Development of onsite and instrument-free recombinase polymerase amplification for smart molecular diagnosis at species level

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Doctoral Dissertation

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Abstract

Introduction: Nucleic acid identification tests based on conventional polymerase chain reaction (PCR) are often instrumental in choosing the correct treatment for the infection due to the rapidity, sensitivity and specificity of these tests and can be used for the detection of asymptomatic infections[1], early stage diagnosis and disease relapse[2]. Very interestingly, recombinase polymerase amplification (RPA)-based molecular tools have attracted great interest since their initial publication in 2006[3] and are continually emerging as an elegant method of choice for performing amplification without the need for complex instrumentation. Additionally, sampling methods such as liquid biopsy are a burden to both patients and physicians, and the DNA extraction and purification steps involved in sampling in DNA-based methods increase the time to diagnosis. Direct sampling via an FTA card reduces the risk of contamination and facilitates the transport and long-term storage of the sample at room temperature. In this work, a way to discriminate true-positive results from false-positive and/or negative results generated during the RPA reaction is explored and an FTA card is used for direct sampling of RPA reactions to eliminate the concerns involved with sample contamination as well as the sample preparation steps and a method for species level analysis of RPA products obtained for leishmaniasis disease is also developed.

Methodology: First, we devised an RPA protocol using seven *Leishmania* species, namely, *Leishmania tarentolae* (noninfective to humans), *Leishmania infantum*, *Leishmania mexicana*, *Leishmania tropica* and *Leishmania major*, belonging to the subgenus *Leishmania*, and *Leishmania lainsoni* and *Leishmania braziliensis*, belonging to the subgenus *Viannia*, to detect leishmaniasis infection. Next, we demonstrated a near-to-patient diagnostic tool utilizing an integrated approach of RPA, using Whatman FTA card as a direct sampling tool and body heat as the source of incubation temperature targeting a 360-bp gene segment of the 18S rRNA gene, and one-inch gel electrophoretic system. Next, Micro-Temperature Gradient Gel Electrophoresis (uTGGE)-type device is utilized, which has a power to distinguish, even a single nucleotide difference between two DNA molecules based on their thermal stabilities. We analyzed 8 *Leishmania* species and constructed a phylogenic tree for subtype differentiation to differentiate the forms of leishmaniasis which are specifically caused by various subtypes of *Leishmania* parasite.

Results: The schematic of the near-to-patient diagnosis is highlighted in Fig.1. First, the sample can be obtained from the patient by aspirating/scraping the margins of active lesions, and the material is then spotted onto the FTA card and dried. Next, a 2.0-mm-diameter disc is punched from the FTA card and immersed in an Eppendorf tube containing liquid RPA reagents supplied with primers targeted to amplify the target gene fragment. This tube is then held in closed fist for 10 minutes to provide the incubation temperature for RPA using body heat. EvaGreen dye is then added for direct detection of amplification, and fluorescence is detected using a simple blue LED-induced fluorescent detection component installed in the palm-sized electrophoretic device to distinguish positive and negative results. Although the process was simple, the weak true-positive signals amplified in the presence of a low template load (10 parasite copies, see Fig. 1a) could not be distinguished from a negative reaction (no parasite copies). Therefore, the amplified products from RPA are subjected to rapid and portable gel-based detection using one-inch gel electrophoresis. Fig. 1b shows the gel electrophoretic analysis of the RPA reaction using the palm-sized electrophoretic device; RPA was performed using FTA cards with 10^4 parasite copies, 10 parasite copies and no parasite copies. The expected 360-bp band was clearly obtained with 10^4 copies. However, byproducts of smaller fragment sizes were also obtained in reactions containing 10 or no copies of template parasites, which could be clearly distinguished from weak true-positive signals using our handheld electrophoretic

device. Next, 10 min- Micro-Temperature Gradient Gel Electrophoresis (uTGGE)-type device is utilized, which has a power to distinguish, even a single nucleotide difference between two DNA molecules based on their thermal stabilities and uses the same type of 1-inch gel electrophoretic system. The melting profiles are obtained and species identification dots are assigned. Pattern similarity scores are then obtained using computer-aided normalization and used to plot a dendrogram. The results can then be submitted to a public database for treatment assessment programmes in epidemic conditions.

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Keywords: Point-of-care diagnostics, leishmaniasis, recombinase polymerase amplification, electrophoresis, species detection

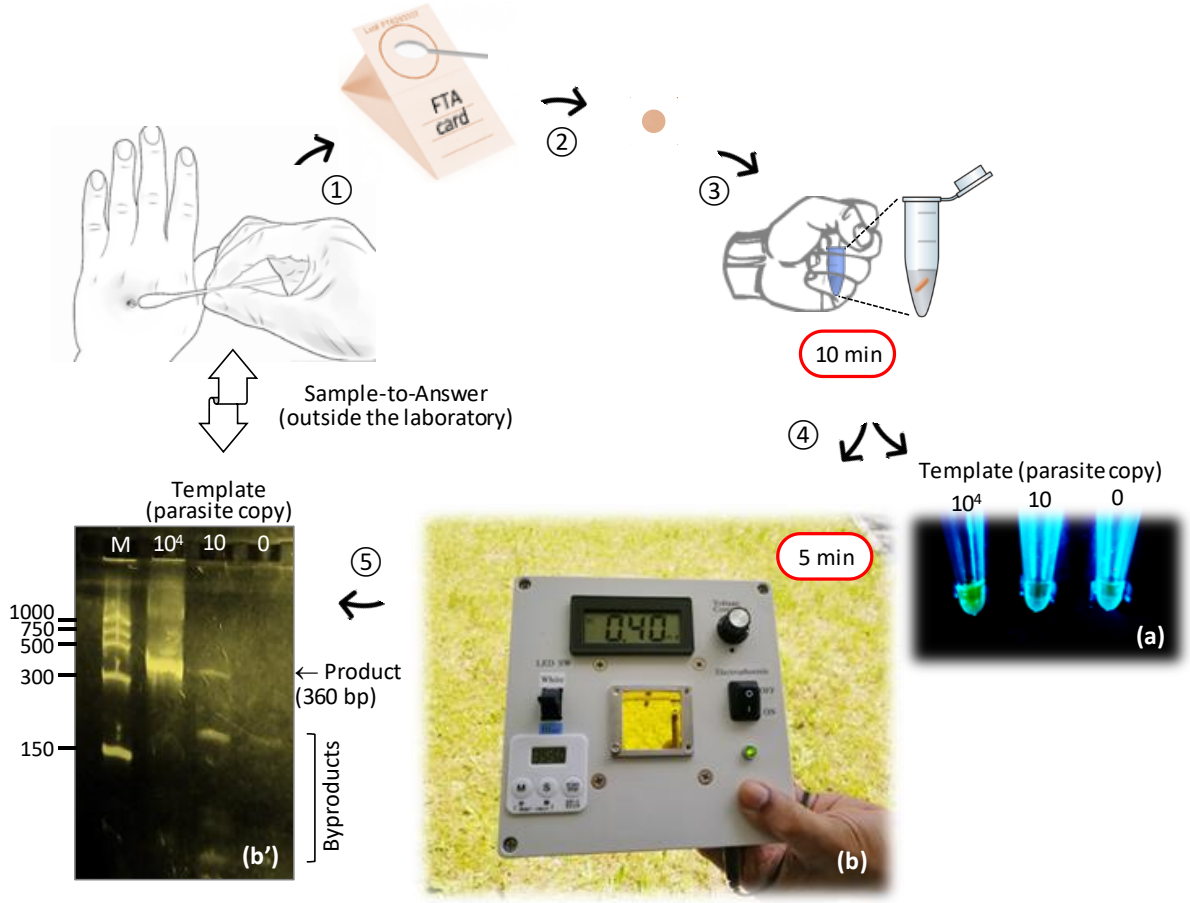

Figure 1: Schematic of near-to-patient nucleic acid-based molecular diagnostic tests.

Achievements

Journals:

1. Himankshi Rathore, Radhika Biyani, Hirotomo Kato, Yuzuru Takamura and Manish Biyani. Palm-size and one-inch gel electrophoretic device for reliable and field-applicable analysis of recombinase polymerase amplification. *Anal. Methods* (2019). doi: 10.1039/C9AY01476D
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4. Himankshi Rathore, Yuzuru Takamura, Manish Biyani. PCR-on-paper for Wearable DNA Sensing Device using Recombinase Polymerase Amplification and Body Heat. Symposium, University of Toyama, Toyama, Japan, Sept 15, 2016 *(Poster Presentation)*

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Contents

1. Chapter 1: General Introduction

1.1. Introduction

Global efforts to combat the emerging infectious diseases are largely based on post-outbreak control, drug and vaccine development, as well as awareness campaigns[1]. However, lack of accessible diagnostics and time-taking and high-end available diagnostic tests and delays in the detection of newly emerging pathogens have resulted in high mortality rates and economic losses worldwide[2]. Microscopic methods[3] such as gram stain, acid-fast and modified acid-fast stains, fluorescent stains, India ink stain, Warthin-Starry stain and Dieterle stain, Wright stain and Giemsa stain, Trichrome stain and iron haematoxylin stain, comprise microscopic examination of tissue to differentiate invasive disease from surface colonization but is limited to be done in certified laboratories[4] and often require highly expensive microscopes which in turn increases the cost of diagnosis. Culture based methods are a gold standard for the identification of organisms but require days or even weeks to get the results which is enough for the pathogens to spread and infect a large population. Moreover, not all pathogens can be cultured, and sample collection is a burden for both patients and physicians. Specimen collection is the most important step to be considered such as for lesions, the leading edge and not the centre should be sampled, the use of the wrong type of swab can give rise to false-negative results and the component of the swab might be toxic to some types of pathogens, blood samples need to be taken from multiple sites simultaneously and some fungi[5], particularly molds cannot be cultured from blood[4]. Moreover special considerations should be taken care of for certain cultures such as anaerobic bacteria should not be cultured from sites where they are part of the normal flora and should be protected from air while transportation to the laboratory, mycobacteria[6] cultures take upto 8-12 weeks to grow, viruses should be inoculated onto tissue cultures within one hour of collection and some do not grow in routine viral cultures, and fungal specimens which take upto 3-4 weeks with antibacterial agents[7]. Therefore, alternatives such as immunologic tests, non-nucleic acid and nucleic acid-based identification tests are rapidly developing for point-of-care diagnostics[8] of infectious diseases. Immunologic tests use either antigen to detect antibodies to a pathogen or vice versa in the patient's specimen. Agglutination tests require the coupling of latex beads[9], gelatine particles or bacteria to an antigen or antibody and the resulting particle complex is mixed with the specimen and the agglutination or cross linking of the particles is measured[10]. Usually, these tests are rapid but less sensitive and the specimens need to be handled carefully to prevent contamination. In complement fixation tests, the specimen is incubated with known quantities of complement and the antigen and the degree of fixation relates to the quantity of the antibody

such as IgG and IgM in the specimen[11]. These tests are usually accurate but are labour intensive and require numerous controls and handling procedures. Enzyme immunoassays such as enzyme-linked immunosorbent assay (ELISA)[12] use antibodies linked to enzymes to detect antigens and to detect and quantify antibodies. Although, the sensitivity of these assays is high, the results can vary according to factors such as patient age, microbial serotype, specimen type, and stage of clinical disease. Precipitation tests such as Ouchterlony double diffusion, counterimmuno-electrophoresis, etc., measure an antigen or antibody in liquid clinical samples by the degree of visible precipitation within a gel or in solution. The sensitivity is usually low and early detection is not possible due to the limitation of the amount of antigen or antibody present in patient specimens. Moreover, diseases such as cutaneous leishmaniasis have less or no antibodies present in the blood stream even if the disease is in progressive stage. Western blot tests and the technical modifications such as the line immunoassay (LIA), the recombinant immunoblot assay (RIBA) and immunochromatographic assays detect antimicrobial antibodies in the patient's sample by their reaction with target antigens such as viral components, synthetic or recombinant-produced antigens immobilized onto a membrane to detect Shiga toxin-producing microorganisms, *Cryptococcus neoformans* capsular antigen and influenza virus[13]. These tests are less sensitive than ELISA and are therefore used for confirmatory purposes in addition to other tests and often pose the same limitations as other immunologic tests. Non-nucleic acid-based identification tests are mainly based on the identification of phenotypic traits such as colony size, colour and shape of pathogen's growth on culture media. These tests are often done sequentially, and the result of the previous test determines the next step. They may involve chromatographic methods where high-performance liquid chromatography (HPLC) or gas chromatography is used to separate and identify microbial components or products. Test accuracy is often dependent on specimen culture which may give rise to false-negatives. Another method is using mass spectrometry to detect various proteins in a specimen and is often used in detection of biological warfare and bioterrorism agents[14]. However, these tests require high-end mass spectrometers which limit the field applicability of this method.

Nucleic acid-based identification tests are often rapid and are instrumental in choosing the correct treatment for the infection due to the rapidity, sensitivity and specificity of these tests. Molecular diagnostic tests are often independent of the type of pathogen and can be easily commercialised for a wide spectrum of pathogens just by changing one or two reagents in the kit[15]. These tests basically comprise of unamplified testing of nucleic acids[16] and tests based on nucleic acid amplification methods such as polymerase chain reaction (PCR). Unamplified testing[17] often requires pre-culture or the presence of the target sequences in high concentration

in the specimen for instance, in infections caused by group A *Streptococcus*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*[18]. Nucleic acid amplification techniques, on the other hand, use small amounts of DNA/RNA and replicate them, thus avoiding the need of culture[19]. Nucleic acid amplification technologies comprise of techniques such as PCR[20], strand displacement amplification (SDA), reverse transcriptase-PCR (RT-PCR), signal amplification such as branched DNA assays, capture of hybridised DNA, transcription amplification and probe amplification such as ligase chain reaction, cleavase-invader, cycling probes. These can also involve postamplification analysis such as sequencing of the amplicon[21], microarray analysis[22] and melting curve analysis[23]. However, specimen should be transported in a low-temperature environment and have to be shielded from contamination. In the next section, the basic mechanism of PCR is highlighted, and the limitations are presented which poses the need of newer amplification methods.

