 7780–7797.
25. Suea-Ngam A, Bezinge L, Mateescu B, Howes PD, deMello AJ, Richards DA, 2020. Enzyme-assisted nucleic acid detection for infectious disease diagnostics: moving toward the point-of-care. *ACS Sens 5:* 2701–2723.
26. Kumar A, Saurabh S, Jamil S, Kumar V, 2020. Intensely clustered outbreak of visceral leishmaniasis (kala-azar) in a setting of seasonal migration in a village of Bihar, India. *BMC Infect Dis 20:* 1–13.
27. Nassar AA, Abdelrazzaq MH, Almahaqri AH, Al-Amad MA, Al Serouri AA, Khader YS, 2021. Cutaneous leishmaniasis outbreak investigation in Hajjah Governorate, Yemen, in 2018: case-control study. *JMIR Public Health Surveill 7:* 1–9.
28. Horrillo L et al., 2019. Clinical aspects of visceral leishmaniasis caused by *L. infantum* in adults: ten years of experience of the largest outbreak in Europe: what have we learned? *Parasit Vectors 12:* 1–11.
29. Bengtson M, Bharadwaj M, Bosch AT, Nyakundi H, Matoke-Muhia D, Dekker C, Diehl JC, 2020. Matching development of point-of-care diagnostic tests to the local context: a case study of visceral leishmaniasis in Kenya and Uganda. *Glob Health Sci Pract 8:* 549–565.
30. Archetti C, Montanelli A, Finazzi D, Caimi L, Garrafa E, 2017. Clinical laboratory automation: a case study. *J Public Health Res 6:* 31–36.
31. Beal SG, Assarzadegan N, Rand KH, 2016. Sample-to-result molecular infectious disease assays: clinical implications, limitations and potential. *Expert Rev Mol Diagn 16:* 323–341.
32. Kulkarni RD, Mishra MN, Mohanraj J, Chandrasekhar A, Ajantha GS, Kulkani S, Bhat S, 2018. Development of a dry-reagent mix-based polymerase chain reaction as a novel tool for the identification of *Acinetobacter* species and its comparison with conventional polymerase chain reaction. *J Lab Physicians 10:* 68–72.
33. Opota O, Brouillet R, Greub G, Jaton K, 2020. Comparison of SARS-CoV-2 RT-PCR on a high-throughput molecular diagnostic platform and the Cobas SARS-CoV-2 test for the diagnostic of COVID-19 on various clinical samples. *Pathog Dis 78:* 1–6.
34. Peeling RW, McNerney R, 2014. Emerging technologies in point-of-care molecular diagnostics for resource-limited settings. *Expert Rev Mol Diagn 14:* 525–534.
35. Klatser P, Kuijper S, van Ingen C, Kolk A, 1998. Stabilized, freeze-dried PCR mix for detection of mycobacteria. *J Clin Microbiol 36:* 1798–1800.
36. Abou Tayoun A, Burchard P, Malik I, Scherer A, Tsongalis G, 2014. Democratizing molecular diagnostics for the developing world. *Am J Clin Pathol 141:* 17–24.
37. Mondal D, Ghosh P, Khan MA, Hossain F, Bohlken-Fascher S, Matlashewski G, Kroeger A, Olliaro P, Abd El Wahed A, 2016. Mobile suitcase laboratory for rapid detection of *Leishmania donovani* using recombinase polymerase amplification assay. *Parasit Vectors 9:* 1–8.
38. Ghosh P et al., 2021. A multi-country, single-blinded, phase 2 study to evaluate a point-of-need system for rapid detection of leishmaniasis and its implementation in endemic settings. *Microorganisms 9:* 1–14.
39. Hin S et al., 2021. Fully automated point-of-care differential diagnosis of acute febrile illness. *PLoS Negl Trop Dis 15:* 1–24.

40. Armstrong M, Harris AR, D'Ambrosio MV, Coulibaly JT, Essien-Baidoo S, Ephraim RKD, Andrews JR, Bogoch II, Fletcher DA, 2022. Point-of-care sample preparation and automated quantitative detection of *Schistosoma haematobium* using mobile phone microscopy. *Am J Trop Med Hyg 106:* 1442–1449.
41. Longoni SS, Pomari E, Antonelli A, Formenti F, Silva R, Tais S, Scarso S, Rossolini GM, Angheben A, Perandin F, 2020. Performance evaluation of a commercial real-time PCR Assay and of an in-house real-time PCR for *Trypanosoma cruzi* DNA detection in a tropical medicine reference center, northern Italy. *Microorganisms 8:* 1–12.
42. Bekdi N, Mansouri R, Bettaieb J, Yaacoub A, Souguir Omrani H, Saadi Ben Aoun Y, Saadni F, Guizani I, Guerbouj S, 2017. Molecular characterization of *Leishmania* parasites in Giemsa-stained slides from cases of human cutaneous and visceral leishmaniasis, eastern Algeria. *Vector Borne Zoonotic Dis 17:* 416–424.
43. Nateghi Rostami M, Dazzi F, Farahmand M, Aghaei M, Parvizi P, 2020. Performance of a universal PCR assay to identify different *Leishmania* species causative of Old World cutaneous leishmaniasis. *Parasit Vectors 13:* 1–12.
44. Sudarshan M, Singh T, Chakravarty J, Sundar S, 2015. A correlative study of splenic parasite score and peripheral blood parasite load estimation by quantitative PCR in visceral leishmaniasis. *J Clin Microbiol 53:* 3905–3907.
45. Taslimi Y, Sadeghipour P, Habibzadeh S, Mashayekhi V, Mortazavi H, Muller I, Lane ME, Kropf P, Rafati S, 2017. A novel non-invasive diagnostic sampling technique for cutaneous leishmaniasis. *PLoS Negl Trop Dis 11:* 1–12.
46. Asfaram S, Fakhar M, Mohebali M, Ziaei Hezarjaribi H, Mardani A, Ghezelbash B, Akhoundi B, Zarei Z, Moazeni M, 2022. A convenient and sensitive kDNA-PCR for screening of *Leishmania infantum* latent infection among blood donors in a highly endemic focus, northwestern Iran. *Acta Parasitol 67:* 842–850.
47. Aronson N, Herwaldt BL, Libman M, Pearson R, Lopez-Velez R, Weina P, Carvalho EM, Ephros M, Jeronimo S, Magill A, 2016. Diagnosis and treatment of leishmaniasis: clinical practice guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Clin Infect Dis 63:* e202–e264.
48. Verrest L et al., 2021. Blood parasite load as an early marker to predict treatment response in visceral leishmaniasis in eastern Africa. *Clin Infect Dis 73:* 775–782.
49. Quig K, Wheatley EG, O'Hara M, 2019. Perspectives on blood-based point-of-care diagnostics. *Open Access Emerg Med 11:* 291–296.