1.2. Polymerase Chain Reaction (PCR)

PCR was invented by Kary Mullis[24] in 1986 and is used to amplify DNA in vitro using enzymes. The target DNA is supplied in small amount in the reaction and is copied using short oligonucleotide primers (18-25bp) complementary to the ends of the template DNA. A thermostable DNA polymerase is used in combination with multiple cycles of heating and cooling in a thermocycler. The method generates exponential amounts of amplicon based on the number of cycles in less than 2 hours. Fig 2 shows the schematic representation of the process of PCR. One cycle of PCR basically comprises of denaturation step at high temperature (94ºC or higher for 15 seconds to 2 minutes), annealing step where temperature is reduced to 40-60 ºC for 15-60 seconds depending upon the primers and extension step where the temperature is set according to the DNA polymerase, for instance, 72ºC for *Taq* DNA polymerase for 1-2 minutes[25]. In the denaturing step, the double stranded template DNA is denatured in two single strands and each strand then proceeds for the amplification separately. In the annealing step, the forward and reverse primer form stable associations to 5' end of one strand and 3' end of another strand by complimentary bases of primer and template. In the extension step, the synthesis of new DNA strand takes place by the addition of nucleotides (dNTPs) to the primers using thermostable DNA polymerase such as *Taq* DNA polymerase, *Tfl* DNA polymerase, *Tth* DNA polymerase, *Tli* DNA polymerase and *Pfu* DNA polymerase. PCR is used with various technical modifications such as RT-PCR, Hot-start PCR[26–28], Long PCR[29], Quantitative endpoint PCR[30], quantitative real-time PCR[31,32], rapid amplified

polymorphic DNA (RAPD) analysis[33], rapid amplification of cDNA ends (RACE)[34], differential display PCR[35], *in situ* PCR[36] and high-fidelity PCR[37]. The PCR reaction conditions have to be optimized for the combination of each template and primers for factors such as magnesium concentration, buffer considerations, enzyme concentration, cycling parameters, template and primer concentrations, etc[38]. However, even with the advantage of being highly sensitive and specific, conventional PCR cannot be used in the point-of-care settings due to the involvement of temperature cycling, time taking process, and requirement of expensive equipment and highly trained personnel[39].

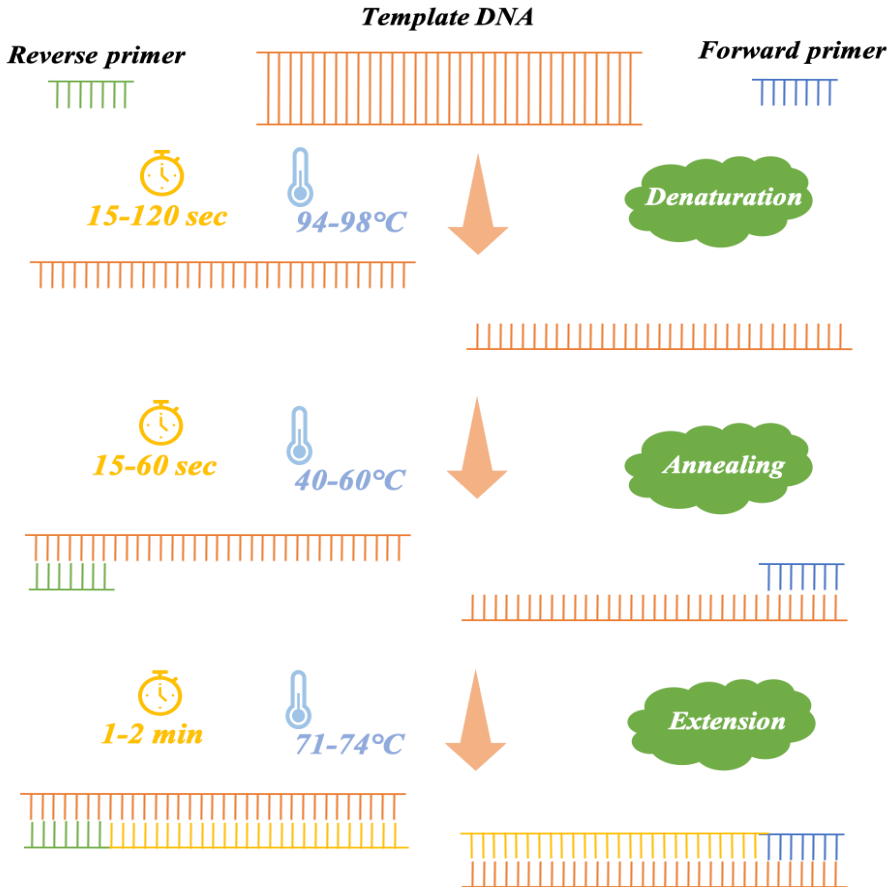

Figure 2: Schematic representation of the process of polymerase chain reaction (PCR) which comprises of three steps, namely, denaturation, annealing and extension.

1.3. Isothermal amplification methods

Routine healthcare check-ups are important for early stage diagnosis of the disease. This needs the development of systems to monitor healthcare regularly. Thus, the development of simple, affordable and sensitive point of care testing has become the need of the hour to bridge the gap between diagnostics and treatment of deadly diseases prevailing in today's world. The unavailability of health care resources mainly in the developing world leads to an ever increasing

rate of spreading infection from one person to another. Point-of-care diagnostics i.e. medical diagnostic testing performed outside the clinical laboratory in close proximity to where the patient is receiving care are an excellent way to fight the epidemics faced by the world every now and then. The development of POCT need to be in consideration with the ASSURED (**A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free and **D**elivered to end user) criteria highlighted by World Health Organisation (WHO). However, due to the limitations highlighted in the preceding sections, conventional diagnostic methods such as microscopy, culture-based methods, immunologic tests, non-nucleic acid-based methods and PCR cannot be used in the manufacturing of ASSURED POCT devices. Molecular approaches coupled with a suitable visual detection method, play an important role in rendering specificity and sensitivity to point of care testing devices. Recently, a milestone in molecular biology research has been the development of isothermal amplification methods based on some new researches in the molecular biology of DNA/RNA synthesis and their interaction with some accessory proteins[40]. These amplification methods require single temperature for target amplification and are best suited for the manufacturing of ASSURED POCT devices as they eliminate the need of equipment for temperature cycling making POCT devices accessible for resource poor settings such as in developing countries. These methods provide excellent specificity, sensitivity and simplicity to be used in the development of POCT devices for rapid and cost-effective health monitoring. In the next section, the mechanisms of few isothermal amplification methods are discussed.

1.3.1. Nucleic acid sequence-based amplification

This method is designed to detect RNA targets and the reaction consists of avian myeloblastosis virus (AMV), reverse transcriptase (RT), T7 RNA polymerase and RNase H with two oligonucleotide primers[41]. The forward primer is composed of two parts, one, complementary to the T7 promoter sequence and other, complementary to the 3' end of target RNA. Step 1 comprises of the synthesis of cDNA from RNA using the part of the primer which is complementary to the 3'end of RNA through reverse transcriptase (AMV-RT) and step 2 comprises of the degradation of RNA using RNase H. Then, the reverse primer binds to the DNA and is elongated by AMV-RT, forming a double-stranded DNA (dsDNA) molecule (step 3). The forward primer is designed in a way that when it forms a dsDNA, it codes for the T7 RNA polymerase promoter site which generates antisense RNA copies using a DNA template (step 4). The same process is followed for another strand except the reverse primer binds first in this case.

The reaction occurs isothermally at 41ºC and more than 1012 fold amplification can take place in 90-12 minutes[42]. A wide range of target sequences such as HIV-1 genomic RNA[43], hepatitis C virus RNA[44], Human Cytomegalovirus RNA[45], 16S RNA of many bacterial species[46–50], and enterovirus genomic RNA[51] have been amplified and detected using this technique.

1.3.2. Strand Displacement Amplification

The original method of strand displacement amplification (SDA) consists of a probe containing a HincII recognition site at the 5'end and another part complementary to the template DNA[52]. First, the primer binds to the template DNA and DNA polymerase then incorporates deoxyadenosine 5'-[α-thio] triphosphate (dATP[αS]). HincII then cleaves the probe at the recognition site in the primer which reveals a 3'-OH. The strand is then extended by DNA polymerase due to the presence of 3'-OH. These steps are repeated several times and the DNA is amplified. Another, later developed variant of SDA uses 4 probes instead of two where two sets of probes bind to two DNA strands simultaneously which makes it more specific[53]. The SDA products can be detected by molecular beacons[54–56] and intercalating dyes[54,57]. Another variant is the combination of ligation and SDNA to detect single nucleotide polymorphisms (SNP) which was developed by Wang et al. by incorporating a G-DNA Quadruplex for DNAzyme/hemin-based chemiluminescence detection[58]. SDA works at 37ºC with initial heating at 95ºC and is widely used over the past 2 decades for both singlex detection for targets such as *Salmonella*, Hepatitis, *E.coli* O157:H7, mycobacterium, *Human papilloma Virus* (HPV), HIV, etc. and multiplex detection for targets such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, *E.coli* O157:H7 and its serotypes, Vibrio and *E.coli* O157:H7, etc[59].

1.3.3. Helicase-dependent amplification

Helicase dependent amplification basically comprises of helicase which is used to separate dsDNA instead of high temperature[60]. The first step comprises of the unwinding of DNA duplexes by helicase and the stabilization of released single stranded DNA (ssDNA) by single strand binding proteins (SSB), which then hybridize to the primers in step 2. DNA polymerase then extends the primers and the newly formed dsDNA acts as template for the next cycle. The assay is performed at 60ºC using a thermally stable helicase which eliminates the need of accessory proteins[61]. It has been used to detect genomic fragments of *Treponema denticola* in cells and *Brugia malayi* in blood samples[60]. HDA can achieve over a million-fold amplification and can be performed on crude samples.

1.3.4. Rolling circle amplification

In rolling circle amplification (RCA), a short DNA or RNA primer is used to generate a long single stranded DNA or RNA using specialized DNA and RNA polymerases containing multiple copies of a given sequence[62–64]. RCA utilizes a probe that contain 3' and 5' end sequences that bind to the target sequences to aid a ligase mediated circularization of the probe if the 5'end is phosphorylated[65]. After circularization, a primer and DNA polymerase are utilized for the production of long repeating DNA sequences. Several genes such as cystic fibrosis transmembrane conductance regulator (CFTR) G542X mutation[63], Epstein-Barr virus (EBV) in human lymphoma specimens[66], H1N1 and H3N2 influenza A mutations[67], porcine circovirus type 2[68] and Listeria monocytogenes[69] have been detected using this technique. Another variant, Target sequence recycled RCA (TR-RCA) is available where the DNA template is already circularized but the primer binding site is sequestered in the duplex region. RCA basically works at 37ºC with a pre-heating step at 95ºC and the process takes upto an hour for amplification[70].

1.3.5. Loop-mediated isothermal amplification

Loop-mediated amplification (LAMP) has been able to achieve sensitivity comparable to PCR with a high target specificity[71]. There are two primer sets in the forward primer namely inner (F1c-F2, c for complementary) and outer (F3) primers. The first step comprises the binding of F2 region (inner primer) to the target which is then extended by DNA polymerase. The second step then consists of the binding of F3 (outer primer) to the same target DNA which is then extended by DNA polymerase to displace the previously formed strand. The third step comprises of the displaced strands forming a stem loop at the 5'end and 3'end of the previously formed strand, and finally, the reverse primer set hybridizes to the target DNA and forms another stem loop in identical fashion. This dumbell shaped structure enters the exponential amplification and strands with several repeats of the target DNA are made by extension and strand displacement. 10^9 copies can be generated using this method in less than an hour, even in the presence of large amount of non-target DNA. LAMP works at 64ºC and has been widely used over the last decade for the detection of pathogens such as dengue[72], Japanese Encephalitis[73], Chikungunya[74], West Nile[75], Severe acute respiratory syndrome (SARS)[76], highly pathogenic avian influenza (HPAI) H5N1[77], Leishmaniasis[78], etc. Although, LAMP is

superior in specificity and sensitivity, the primer design is rather complicated due to primers complementary to 6 regions of the target sequence. LAMP products can be easily detected by turbidity and dyes such as malachite green[78], etc.

1.3.6. Recombinase Polymerase Amplification

Recombinase Polymerase Amplification (RPA) is an isothermal nucleic acid amplification technique which amplifies the target at 37 ˚C for the detection of infection in just 10-20 minutes[79]. The basic mechanism of RPA is shown in fig 3. Firstly, gp32, which is an SSB, binds to the primers and resolves the secondary structures formed by the primer, if any. Then, Uvsy loading factor binds to the primers and gp32 is released simultaneously. The binding of the Uvsy loading factor leads to the binding of Uvsx recombinase and results in the formation of Uvsy-primer-Uvsx complex. This complex then performs a homology search according to the complementarity of the primer sequence to the target DNA. This results in the formation of D-loop and strand exchange which releases one strand of DNA. This ssDNA is stabilized by gp32 and DNA polymerase extends the primer and the synthesis of new DNA strand takes place. This process repeats itself over and over to create multiple copies of the target DNA.

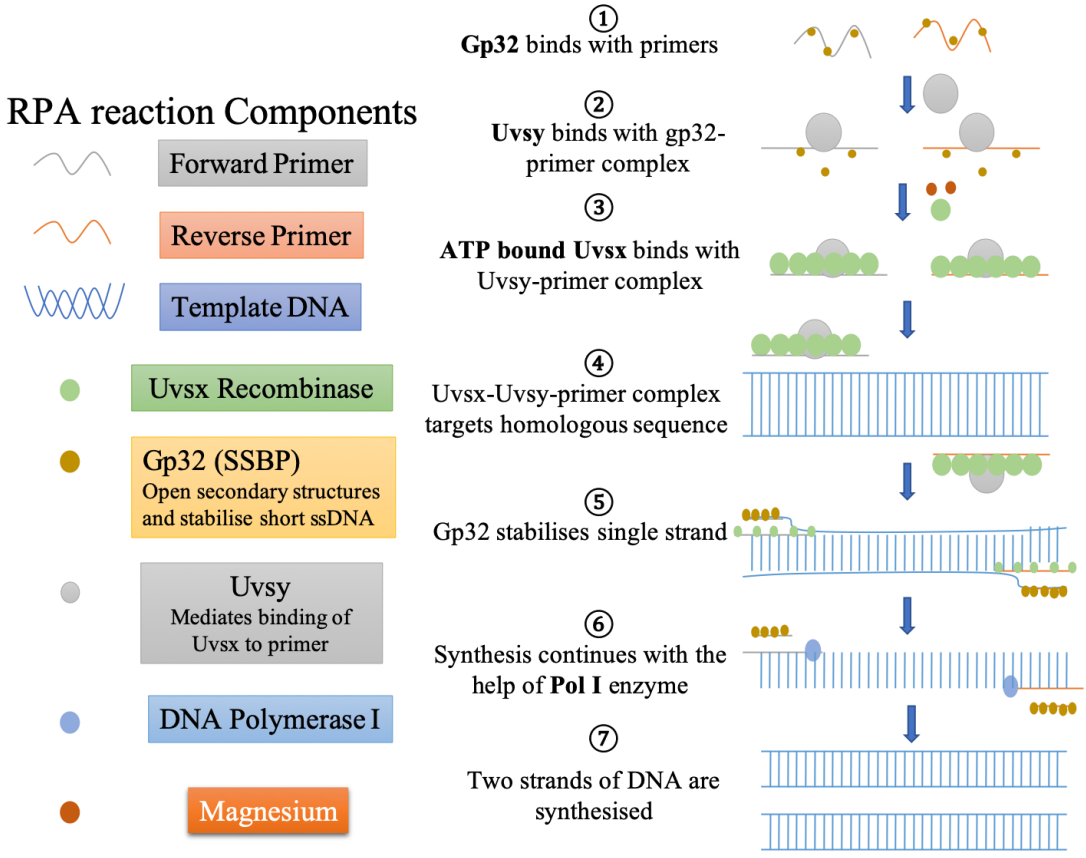

Figure 3: Schematic representation of the process of recombinase polymerase amplification (RPA).

RPA primer design is crucial but the availability of specialised software for designing RPA primers in the past year has made the process automated and easier. RPA has been modified and used in combination with various strategies to increase the sensitivity and specificity and facilitate the detection process of the amplicon. In this work, RPA is used as a tool to amplify target DNA and develop a point-of-care tool for resource-poor settings. The use of RPA holds a great importance as it renders the ability of using human body temperature (37ºC) for the amplification of DNA thus eliminating the use of specialized equipment to provide required temperature. The detailed mechanism and assay specifications will be highlighted in chapter 2. Here, some point-of-care tools developed in the last decade using RPA are reviewed. Over 350-peer reviewed publications have been written from the time of the development of RPA till today, which highlights the importance of this tool as a molecular diagnostic approach. Clinical trials with RPA have already been performed with organisms as summarised in the following table[80]:

Target gene	Organism	Detection method	References
Nucleocapsid gene	Bovine coronavirus	Real-time fluorescent detection	[81]
CDS2 gene	*Chlamydia trachomatis*	Lateral flow strip detection	[82]
cAMP factor (*cfb*) gene	Group B Streptococci	Real-time fluorescent detection	[83]
	Cryptosporidium spp.	Lateral flow strip detection	[84]
5'Untranslated region (UTR)	Yellow fever virus (YFV)	Real-time fluorescent detection on the tube scanner	[85]
IS6110 gene	*Mycobacterium tuberculosis*	Real-time fluorescent detection on microfluidic platform	[86]
IS1081 gene	*Mycobacterium tuberculosis*	Real-time fluorescent detection	[86]

Beta giardin gene	*Giardia*	Lateral flow strip detection	[87]
IS6110 gene	*Mycobacterium tuberculosis*	Real-time photonic detection	[88]
Highly conserved 3' UTR	DENV 1-4	Real-time fluorescent detection	[89]
47kDa gene from Karp strain	*Orientia tsutsugamushi*	Lateral flow strip detection	[90]
17 kDa gene from Wilmington strain	*Rickettsia typhi*	Lateral flow strip detection	[90]
Ribosomal 18S DNA	*Entamoeba histolytica*	Lateral flow strip detection	[91]
ITS sequences of strain CBS 109801	*Madurella mycetomatis*	Gel electrophoresis detection	[92]
Nucleocapsid sequence	Ebola virus (EBOV)	Real-time fluorescent detection	[93]
DNA polymerase	Orf virus (ORFV)	Real-time fluorescent detection	[94]
Leader peptidase A (LepA) gene	*Streptococcus pneumoniae*	Real-time fluorescent detection	[95]
DNA polymerase	ORFV	Lateral flow strip detection	[96]
Kinetoplast minicircle DNA	*Leishmania donovani*	Real-time fluorescent detection	[97]
NSP2 gene	Highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV)	Real-time fluorescent detection	[98]
Major capsid protein gene	Cyprinid herpesvirus 3	Gel-electrophoresis detection	[99]
cfb gene conserved region	Group B Streptococci	Real-time fluorescent detection	[100]

Non-structure protein 1 (nsP1)	Chikungunya virus (CHIKV)	Real-time fluorescent detection	[101]
NS2A conserved region	Zika virus	Real-time fluorescent detection	[102]
G-protein coupled chemokine receptor (GPCR) gene	Lumpy skin disease virus (LSDV)	Real-time fluorescent detection	[103]
IS900 gene	*Mycobacterium avium* subsp. *paratuberculosis*	Real-time fluorescent detection	[104]
T1E4 gene	Prostate cancer	Real-time fluorescent detection	[105]
NS1 gene	Porcine parvovirus (PPV)	Real-time fluorescent detection	[106]
Nucleocapsid gene	type 2 porcine reproductive and respiratory syndrome virus (PRRSV)	Real-time fluorescent detection	[107]
Cytochrome b gene	*Theileria annulata*	Lateral flow strip detection	[108]
pirA-like gene	*Vibrio owensii*	Real-time fluorescent detection	[109]
rRNA gene	*Fasciola hepatica*	Gel electrophoresis detection Lateral flow strip detection	[110]
N gene	Pest des petits ruminants virus (PPRV)	Real-time fluorescent detection Lateral flow strip detection	[111]
ITS2 gene	*Phytophthora infestans*	Real-time fluorescent detection	[112]
ORF2 gene	Porcine circovirus type 2 (PCV2)	Real-time fluorescent detection	[113]

		Lateral flow strip detection	
gD gene	*Pseudorabies virus*	Real-time fluorescent detection Lateral flow strip detection	[114]
B1 gene	*Toxoplasma gondii*	Lateral flow strip detection	[115]
RNA transcript of TMPRSS2:ERG fusion gene	Prostate cancer	RPA fluocculation assay	[116]
VP2 gene	Porcine parvovirus	Real-time fluorescent detection	[117]
GPCR gene	*Capripoxvirus*	Real-time fluorescent detection Lateral flow strip detection	[118]
Nucleocapsid protein gene	Canine distemper virus	Real-time fluorescent detection	[119]
imp gene	*Candidatus* Phytoplasma oryzae	Real-time fluorescent detection Lateral flow strip detection	[120]
imp gene	*Candidatus* Phytoplasma mali	Real-time fluorescent detection Lateral flow strip detection	[121]
N gene	Rabies	Real-time fluorescent detection	[122]
KRAS Oncogenic mutation gene G12D on exon 12	Colorectal cancer	Real-time silicon photonic microring-based detection	[123]

KRAS Oncogenic mutation gene G12D on exon 13	Colorectal cancer	Real-time silicon photonic microring-based detection	[123]
Consensus region covering all 7 S-segment clades	Crimean-Congo Hemorrhagic fever virus (CCHFV)	Real time fluorescent detection	[124]
Nucleocapsid protein gene	Canine parvovirus 2	Real time fluorescent detection	[125]
G gene	Bovine ephemeral fever virus (BEFV)	Lateral flow strip detection	[126]
IS900 gene	*Mycobacterium avium* subsp. *paratuberculosis*	Lateral flow strip detection	[127]
Fno FSC771 hypothetical protein gene	*Francisella noatunensis* subsp. *Orientalis*	Real time fluorescent detection	[128]
VP1 gene	Enterovirus 71 subgenotype C4	Real time fluorescent detection	[129]
56 Kda gene of Karp-like strain	*Orientia tsutsugamushi*	Lateral flow strip detection	[130]
23S rRNA gene	*Coxiella burnetti*	Lateral flow strip detection	[131]

Table 1: Clinical trials performed using RPA: The table shows the clinical trials performed using RPA, the target sequence used, and the detection method used.

1.4. Purpose of dissertation

The recombinase polymerase amplification (RPA)-based isothermal nucleic acid amplification strategy is one of the most rapidly developing methods for performing nucleic acid tests outside laboratory settings. However, the reaction mechanism of RPA results in unavoidable concerns related to nonspecific and off-target binding of the recombinase enzyme that lead to false-positive results, and thus successful RPA signal detection outside the laboratory has yet to be demonstrated. Additionally, sampling methods such as liquid biopsy are a burden to both patients and physicians, and the DNA extraction and purification steps involved in sampling in DNA-based methods increase the time to diagnosis. Direct sampling via an FTA card reduces

the risk of contamination and facilitates the transport and long-term storage of the sample at room temperature.

In this work, a way to discriminate true-positive results from false-positive and/or negative results generated during the RPA reaction is explored and an FTA card is used for direct sampling of RPA reactions to eliminate the concerns involved with sample contamination as well as the sample preparation steps. First, a suitable target sequence is selected for the detection of leishmaniasis using RPA and then the assay is optimized by evaluating various parameters. Then, RPA is performed for seven species, namely, *Leishmania tarentolae* (noninfective to humans), *Leishmania infantum*, *Leishmania mexicana*, *Leishmania tropica* and *Leishmania major*, belonging to the subgenus *Leishmania*, and *Leishmania lainsoni* and *Leishmania braziliensis*, belonging to the subgenus *Viannia*, targeting a 360-bp gene segment of the 18S rRNA gene to evaluate the versatility of RPA to detect all the three forms of leishmaniasis. Next, a rapid gel electrophoretic analysis of RPA results is done using in-lab developed palm-sized gel electrophoretic device based on the formal micro-gel electrophoretic design to enable field applicable biosensing, particularly for resource-poor settings. Next, Micro-Temperature Gradient Gel Electrophoresis (uTGGE)-type device is utilized, which has a power to distinguish, even a single nucleotide difference between two DNA molecules based on their thermal stabilities. We analyzed 8 *Leishmania* species and constructed a phylogenic tree for subtype differentiation to differentiate the forms of leishmaniasis which are specifically caused by various subtypes of *Leishmania* parasite. The schematic of the near-to-patient diagnosis is highlighted in Fig.1. First, the sample is obtained from the patient by aspirating/scraping the margins of active lesions, and the material is then spotted onto the FTA card and dried. Next, a 2.0-mm-diameter disc is punched from the FTA card and immersed in an Eppendorf tube containing liquid RPA reagents supplied with primers targeted to amplify the target gene fragment. This tube is then held in the palm for 10 minutes to provide the incubation temperature for RPA using body heat. Primary screening is performed using a palm-sized electrophoretic device to perform a rapid 1-inch gel electrophoretic analysis of RPA products. Next, 10 min- Micro-Temperature Gradient Gel Electrophoresis (uTGGE)-type device is utilized, which has a power to distinguish, even a single nucleotide difference between two DNA molecules based on their thermal stabilities and uses the same type of 1-inch gel electrophoretic system. The melting profiles are obtained and species identification dots are assigned. Pattern similarity scores are then obtained using computer-aided normalization and used to plot a dendrogram. The results can then be submitted to a public database for treatment assessment programmes in epidemic conditions.

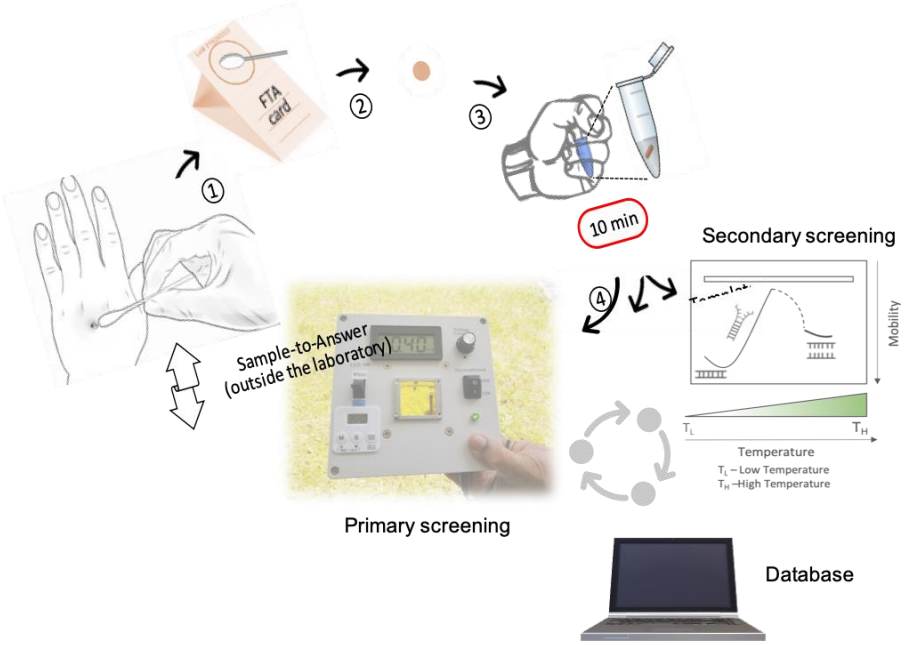

Figure 1: Schematic of near-to-patient nucleic acid-based molecular diagnostic tests. Steps involved in the strategy as highlighted in the text for on-site gel electrophoresis-based genotyping are used for field applicable biosensing (primary screening) as well as rapid subtype identification (secondary screening) using micro-Temperature Gradient Gel Electrophoresis.

1.5. References

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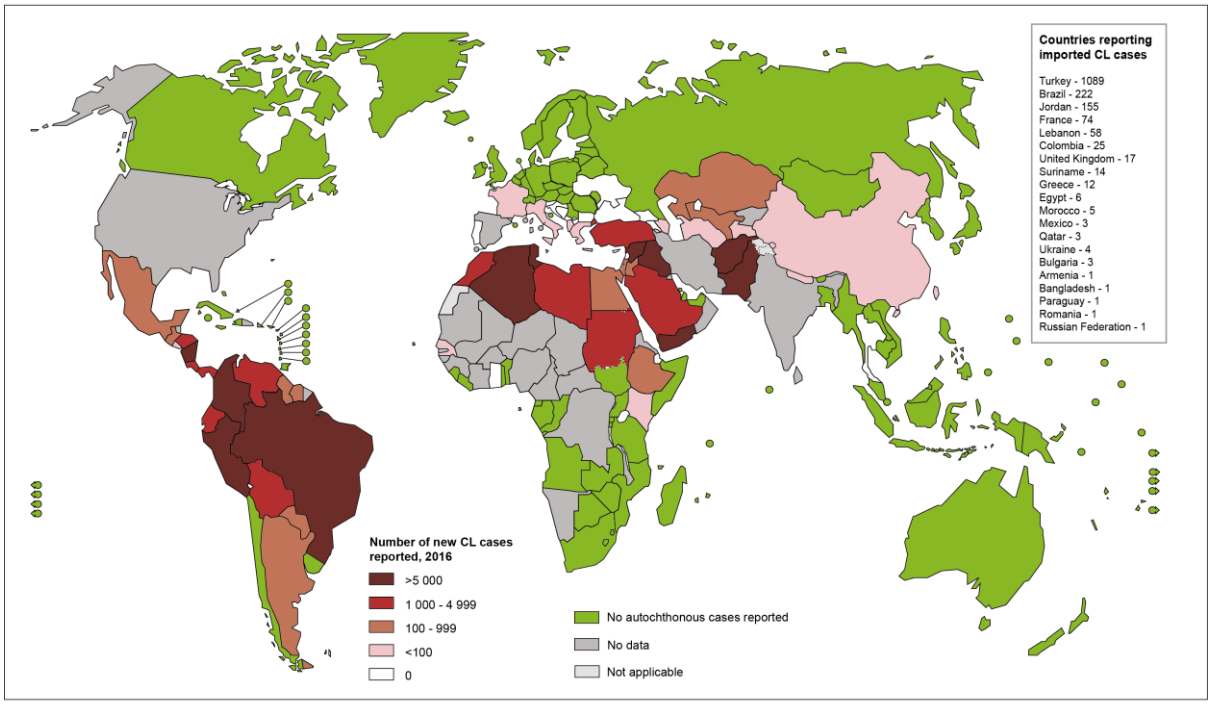
2. Chapter 2: Protocol development of Recombinase polymerase amplification (RPA) for improved molecular analysis

As highlighted in table 1 (chapter 1) approximately 50 analytes have been used for amplification and detection through RPA. Such a wide application highlights the versatility of RPA in terms of the template. The World Health Organization (WHO) recognized 20 major neglected tropical diseases (NTDs) under the global elimination program, of which 13, including leishmaniasis, lack field-friendly diagnostic tools[1]. In this research, I aim to use RPA in combination with Whatman FTA card as sampling tool to detect Leishmaniasis which is a skin infection caused by protozoan *Leishmania* parasite. In the next section, the epidemiology and the available diagnostic tools for leishmaniasis are discussed. Then, the protocol for the amplification of the target for the detection of leishmaniasis is optimized and various parameters of the RPA assay are evaluated accordingly.

2.1. Leismaniasis: Epidemiology

Leishmaniasis is a parasitic disease transmitted by infected female sand flies of the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World)[2] and is endemic in more than 98 countries[3]. WHO has maintained a database for the epidemiology of leishmaniasis according to the regions in the world, namely, the Americas, South-East Asia, European region, Eastern Mediterranean region and Western Pacific Region. The disease is associated with malnutrition, weak immune system and lack of healthcare facilities and mainly affects below poverty line population in Africa, Asia and Latin America. In 2017, WHO revealed that out of 200 countries and territories, 97 countries and territories are endemic for leishmaniasis which includes 65 countries (endemic for visceral and cutaneous leishmaniasis), 10 countries (endemic for visceral leishmaniasis only) and 22 countries (endemic for cutaneous leishmaniasis only). In 2016, over 90% of global visceral leishmania cases were reported from Brazil, India, Ethiopia, Somalia, South Sudan, Kenya and Sudan and 84% of global cutaneous leishmaniasis cases were from Algeria, Brazil, Afghanistan, Pakistan, Iraq, Columbia Peru, Tunisia, Yemen and the Syrian Arab Republic. As of October 2018, the map in figure 2(a) shows the reported data from 50 VL-endemic countries (66% global cases) and in figure 2(b) shows the reported data from 52 CL-endemic countries (60% global cases)[3].

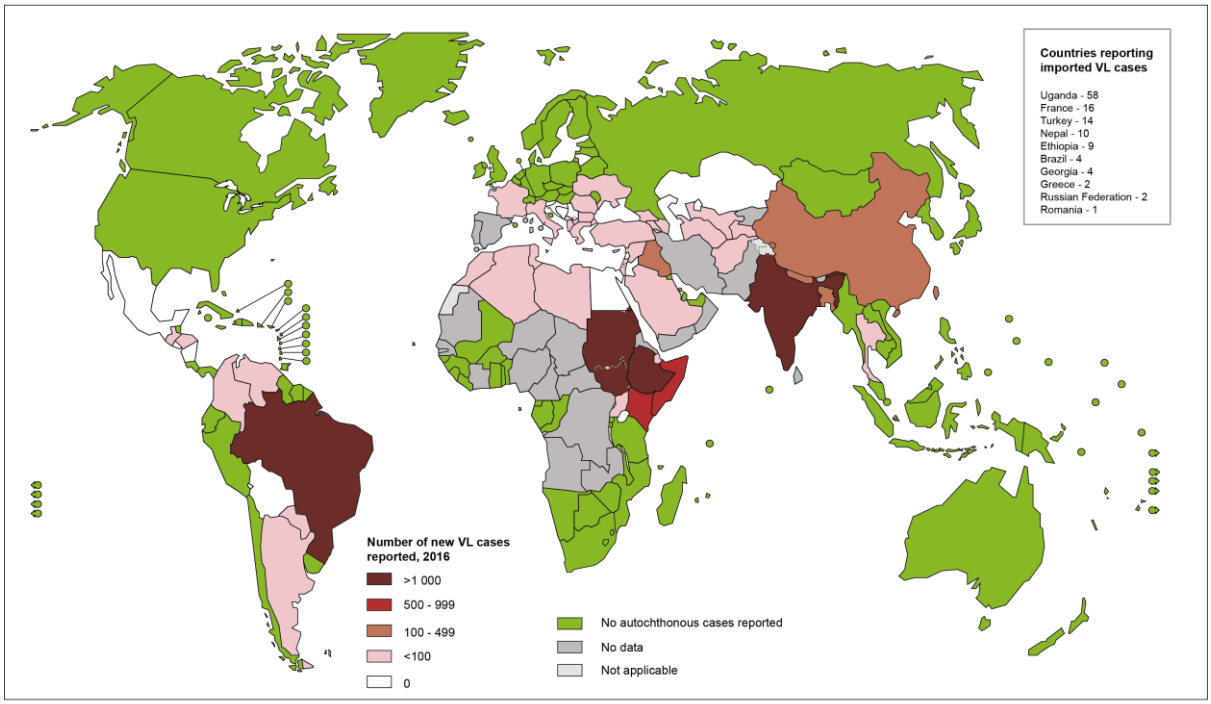
Status of endemicity of cutaneous leishmanisis worldwide, 2016



Data Source: World Health Organization
Map Production: Control of Neglected Tropical Diseases (NTD)
World Health Organization

World Health Organization

Status of endemicity of visceral leishmaniasis worldwide, 2016



Data Source: World Health Organization
Map Production: Control of Neglected Tropical Diseases (NTD)
World Health Organization

World Health Organization

Figure 4: Status of endemicity of cutaneous leishmaniasis (upper) and visceral leishmaniasis (lower) worldwide, 2016 as reported by WHO[3].

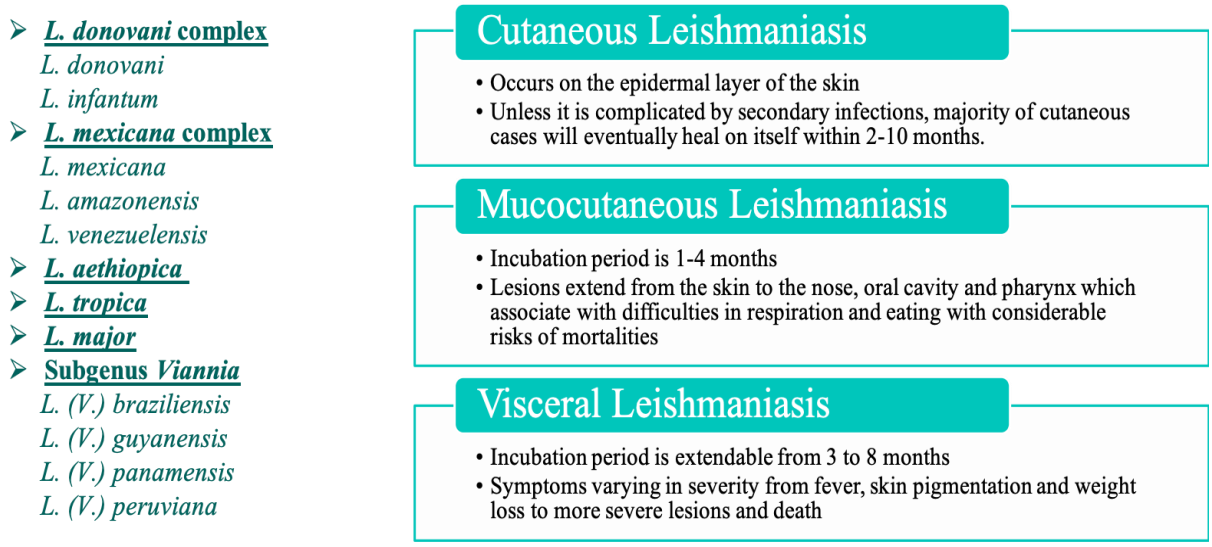


Figure 5: Species causing human infection of leishmaniasis and the three forms of leishmaniasis, namely, cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis.

2.2. Leishmaniasis: Diagnostic tests

Leishmaniasis is generally diagnosed by microscopic examination of spleen aspirates[4] (VL in immunocompetent patients), liver biopsy samples (40% sensitivity when most of the amastigotes are colonized within the Kuffer cells to 90% sensitivity), bone marrow aspirates[5] (76-85% sensitive) and other relevant tissue aspirates for *Leismania* amastigotes. The stains used for the smears can be Romnowsky's, hemotoxyline eosine or immunoperoxidase stains (increased sensitivity for cutaneous and mucocutaneous leishmaniasis) and Giemsa stain at pH 7.2. The amastigote stage which is seen in clinical specimens are known as Leishman-Donovan (LD) bodies. The promastigote form can be cultured and isolated from the specimens on solid NNN medium having 20-30% rabbit blood or liquid Schneider's insect medium. Culture examination[6,7] is then done using any of the stains, however, this technique has proved of low sensitivity for mucocutaneous leishmaniasis. Another alternative is the isolation in experimental animals[8] where the clinical sample is inoculated within a susceptible BALB/c mouse or into a hamster footpad or nose. A number of immunological methods based on host-parasite interaction such as Fluorescent Antibody Test (FAT) is based on detecting antibodies demonstrated in the early stages of infection but are undetectable after 6-9 months of cure. Direct Agglutination Test (DAT)[9] is a highly specific and sensitive test which uses whole promastigotes but the incubation time is 18 hwhicgh is quite long and the need of serial dilutions of blood or serum makes it tedious. Enzyme Linked Immunosorbent Assay (ELISA)[10] is another method for diagnosis but the sensitivity and specificity is highly dependent upon the

antigen used. Few antigens such as gp63 have been cloned early but failed in early stage diagnosis. Leishmanin Skin Test (LST)[11] is used as an indicator of delayed hypersensitivity in cases of cutaneous and mucocutaneous leishmaniasis but the Leishmanin antigen is not available commercially. Immunological methods are helpful in the case of visceral leishmaniasis but fail to diagnose cutaneous and mucocutaneous leishmaniasis due to low antibody response[12,13]. On the other hand, DNA-based molecular typing methods[14–18] such as Polymerase Chain Reaction (PCR)[19] are highly reliable, sensitive and specific and can be used for the detection of asymptomatic infections[20], early stage infections and in cases of disease relapse[21] of all the forms of leishmaniasis due to high copy number of target genes in the parasite[22]. Although these methods are highly reliable but unsuited for implementation at primary or secondary health-care facilities due to the obvious requirements of highly sophisticated laboratory and trained personnel. An alternative of conventional PCR by adaptation of existing isothermal amplification technologies could be a smart solution to perform DNA amplification outside laboratory settings[23]. Loop mediated isothermal amplification (LAMP) combined with an FTA card and colorimetric visual detection approach has recently been reported for a rapid identification of CL[24,25]. However, the pre-heating step (95°C), reaction incubation temperature (65°C) and the post-heating step (80°C) prevent these methods to be used as Rapid Diagnostic Tests (RDTs) for Point of care (POC) applications.

2.3. RPA: Assay specifications

The RPA kit is commercially available from TwistDx, UK and the manufacturer has highlighted some specifications for the efficient amplification of the target using RPA. The standard temperature range for the process of RPA is advised to be 37-42°C but RPA can work below this range with reduced reaction rate. Although, the energy consumption rates for the reagents used in the kit lengthen less rapidly than template doubling times which can lead to premature fuel consumption and in turn reduces the efficiency of RPA. Detectable amplification can be achieved in shorter times if the starting template concentration is high and vice versa. Although, the reactions should be onset at the same time either by using magnesium start or delaying the reaction by keeping on ice. The RPA Assay can be optimized by exploring the permitted ranges of temperature, magnesium acetate concentrations (12mM-20mM), agitation time (3-6 minutes) and primer concentration (150nM-600nM). RPA often amplifies target sequences less than 500 bp efficiently. Longer sequences lead to the onset of background amplification as the reactions principally rely upon viscous crowding agents and homology

searches at low temperature, and unavoidable concerns thus arise related to nonspecific and off-target binding of the recombinase enzyme, which may lead to false-positive results[26]. RPA primers typically range from 30-35 nucleotides, although successful results have been obtained with shorter primers also. Moreover, specifications such as long tracks of guanines at the 5'end may deter the reaction, while cytidines may be beneficial as this might increase the formation of recombinase filaments. Also, G and C at the 3' end improve the performance and provide a clamped target for polymerase. GC content ranging from 40-60% is the best suited and artefact generation has to be evaluated using available softwares. Moreover, noise reactions or background amplification are in competition with the genuine amplification process, therefor the selection of primers and the optimization of assay becomes a crucial step in RPA.

2.4. Objective

The aim of this section is to select the target sequence for early stage and sensitive detection of leishmaniasis. After the selection of the target, the assay parameters are optimized for efficient amplification using RPA and detection using rapid gel electrophoresis (5-7 minutes) previously fabricated in the laboratory. Then, the versatility of the target is evaluated for the detection of various species of *Leishmania* responsible for different types of forms, namely, cutaneous leishmaniasis, visceral leishmaniasis and mucocutaneous leishmaniasis.

2.5. Materials and Methods

2.5.1. RPA primers

The primers used for different targets for leishmaniasis are outlined in the table below:

Name	Sequence	Target (amplicon size)
RPA-L.rRNA-S	5'-TTCTCCACTCCAGACGGTGGGCAACCATCGT-3'	18S rRNA (360 bp)
RPA-L.rRNA-R	5'-AATGACAATCCAAATGGACTGACAACTCTA-3'	18S rRNA (360 bp)
RPA-L.mpi-S	5'-AGTACCCSGATGATGTCGGGTGCTGGATGGT-3'	mpi (165 bp)
RPA-L.mpi-R	5'-GTGAGTCCAGCACGCACGACGTTGTCGCTGC-3'	mpi (165 bp)
L.MC-1S	5'-CTRGGGGTTGGTGTAAAATAG-3'	kDNA minicircle (700 bp)
L.MC-1R	5'-TWTGAACGGGRTTTCTG-3'	kDNA minicircle (700 bp)
L.Cyt-S	5'-GGTGTAGGTTTTAGTYTAGG-3'	Cytochrome B (900 bp)
L.Cyt-R	5'-CTACAATAAACAAATCATAATATRCAATT-3'	Cytochrome B (900 bp)

Table 2: Primer sequences used for the RPA assay of different targets with the size of the amplicon they yield.

2.5.2. Reaction protocol

A set of forward and reverse primers (Table 1) was designed for RPA of the target genes- 18S rRNA, mannose phosphate isomerase (mpi), cytochrome B and kDNA minicircle sequences[27]. For RPA reactions without an FTA disc, RPA was performed in a 12.5-µl reaction volume

using a TwistAmp Basic kit (TwistDX, Cambridge, United Kingdom) consisting of 0.24 μM each of the primers (as and where indicated), TwistAmp rehydration buffer, and RPA enzyme pellet and 14 mM magnesium acetate (MgAc). All reagents except the template DNA and magnesium acetate were prepared in a master mix, which was divided into four aliquots. Template DNA for 10^4-10 parasites/reaction was added for the evaluation of sensitivity, and 10 ng of purified genomic DNA of *L. tarentolae* (1) (noninfective to humans), *L. infantum* (2), *L. lainsoni* (3), *L. mexicana* (4), *L. tropica* (5), *L. major* (6) and *L. braziliensis* (7) was added for the amplification of target gene segments from different species. Magnesium acetate was pipetted in Eppendorf tube lids and mixed by vortexing. Reactions were incubated for 10 minutes at 37℃ as and where indicated.

2.5.3. Downstream analysis

For direct (non-gel-based) detection, EvaGreen fluorescent dye (0.5X concentration) was added to the RPA reactions, and fluorescence was visualized using UV transilluminator. For gel-based detection, micro gel electrophoresis designed and fabricated in the laboratory was used. A 1-inch (2.5 × 2.5 cm) precast 6% polyacrylamide gel was used to analyze the RPA products. Paper pads (1.0 × 2.0 cm) were soaked in 2 ml of 1X TBE buffer and used as a source of running buffer. A 1-μl RPA reaction containing 0.5 μl of 6X gel loading dye was loaded into the wells of a precast gel (6% T PAGE gel) and electrophoresed for 5-7 minutes at 100 V. A 300 μl volume of SYBR gold dye was poured on top of the gel, and the bands were visualized using UV transilluminator and the images were obtained from the instrument.

2.6. Results and Discussion

2.6.1. Optimization of target sequence

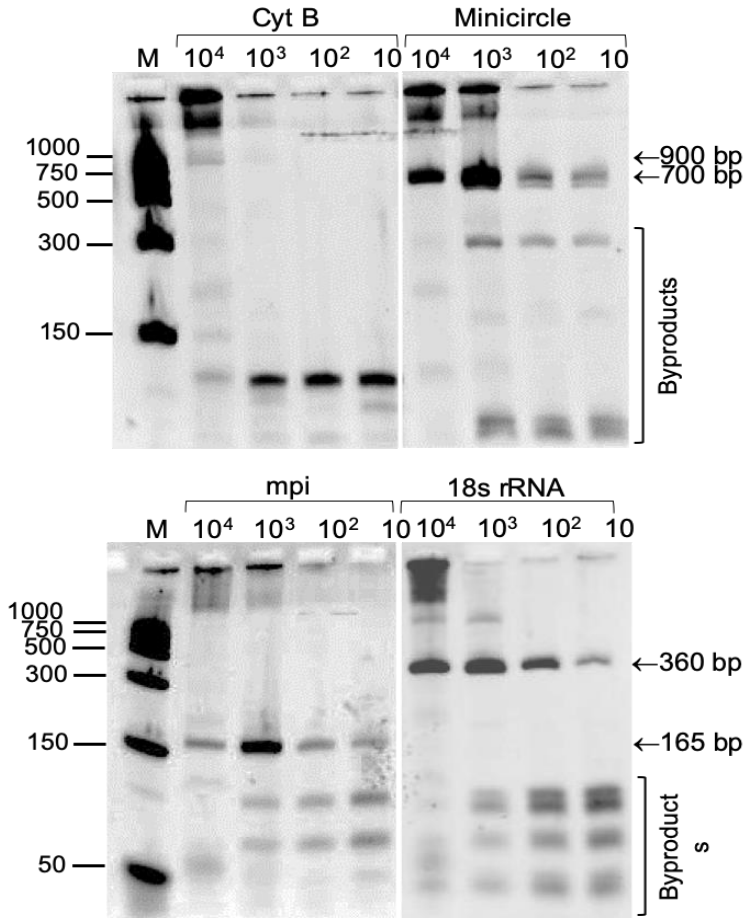


Figure 6: Primers targeting 4 target gene segments, namely, cytochrome B (900 bp), minicircle (700 bp), mannose phosphate isomerase (mpi) (165 bp) and 18S rRNA (360 bp), were used for amplification via RPA. The sensitivity for each target was evaluated using total DNA from 10^4 to 10 parasite copies of *Leishmania major* and analyzed by the portable polyacrylamide gel electrophoresis device.

The sensitivity of the RPA assay for different target sequences was evalutated to optimize the protocol for the detection of low amounts of template. In general, the RPA method results in the generation of short fragments (<150 bp) or highly branched long DNA products in addition to the desired target fragment. Targets such as cytochrome *b* (*cyt* b), kDNA minicircle sequences, mpi, and 18S rRNA genes have been used to detect *Leishmania* infection using PCR and other isothermal amplification methods such as LAMP and nucleic acid sequence-based amplification (NASBA). Therefore, in this study, we first analyzed RPA to amplify these four target gene regions and evaluated the efficiency of RPA. As shown in Fig. 6, the amplification of minicircle sequences (700 bp), mpi (165 bp) and 18S rRNA (360 bp) were successful, but *cyt* b (900 bp) amplification was not. This failure can be attributed to the low

copy number of the *cyt* b gene in the *Leishmania major* genome used in this reaction compared to that of the other targets. In addition, as shown in figure 7, since RPA and other isothermal methods are limited to amplification of discrete (i.e., a single DNA species, <150 bp) and long (>500 bp) DNA fragments, we decided to use 18S rRNA fragments (360 bp) for further analysis. As highlighted in the assay specifications section, the doubling time for the template becomes longer with longer template but the process of RPA is optimized as such the energy consumption rates are unaffected by template concentration which leads to premature fuel burn-out. Moreover, background amplification is in competition with specific amplification which leads to the formation of byproducts.

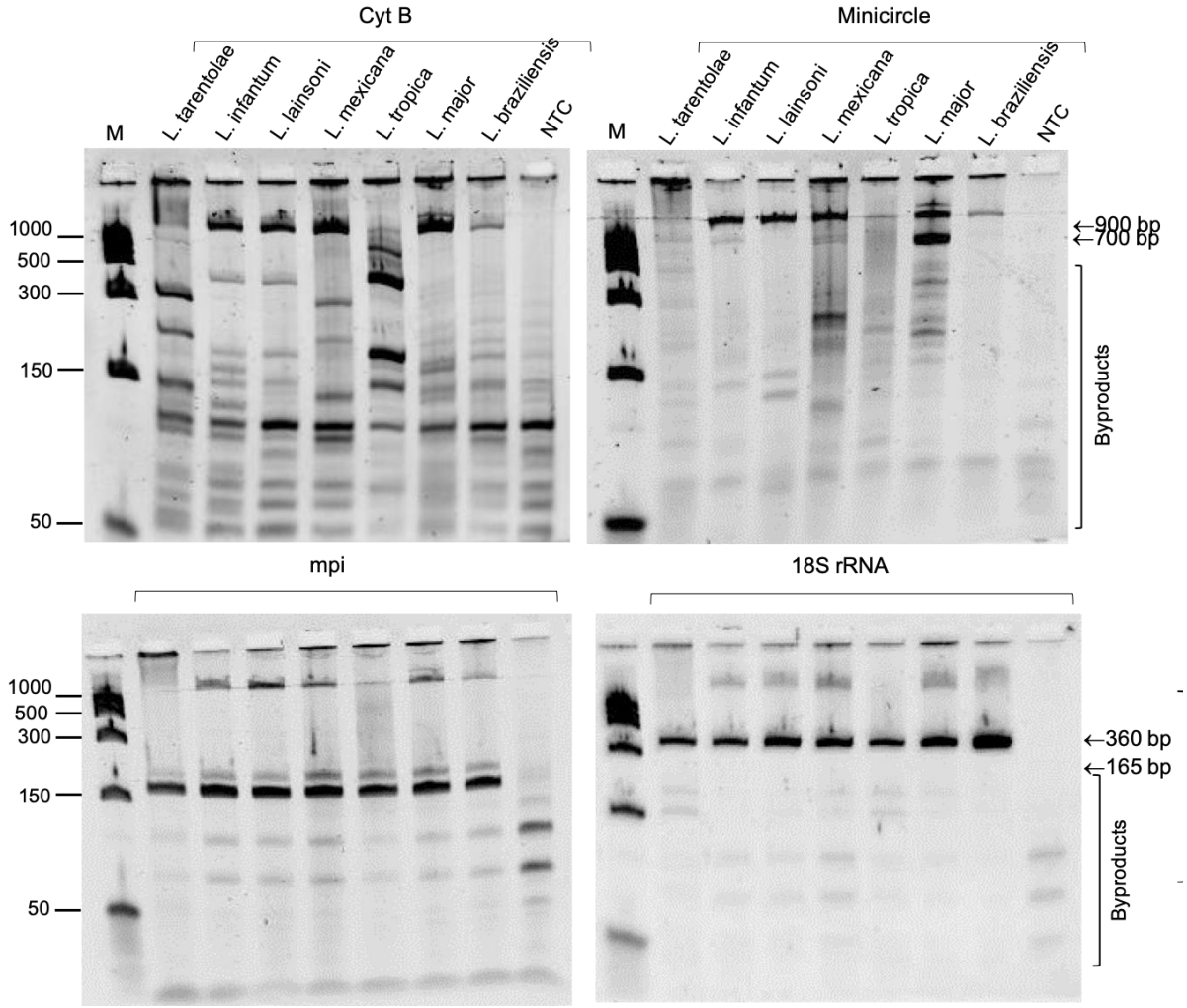


Figure 7: RPA was performed using genomic DNA from 7 species, namely, *L. tarentolae* (noninfective to humans), *L. infantum*, *L. lainsoni*, *L. mexicana*, *L. tropica*, *L. major* and *L. braziliensis*, with 240 nM each primer and a 15 minutes incubation time, followed by proteinase K treatment for all the 4 targets. All reaction products were next analyzed by the portable gel electrophoretic device.

2.6.2. RPA Assay optimization

Primer concentration and incubation time:

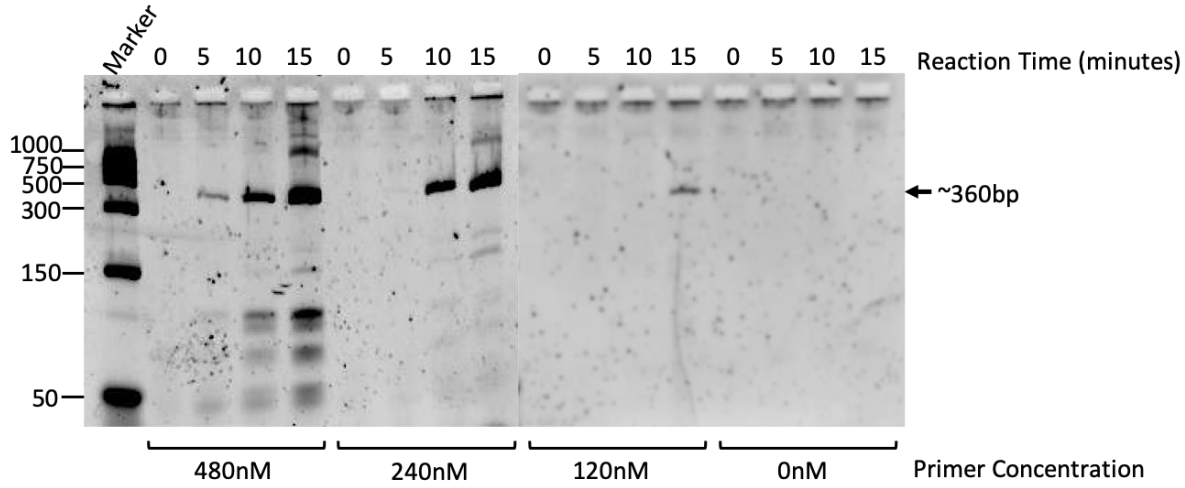


Figure 8: RPA reactions with incubation times ranging from 0 to 15 minutes were performed to amplify 18S rRNA gene fragments (360 bp) using primer concentrations of 480, 240, 120 and 0 nM. The amplification products were analyzed by a portable electrophoresis device after treatment with proteinase K.

Template concentration:

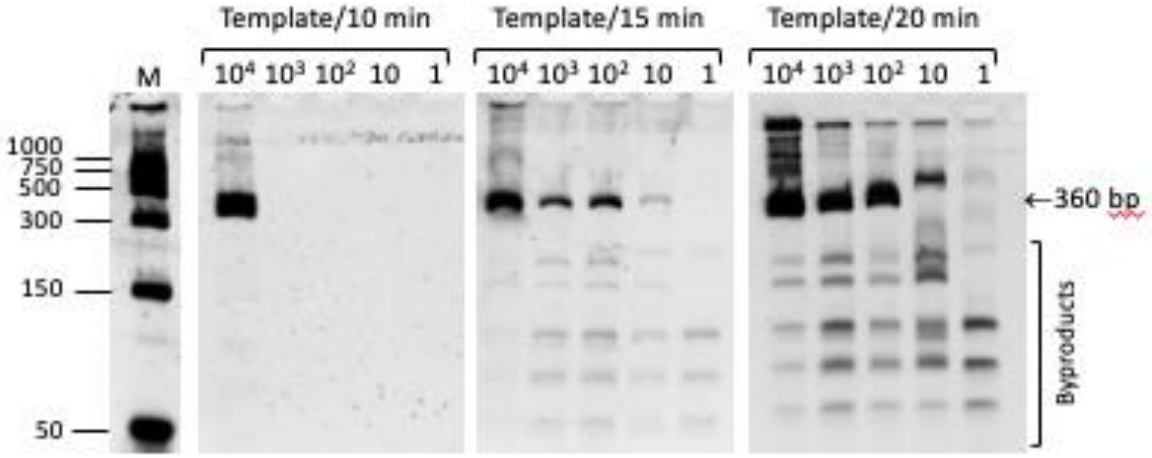


Figure 9: A sensitivity evaluation was performed for parasite copy numbers of *Leishmania major* ranging from 10^4 to 1 for different incubation times (10, 15, and 20 minutes). The amplification products were analyzed by a portable electrophoresis device after treatment with proteinase K.

Next, optimization of the primer concentration and time of the RPA reaction to amplify 18S rRNA was performed. As shown in Fig. 8, primer concentrations ranging from 480 nM to 0 nM were used in the RPA reaction, and the reaction was incubated for durations ranging from 0 to 15 minutes for each primer concentration. The RPA reaction products were electrophoresed for each condition. RPA was able to amplify the target gene in as few as 5

minutes when the primer concentration was 480 nM, but a similar intensity of nonspecific byproducts was also generated, which may result in false-positive signals. For 240 nM primer, a distinct target band of 360 bp was produced in just 10 minutes without any significant byproducts, which showed that the primer concentration in the RPA reaction was crucial. However, primer concentrations lower than 240 nM were not able to yield enough product even with longer reaction times. Next, 240 nM primer was used with a template ranging from 10^4 copies to 1 copy of the parasite and incubated for reaction times ranging from 10 to 20 min. As shown in Fig. 9, a clear band for the target gene, without any byproducts, could be obtained for higher template concentrations (10^4 copies) in as little as 10 minutes. However, the ratio of byproducts gradually increased during attempts to amplify target genes from lower template concentrations by increasing the reaction time from 10 to 20 minutes. This finding confirms that low-copy-number templates require longer reaction times to produce detectable amounts of the target genes and that the presence of byproducts can be clearly distinguished by utilizing our palm-sized electrophoretic device. Therefore, the optimization conditions for the RPA reaction vary according to the template concentration supplied in the reaction. Particularly when sufficient copies of DNA template are not available.

2.6.3. Demonstration of the optimized RPA Assay:

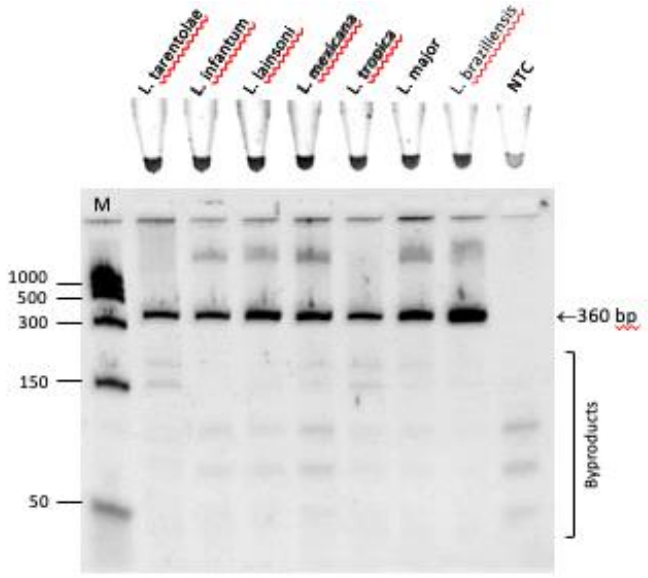


Figure 10: RPA was performed using genomic DNA from 7 species, namely, *L. tarentolae* (noninfective to humans), *L. infantum*, *L. lainsoni*, *L. mexicana*, *L. tropica*, *L. major* and *L. braziliensis*, with 240 nM each primer and a 15 minutes incubation time, followed by proteinase K treatment. The amplification products were mixed with 0.5X EvaGreen dye and visualized using a blue LED flashlight (upper panel). All reaction products were next analyzed by the portable gel electrophoretic device (bottom panel).

To evaluate the utility of this assay for the rapid detection of *Leishmania* species, genomic DNA from 7 species, namely, *L. tarentolae* (noninfective to humans), *L. infantum, L. lainsoni, L. mexicana, L. tropica, L. major* and *L. braziliensis*, was used to perform RPA reactions using primers targeting 18S rRNA. As shown in Fig. 10, successful amplification reaction signals for 18S rRNA were achieved for all seven species and were visually discriminated by both direct visualization of fluorescence and electrophoretic analysis. A very faint background fluorescence in the tube or an absence of the band representing the desired product in electrophoresis was observed for the no-template control (NTC) reaction. The gel electrophoresis analysis results revealed that the primers targeting the 18S rRNA target gene segment produced a distinct band of 360 bp in the case of all species of *Leishmania* regardless of whether they belonged to the subgenus *Leishmania* or *Viannia*. Therefore, the 18S rRNA target gene can be the target in RPA reactions to detect all forms of leishmaniasis caused by different species of *Leishmania*. However, this universal detection did not occur when the RPA reaction was performed to amplify cytochrome *b* (*cyt* b) or kDNA minicircle target genes as shown in Fig. 7. The electrophoresis results showed a high background of byproducts and/or low signals of target gene products.

2.7. Conclusion

The sensitivity of the RPA assay for different target sequences was evaluated to optimize the protocol for the detection of low amounts of template. In general, the RPA method results in the generation of short fragments (<150 bp) or highly branched long DNA products in addition to the desired target fragment. the amplification of minicircle sequences (700 bp), mpi (165 bp) and 18S rRNA (360 bp) were successful, but *cyt* b (900 bp) amplification was not. This failure can be attributed to the low copy number of the *cyt* b gene in the *Leishmania major* genome used in this reaction compared to that of the other targets. In addition, as shown in figure 7, since RPA and other isothermal methods are limited to amplification of discrete (i.e., a single DNA species, <150 bp) and long (>500 bp) DNA fragments, we decided to use 18S rRNA fragments (360 bp) for further analysis. A clear band for the target gene, without any byproducts, could be obtained for higher template concentrations (10^4 copies) in as little as 10 minutes. However, the ratio of byproducts gradually increased during attempts to amplify target genes from lower template concentrations by increasing the reaction time from 10 to 20 minutes. This finding confirms that low-copy-number templates require longer reaction times to produce detectable amounts of the target genes and that the presence of byproducts can be clearly distinguished by utilizing our palm-sized electrophoretic device. Therefore, the optimization conditions for the RPA reaction vary according to the template concentration supplied in the reaction. Particularly when sufficient copies of DNA template are not available. The gel electrophoresis analysis results revealed that the primers targeting the 18S rRNA target gene segment produced a distinct band of 360 bp in the case of all species of *Leishmania* regardless of whether they belonged to the subgenus *Leishmania* or *Viannia*. Therefore, the 18S rRNA target gene can be the target in RPA reactions to detect all forms of leishmaniasis caused by different species of *Leishmania*.

2.8. References

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3.Chapter 3: Design and demonstration of RPA for field applicable biosensing of leishmaniasis using portable gel electrophoretic system

3.1. Point-of-care diagnostics

Global efforts to combat the emerging infectious diseases are largely based on post-outbreak control, drug and vaccine development, as well as awareness campaigns[1]. However, lack of accessible diagnostics and time-taking and high-end available diagnostic tests and delays in the detection of newly emerging pathogens have resulted in high mortality rates and economic losses worldwide[2]. Routine healthcare check-ups are important for early stage diagnosis of the disease. This needs the development of systems to monitor healthcare regularly. Thus, the development of simple, affordable and sensitive point of care testing has become the need of the hour to bridge the gap between diagnostics and treatment of deadly diseases prevailing in today's world. The unavailability of health care resources mainly in the developing world leads to an ever increasing rate of spreading infection from one person to another. Point-of-care diagnostics i.e. medical diagnostic testing performed outside the clinical laboratory in close proximity to where the patient is receiving care are an excellent way to fight the epidemics faced by the world every now and then. The development of POCT need to be in consideration with the ASSURED (**A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free and **D**elivered to end user) criteria highlighted by World Health Organisation (WHO). However, due to the limitations highlighted in the preceding sections, conventional diagnostic methods such as microscopy, culture-based methods, immunologic tests, non-nucleic acid-based methods and PCR cannot be used in the manufacturing of ASSURED POCT devices. As highlighted in chapter 1, isothermal amplification methods require single temperature for target amplification and are best suited for the manufacturing of ASSURED POCT devices as they eliminate the need of equipment for temperature cycling making POCT devices accessible for resource poor settings such as in developing countries. These methods provide excellent specificity, sensitivity and simplicity to be used in the development of POCT devices for rapid and cost-effective health monitoring.

3.2. Current methods and challenges

Microscopic methods[3] such as gram stain, acid-fast and modified acid-fast stains, fluorescent stains, India ink stain, Warthin-Starry stain and Dieterle stain, Wright stain and Giemsa stain, Trichrome stain and iron haematoxylin stain, comprise microscopic examination of tissue to differentiate invasive disease from surface colonization but is limited to be done in certified laboratories[4] and often require highly expensive microscopes which in turn increases the cost of diagnosis. Culture based methods are a gold standard for the identification of organisms but

require days or even weeks to get the results which is enough for the pathogens to spread and infect a large population. Moreover, not all pathogens can be cultured, and sample collection is a burden for both patients and physicians. Specimen collection is the most important step to be considered such as for lesions, the leading edge and not the centre should be sampled, the use of the wrong type of swab can give rise to false-negative results and the component of the swab might be toxic to some types of pathogens, blood samples need to be taken from multiple sites simultaneously and some fungi[5], particularly molds cannot be cultured from blood[4]. Moreover special considerations should be taken care of for certain cultures such as anaerobic bacteria should not be cultured from sites where they are part of the normal flora and should be protected from air while transportation to the laboratory, mycobacteria[6] cultures take upto 8-12 weeks to grow, viruses should be inoculated onto tissue cultures within one hour of collection and some do not grow in routine viral cultures, and fungal specimens which take upto 3-4 weeks with antibacterial agents[7]. Therefore, alternatives such as immunologic tests, non-nucleic acid and nucleic acid-based identification tests are rapidly developing for point-of-care diagnostics[8] of infectious diseases. Immunologic tests use either antigen to detect antibodies to a pathogen or vice versa in the patient's specimen. Agglutination tests require the coupling of latex beads[9], gelatine particles or bacteria to an antigen or antibody and the resulting particle complex is mixed with the specimen and the agglutination or cross linking of the particles is measured[10]. Usually, these tests are rapid but less sensitive and the specimens need to be handled carefully to prevent contamination. In complement fixation tests, the specimen is incubated with known quantities of complement and the antigen and the degree of fixation relates to the quantity of the antibody such as IgG and IgM in the specimen[11]. These tests are usually accurate but are labour intensive and require numerous controls and handling procedures. Enzyme immunoassays such as enzyme-linked immunosorbent assay (ELISA)[12] use antibodies linked to enzymes to detect antigens and to detect and quantify antibodies. Although, the sensitivity of these assays is high, the results can vary according to factors such as patient age, microbial serotype, specimen type, and stage of clinical disease. Precipitation tests such as Ouchterlony double diffusion, counterimmuno-electrophoresis, etc., measure an antigen or antibody in liquid clinical samples by the degree of visible precipitation within a gel or in solution. The sensitivity is usually low and early detection is not possible due to the limitation of the amount of antigen or antibody present in patient specimens. Moreover, diseases such as cutaneous leishmaniasis have less or no antibodies present in the blood stream even if the disease is in progressive stage. Western blot tests and the technical modifications such as the line immunoassay (LIA), the recombinant immunoblot assay (RIBA) and

immunochromatographic assays detect antimicrobial antibodies in the patient's sample by their reaction with target antigens such as viral components, synthetic or recombinant-produced antigens immobilized onto a membrane to detect Shiga toxin-producing microorganisms, *Cryptococcus neoformans* capsular antigen and influenza virus[13]. These tests are less sensitive than ELISA and are therefore used for confirmatory purposes in addition to other tests and often pose the same limitations as other immunologic tests. Non-nucleic acid-based identification tests are mainly based on the identification of phenotypic traits such as colony size, colour and shape of pathogen's growth on culture media. These tests are often done sequentially, and the result of the previous test determines the next step. They may involve chromatographic methods where high-performance liquid chromatography (HPLC) or gas chromatography is used to separate and identify microbial components or products. Test accuracy is often dependent on specimen culture which may give rise to false-negatives. Another method is using mass spectrometry to detect various proteins in a specimen and is often used in detection of biological warfare and bioterrorism agents[14]. However, these tests require high-end mass spectrometers which limit the field applicability of this method. Nucleic acid-based identification tests are often rapid and are instrumental in choosing the correct treatment for the infection due to the rapidity, sensitivity and specificity of these tests. Molecular diagnostic tests are often independent of the type of pathogen and can be easily commercialised for a wide spectrum of pathogens just by changing one or two reagents in the kit[15]. These tests basically comprise of unamplified testing of nucleic acids[16] and tests based on nucleic acid amplification methods such as polymerase chain reaction (PCR). Unamplified testing[17] often requires pre-culture or the presence of the target sequences in high concentration in the specimen for instance, in infections caused by group A *Streptococcus*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*[18]. Nucleic acid amplification techniques, on the other hand, use small amounts of DNA/RNA and replicate them, thus avoiding the need of culture[19]. These tests may involve techniques for target amplification such as PCR[20], reverse transcriptase-PCR (RT-PCR), strand displacement amplification (SDA), transcription amplification, signal amplification such as branched DNA assays, capture of hybridised DNA, probe amplification such as ligase chain reaction, cleavase-invader, cycling probes, and postamplification analysis such as sequencing of the amplicon[21], microarray analysis[22] and melting curve analysis[23]. However, specimen should be transported in a low-temperature environment and have to be shielded from contamination.

3.3. Whatman FTA cards

FTA cards are proprietary paper-based storage platforms from GE Healthcare Life Sciences[24]. These cards enable easy collection and isolation of nucleic acids and are stable in extreme environmental conditions[25]. These cards preserve the nucleic acids from degradation at room temperature which is otherwise impossible for liquid clinical samples[26]. DNA is readily immobilized on the matrix and can be easily purified by washing with appropriate buffer in a single tube[27–30]. FTA card punches of 2mm are easy to handle and can be readily used for sampling of diagnostic tests for resource-poor settings. These cards can be used by virtually any type of cells such as blood, plasma, cultured cells, solid tissue, buccal cells, etc. The availability of these cards in two colours, white for blood and pink for other colourless samples facilitate the sample addition and distribution over the whole surface of the card. Genomic DNA stored for more than 17.5 years has successfully been used for PCR which highlights the long term preservation of clinical samples[31]. These cards have already been used for various applications such as genetic identification, animal breeding studies, molecular biology, transgenic identification, plasmid screening, drug discovery, genomics and whole genome amplification[32–39]. Recently, Nzelu and coworkers reported a diagnostic tool utilizing loop-mediated isothermal amplification (LAMP) combined with an approach utilizing an FTA card and colorimetric visual detection for rapid identification of cutaneous leishmaniasis[40]. Direct sampling via an FTA card reduces the risk of contamination and facilitates the transport and long-term storage of the sample at room temperature.

3.4. Objective

In chapter 2, the RPA assay has already been optimized and the versatility of the method is evaluated and it was found that the method can be used for the detection of all the forms of leishmaniasis. In this chapter, the aim is to use Whatman FTA card for the direct sampling of the RPA assay to eliminate sample contamination and tedious sample preparation steps and also use human body temperature as the source of incubation temperature for RPA. Moreover, a way to discriminate true-positive results from false-positive and/or negative results generated during the RPA reaction is explored for field applicable biosensing of leishmaniasis.

3.5. Materials and methods

3.5.1. RPA primers

Name	Sequence	Target (amplicon size)
RPA-L.rRNA-S	5'-TTCTCCACTCCAGACGGTGGGCAACCATCGT-3'	18S rRNA (360 bp)
RPA-L.rRNA-R	5'-AATGACAATCCAAATGGACTGACAACTCTA-3'	18S rRNA (360 bp)

Table 3: Primer sequences used for the RPA assay of target gene sequence of 18S rRNA.

3.5.2. FTA card preparation

FTA cards were prepared by applying the cultured parasites of a *L. major* strain to the cards (Whatman, Newton Center, MA, USA), and the cards were allowed to air dry overnight and were stored at room temperature. These cards were obtained from Prof. Hirotomo Kato, Jichi Medical University. For downstream applications, 2.0-mm-diameter discs were punched from each FTA card using a Harris micropunch tool (Whatman) and washed twice with an FTA purification reagent (Whatman) followed by another wash using TE buffer (1 M Tris and 0.5 M EDTA, pH 8) as and when required. The discs were then used directly as the DNA template for the positive (with template) RPA assay. Discs for the no-template control (NTC) reaction were punched from a new FTA card and used. When the parasite sample is transferred onto the FTA card, a uniform distribution of the sample over all areas of the card does not happen. Therefore, discs were punched from several locations on the FTA card to ensure a sufficient amount of template for the RPA reaction.

3.5.3. Reaction protocol

A set of forward and reverse primers was designed for RPA of the target genes- 18S rRNA. For RPA reactions using FTA discs, the reactions were prepared in a 50-µl reaction volume using a TwistAmp Basic kit (TwistDX, Cambridge, United Kingdom) consisting of 0.24 µM each of the primers, TwistAmp rehydration buffer, and RPA enzyme pellet and 14 mM magnesium acetate (MgAc). All reagents except magnesium acetate were prepared in a master

mix and instead of template DNA, 2.0-mm-diameter FTA discs were added in a 50-µl reaction and incubated for 15 minutes at 37℃. For RPA assays with multiple FTA discs, the reactions were scaled to 50 µl/disc. After the incubation was completed, proteinase K was added to the reaction and incubated for 10 minutes at 37℃ to stop the reaction by degrading the enzymes used in RPA.

3.6. Results and Discussion

3.6.1. Schematic of the strategy:

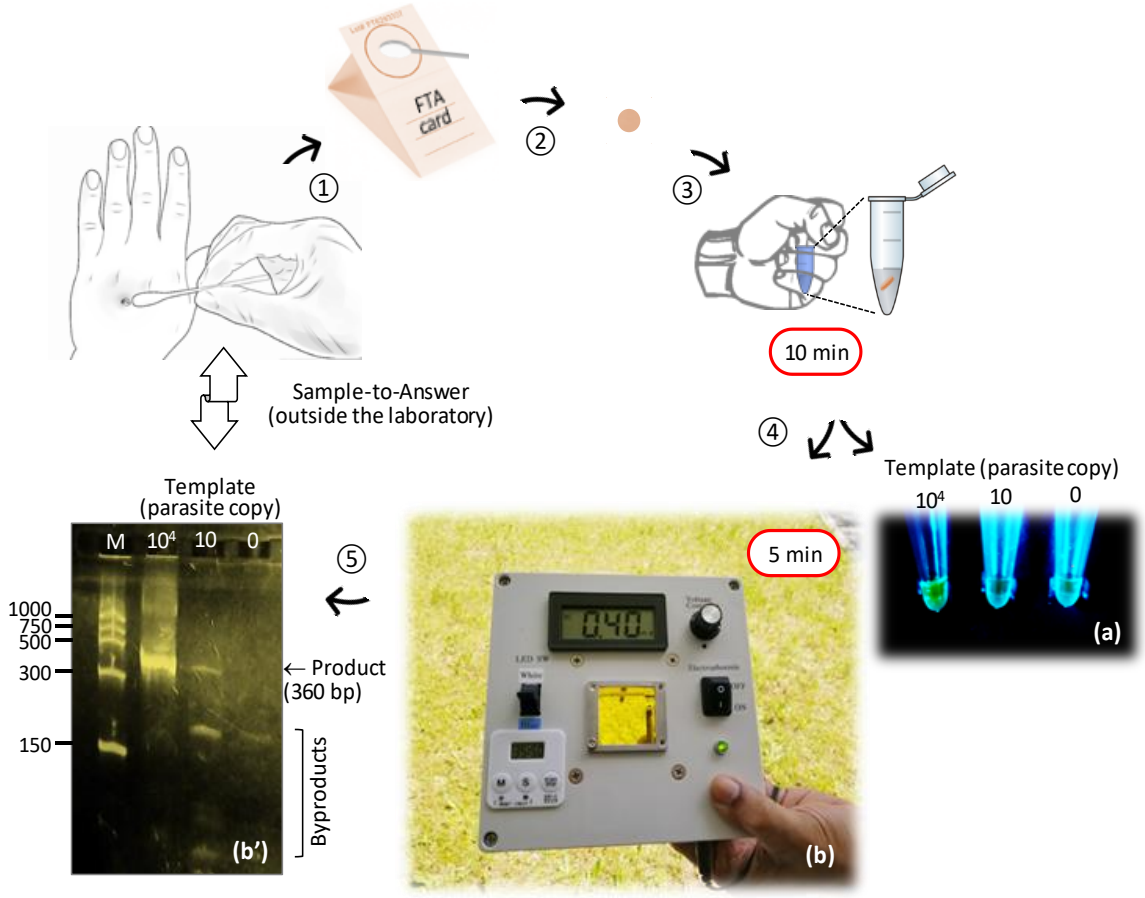

Figure 11: Schematic of near-to-patient nucleic acid-based molecular diagnostic tests. (a) Steps involved in the strategy as highlighted in the text. (b') For on-site gel electrophoresis-based genotyping, a handheld electrophoretic device is used to distinguish true-/false-positive results. (b) A photograph of a portable electrophoretic device is shown.

In this work, a way to discriminate true-positive results from false-positive and/or negative results generated during the RPA reaction is explored and an FTA card is used for direct sampling of RPA reactions to eliminate the concerns involved with sample contamination as well as the sample preparation steps. First, a suitable target sequence is selected for the detection of leishmaniasis using RPA and then the assay is optimized by evaluating various parameters. Then, RPA is performed for seven species, namely, *Leishmania tarentolae* (noninfective to humans), *Leishmania infantum*, *Leishmania mexicana*, *Leishmania tropica* and *Leishmania major*, belonging to the subgenus *Leishmania*, and *Leishmania lainsoni* and *Leishmania braziliensis*, belonging to the subgenus *Viannia*, targeting a 360-bp gene segment

of the 18S rRNA gene to evaluate the versatility of RPA to detect all the three forms of leishmaniasis. Next, a rapid gel electrophoretic analysis of RPA results is done using in-lab developed palm-sized gel electrophoretic device based on the formal micro-gel electrophoretic design to enable field applicable biosensing, particularly for resource-poor settings. The scheme for the field-applicable biosensing of leishmaniasis is highlighted in Fig. 11. First, the sample is obtained from the patient by aspirating/scraping the margins of active lesions, and the material is then spotted onto the FTA card and dried. Next, a 2.0-mm-diameter disc is punched from the FTA card and immersed in an Eppendorf tube containing liquid RPA reagents supplied with primers targeted to amplify the target gene fragment. This tube is then held in the palm for 10 minutes to provide the incubation temperature for RPA using body heat. EvaGreen dye is then added for direct detection of amplification, and fluorescence is detected using a simple blue LED-induced fluorescent detection component installed in the palm-sized electrophoretic device to distinguish positive and negative results. Although the process was simple, the weak true-positive signals amplified in the presence of a low template load (10 parasite copies, see Fig. 11a) could not be distinguished from a negative reaction (no parasite copies). In other words, false-positive signals that can be generated by off-target amplification during the RPA reaction mainly because of primer-primer interactions could not be distinguished with this approach. Therefore, to discriminate true-positive results from false-positive and/or negative results generated during the RPA reaction, a palm-sized electrophoretic device was designed, fabricated and used to perform a rapid gel electrophoretic analysis of RPA products. Fig. 11b) shows the gel electrophoretic analysis of the RPA reaction using the palm-sized electrophoretic device; RPA was performed using FTA cards with 10^4 parasite copies, 10 parasite copies and no parasite copies. The expected 360-bp band was clearly obtained with 10^4 copies. However, byproducts of smaller fragment sizes were also obtained in reactions containing 10 or no copies of template parasites, which could be clearly distinguished from weak true-positive signals using the handheld electrophoretic device.

3.6.2. FTA card washing optimization:

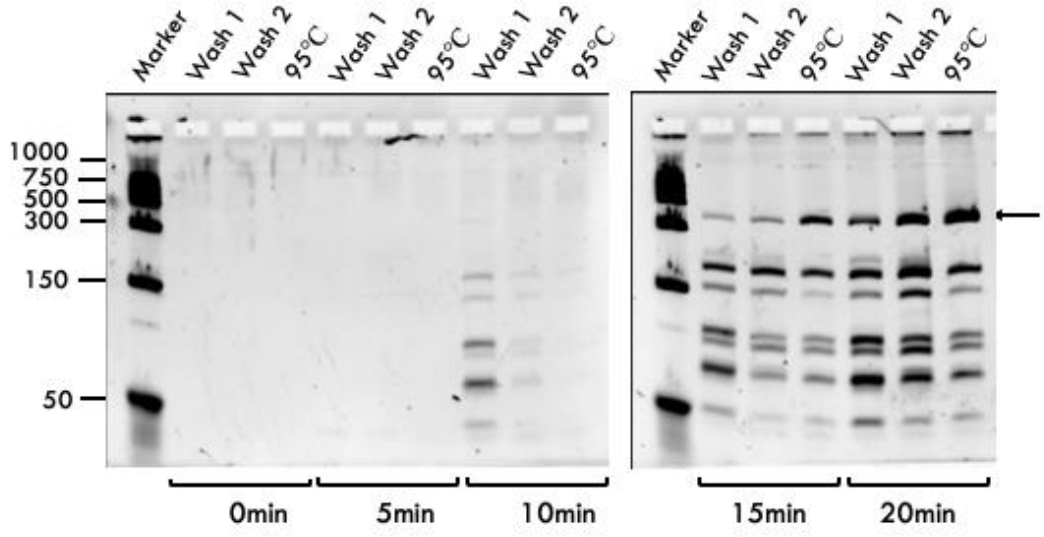

Figure 12: *L.major* 5ASKH culture was spotted onto the FTA card and three 2mm disks were punched. All the disks were washed with FTA purification reagent and 2 disks were then washed with 500ul TE buffer and one disk was heated at 95°C for 4min in 50ul TE buffer. Then, the FTA card disks were directly used for amplification using RPA for 0 to 20 min.

All the FTA disks were washed with FTA purification reagent (proprietary) and 2 disks were then washed with 500ul Tris-EDTA (TE) buffer and one disk was heated at 95°C for 4min in 50ul TE buffer. Then, the FTA disks were directly used for amplification using the optimized RPA assay to amplify 18S rRNA gene segment for 0 to 20 min. As shown in Fig. 12, it was found that in case of the FTA purification reagent might result in byproduct formation which can then compete with specific amplification. Moreover the byproducts decreased when the purification reagent was removed using TE buffer and heating further helps in maintaining the reaction rate of RPA. However, for the purpose of this work, heat equipment cannot be used in point-of-care settings therefore alternate method of chemical treatment was evaluated. As shown in Fig. 13, only wash 2, that is, removing the FTA purification reagent by TE buffer, yielded the specific band for RPA. Therefore, it was concluded that chemical denaturation inhibited the RPA reaction. This finding suggests that any remaining components of the FTA wash buffer might inhibit and/or decrease the yield of the RPA reaction.

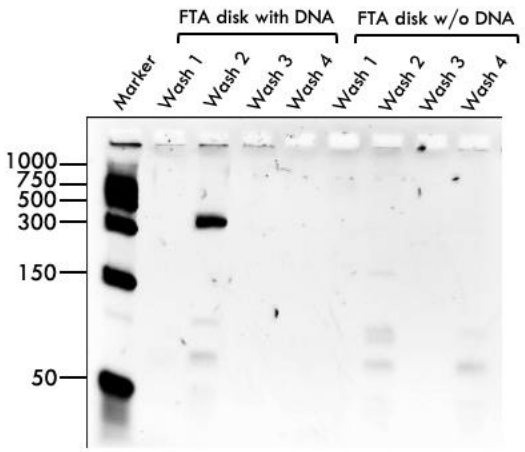

Figure 13: *L.major* 5ASKH culture was spotted onto the FTA card and four 2mm disks were punched. All the disks were washed with 50ul FTA purification reagent, 3 disks were then washed with 500ul TE buffer, 2 disks were then washed with 50ul 1M NaOH and one disk was washed with 500ul TE buffer. Then, the FTA card disks were directly used for amplification using RPA for 15 min.

3.6.3. Template distribution evaluation

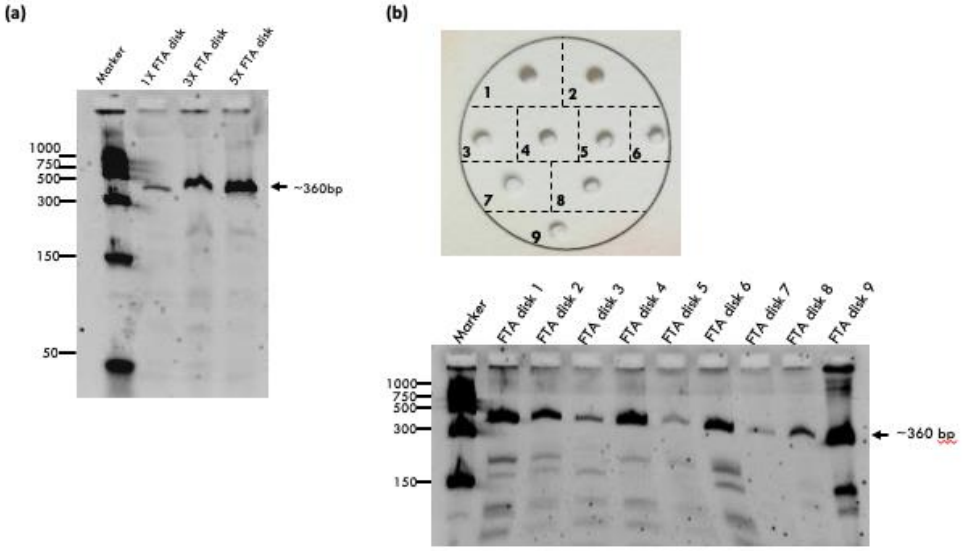

Figure 14: *L.major* 5ASKH culture was spotted onto the FTA card and 2.0-mm-diameter discs were punched at 9 different positions from areas as shown in (b). All the disks were washed with 50 µl FTA purification reagent, then 2 times with 500 µl TE buffer. Then, the FTA card disks were directly used for amplification using RPA for 15 min. Wash 1: Wash with FTA wash buffer (1X); Wash 2: Wash with FTA wash buffer (1X) and TE buffer (2X).

FTA cards provide a simple and field-applicable solution for collecting, preserving, and isolating parasite DNA samples for on-site downstream DNA analysis. However, the use of FTA cards as a source of parasite template DNA for the RPA reaction may be critical, since the parasite DNA is not evenly spread across the FTA matrix, and thus, the probability of

finding parasite DNA in a punch from an FTA card can be very low or very high. If enough template is not present in the RPA reaction, then there is a greater chance of nondesired amplification, mainly due to primer-primer interaction. Since the concentration of parasite template DNA in the FTA card punch is unknown, the effect of the template concentration and the possibility of inhibition of the RPA reaction by utilizing FTA cards as the template were also evaluated. Next, to assess the effect of the available template concentration on the FTA card, a number of 2.0-mm-diameter discs were punched at 9 different locations, and each disc was then used as a template for the RPA reaction. The gel electrophoresis analysis results revealed that the yield of the RPA reaction was not the same for discs punched from different locations. Therefore, we decided to use multiple FTA disks for the RPA reaction to supply enough template for amplification. A gradual increase in the band intensity corresponding to the 360-bp DNA fragment was observed with an increasing number of discs. This finding clearly indicated the need for a sensitive system to discriminate true-positive results from false-positive and/or negative results in case of a low template load in the reaction. Particularly when sufficient copies of DNA template are not available, such as for reactions with <10 parasite copies, which can result from using an FTA card as the template, it is advisable to incubate the RPA reaction for a longer duration (i.e., more than 15 minutes) to obtain a detectable amount of target DNA product.

3.7. Conclusion

A rapid, portable and reliable RPA detection platform was designed for a sample-to-answer nucleic acid test in resource-poor settings and demonstrated the detection of *Leishmania* species. The FTA card was used as the direct sampling tool and the washing optimizations suggested that FTA cards as a source of parasite template DNA for the RPA reaction may be critical, since the parasite DNA is not evenly spread across the FTA matrix, and thus, the probability of finding parasite DNA in a punch from an FTA card can be very low or very high. If enough template is not present in the RPA reaction, then there is a greater chance of non-desired amplification, mainly due to primer-primer interaction. Since the concentration of parasite template DNA in the FTA card punch is unknown, the effect of the template concentration and the possibility of inhibition of the RPA reaction by utilizing FTA cards as the template were also evaluated. First, the possibility of any inhibitory effect due to the composition of the FTA matrix on the RPA reaction was investigated, and an additional wash of the FTA card with TE buffer was observed to be necessary for a successful RPA reaction. This finding suggests that any remaining components of the FTA wash buffer might inhibit and/or decrease the yield of the RPA reaction. successfully used for direct sampling, DNA storage and RPA assays targeted at the 18S rRNA gene of a total of 7 species of *Leishmania*. The gel electrophoresis analysis results revealed that the yield of the RPA reaction was not the same for discs punched from different locations. Therefore, we decided to use multiple FTA disks for the RPA reaction to supply enough template for amplification. A gradual increase in the band intensity corresponding to the 360-bp DNA fragment was observed with an increasing number of discs. This finding clearly indicated the need for a sensitive system to discriminate true-positive results from false-positive and/or negative results in case of a low template load in the reaction. The described approach can be readily used for nucleic acid testing outside the laboratory, with as little as 15 minutes to a yes/no answer, to guide treatment decisions in patients with cutaneous leishmaniasis.

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4. Chapter 4: Rapid temperature gradient gel electrophoretic (TGGE)-typing of Leishmania species for subtype identification

4.1. Species identification of *Leishmania*

As highlighted in figure 5, different forms of leishmaniasis are caused by different subtypes of the parasite. Moreover, the same subtype causes a different form on the disease in two geographical regions, for instance, CL is caused by *L.major*, *L.tropica* or *L.infantum* in southern Mediterranean Basin, while in South America, it is caused by *L.mexicana* and *L.amazonensis* as well as the species of the subgenus *L.(Viannia)*. Moreover, species identification is crucial to differentiate in cases of *Leishmania* species and lower trypanosomatids which are also considered to cause VL in Southern Europe, South America and in the Indian subcontinent[1]. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis which affects near 1.5 million patients every year [2]. CL is caused by several *Leishmania* species that are associated with a wide spectrum of clinical forms and so thus with variable outcomes before and after therapy. For example, *L. major* and *L. mexicana* are associated with frequent spontaneous cure [3] but *L. braziliensis* and *L. guyanensis* are associated with risk of delayed mucosal metastatis [4]. Therefore, considering the variable severity of CL, identification of the infecting *Leishmania* species for species-oriented clinical prognosis and treatment are of utmost clinical importance [5]. The next challenge is obviously to access the outside laboratory facilities that enables to identify type/subtypes of *Leishmania* species near-to-patient and in low-resource settings. Since, it is impossible to differentiate the *Leishmania* species microscopically; the identification requires molecular typing methods for neglected tropical diseases (NTDs) [6]. In this stream, Rapid Diagnostic Tests (RDTs) are promising tools to improve diagnosis of NTDs in remote health centre[7,8]. However, the conventional immunochromatographic-based RDTs do not give information on the infecting species/genotypes that are of epidemiological importance. In addition, serodiagnosis is not effective in several NTDs such as CL due to low antibody responses. On the other hand, DNA-based molecular typing method which is the 'gold standard' are generally performed by two major steps: DNA amplification by PCR followed by sequencing of a targeted conserved gene regions such as *hsp70* genes [9], cytochrome *b* (*cyt* b) [10] in case of typing *Leishmania* species [11]. Although these methods are highly reliable but unsuited for implementation at primary or secondary health-care facilities due to the obvious requirements of highly sophisticated laboratory and trained personnel. An alternative of conventional PCR by adaptation of existing isothermal amplification technologies could be a smart solution to perform DNA amplification outside laboratory settings [12], therefore, in this section RPA is evaluated for specific

discrimination of sequence mismatches using micro-temperature gradient gel electrophoresis for subtype differentiation.

4.2. Micro-temperature gradient gel electrophoresis

Many groups have used random-PCR based strategies with targets such as kinetoplast DNA minicircle[13], small subunit ribosomal RNA[14], mini exon gene[15], hsp70 genes[9], cytochrome B gene[10], etc. for *Leishmania* species detection as well as sequencing and are often combined with molecular techniques such as Restriction Fragment Length Polymorphism (RFLP) or DNA sequencing for highly sensitive and specific detection[16,17]. RFLP patterns are obtained by subjecting amplified DNA to restriction enzymes and analysis using gel electrophoresis. However, dendrograms obtained using these strategies are based on one-dimensional (1D) information such as size of the DNA fragment thus limiting the specificity of the obtained dendrograms. In addition, almost identical RFLP patterns are obtained with different species such as *L.donovani, L.infantum, L.braziliensis, L.guyanensis, L.panamensis, L.peruviana,* etc. and render inadequate for subtype level identification. Temperature gradient gel electrophoresis (TGGE) is a well established method to differentiate between different species where PCR amplified DNA is subjected to denaturing polyacrylamide gel electrophoresis with a suitable temperature gradient to obtain melting profiles based on two-dimensional information such as size and sequence of the DNA fragment. However, conventional TGGE is time taking and involves complex procedures and equipment, and therefore, is limited to be used in point-of-care settings. Another alternative, Micro Temperature Gradient Gel Electrophoresis (μTGGE), which has already been established as a rapid and cost-effective method for TGGE analysis, has been shown to be of hundred-fold performance by attaining size minimization of the apparatus [18]. The minimized setup is able to perform TGGE with comparable performance in less than 15 minutes for each sample. Here, we utilise RPA and micro temperature gradient gel electrophoresis for portable, affordable and reliable DNA fingerprinting. If the infection is confirmed by near-to-patient diagnostic method proposed in chapter 3 by observing the target band in the sample lane, the sample is subjected to secondary screening using micro-TGGE for species analysis and the melting profiles are subjected to computer aided normalization and the pattern similarity scores are used to plot the dendrograms which are compared with the standard plots to confirm the species causing the infection, the data can then be further submitted to public database for central monitoring for further planning and awareness. Figure shows the principle of micro-temperature gradient gel electrophoresis, where, DNA is analysed based on two dimensions: x-axis, i.e., electrophoretic migration axis

for the size-based separtion of DNA and y-axis, i.e., temperature gradient axis for the sequence-based separation of DNA. Structural changes of partially melted DNA under a temperature gradient (from T_L= 20°C to T_H= 65°C) are based on the sequence composition of the analysed DNA as all the four bases have different melting points.

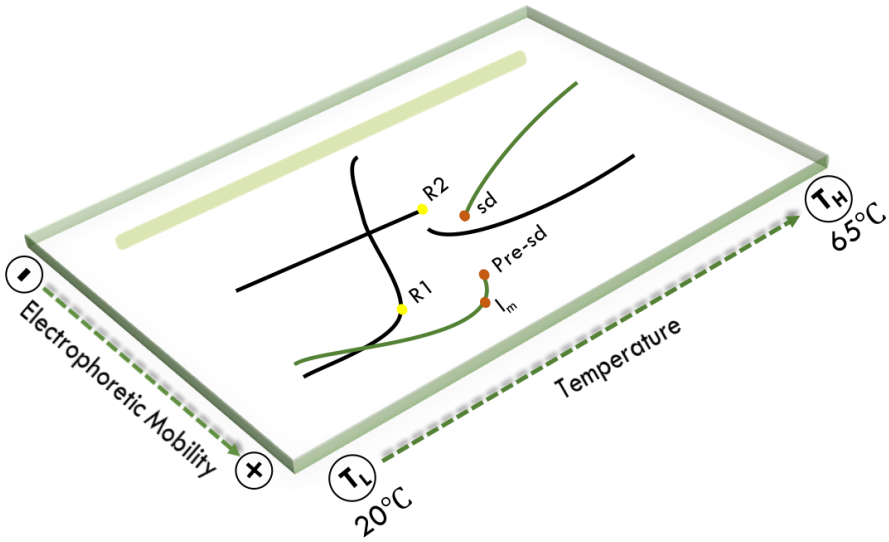

Figure 15: Principle for the basis of separation of DNA sequences based on their melting temperature using micro-TGGE.

This can be used to detect single base sequence mismatches and can act as a tool for species level detection. This structural change can be characterized by the electrophoretic mobility of DNA in the gel which is based on the size of DNA. The DNA is electrophoresed under denaturing condition and temperature gradient T_L to T_H where it passes through the polyacrylamide gel sieves in vertical direction based on size and in horizontal direction based on the melting temperature. The melting profiles obtained are normalized using internal references (R_1 and R_2) and species identification dots are assigned to calculate pattern similarity scores (*PaSS*) which can facilitate as a measure of the genomic distance between two species [19]. The *PaSS* values obtained through computer-aided normalization can then be used to plot the dendrograms for the phylogenetic analysis of the target species.

4.3. Objective

In this chapter, species level identification is performed for the differentiation of differet Leishmania subtypes based on the melting of the target sequence under a temperature gradient using a miniaturized TGGE system. Being conserved in all the subtypes, 18S rRNA gene target cannot be used for subtype identification, therefore, another target heat shock protein 70 (hsp70) is evaluated and the RPA assay is first optimized for this target. Then the obtained amplicons are subjected to micro-TGGE to produce specific melting profiles. Then species identification dots are assigned to the profiles and the Pattern Similarity Scores (PaSS) values are calculated to obtain a dendrogram plot.

4.4. Materials and Methods

4.4.1. Primers

The two sets of primers used are highlighted:

Primer RL_1	5'TCCTGGTTGCTGTTCAGCCACTCCAGCGCC3'
Primer RS_1	5'TGCTCGTACTCTTCCTTCG3'
Primer FL_2	5'TCGAAGTACGAGCAGGCCGACAAGATGCAGCGC3'
Primer FS_1	5'CATATCACCATCACCAACG3'

Table 4: Primer sequences used for the RPA assay of hsp70 where F is forward, R is reverse, L is long, and S is short.

4.4.2. RPA Protocol

Genomic DNA samples were extracted from 8 species of *Leishmania*, namely, *L. braziliensis* INH03, *L. mexicana* PT103 , *L. donovani*, *L. guyanensis*, *L. major* 5ASKH, *L. lainsoni* M6426, *L. peruviana* LC26 and *L. braziliensis* LC53. The target DNA was amplified using either RPA or conventional PCR and used for primary and secondary screening. Rehydration solution was prepared using 29.5µl rehydration buffer, 240nM forward (5'CATATCACCATCACCAACG3') and reverse primer (5'TGCTCGTACTCTTCCTTCG3') each, 2ng/µl target DNA (genomic DNA from *Leishmania* species) and nuclease free water upto 47.5µl. The prepared rehydration solution was used to rehydrate the lyophilised RPA pellet and the reaction was started by adding 2.5µL magnesium acetate. The reaction was incubated at 37°C for 10 minutes. Next, 3mAU Proteinase K was added to the reaction and incubated at 37°C for another 10 minutes.

4.4.3. Micro-gel electrophoresis

10-well Micro-casettes with 6% T Polyacrylamide gel were prepared using 40(w/v)%-Acrylamide/Bis (19:1) (Nacalai Tesque), 5X TBE, 10% Ammonium Persulfate (Wako Pure Chemicals) and Tetramethylethylenediamine (Wako Pure Chemicals). 1.5µl of each sample was loaded in the micro-gel wells with 0.5µl of loading dye. A 50bp DNA ladder was also loaded in one well to confirm the size of the obtained DNA fragments and was co-migrated

with the samples at 100V for 5 mins. The gel was stained with 10X SYBR Gold nucleic acid gel stain (Thermo Fisher Scientific) and visualized using UV transilluminator.

4.4.4. Micro-temperature gradient gel electrophoresis

Single-well Micro-casettes with 6% T Polyacrylamide gel were prepared using 40(w/v)%-Acrylamide/Bis (19:1) (Nacalai Tesque), 5X TBE, 10% Ammonium Persulfate (Wako Pure Chemicals) and Tetramethylethylenediamine (Wako Pure Chemicals). 2µl each internal reference DNAs (600bp and 200bp)[9] were co-migrated with 2µL RPA product at 100V for 13 minutes at a linear temperature gradient from 20°C-65°C in 1X TBE buffer. The gel was stained with 10X SYBR Gold nucleic acid gel stain (Thermo Fisher Scientific) and visualized using UV transilluminator. Next, computer-aided normalization was performed by assigning species identification dots (spiddos) derived from featuring points (where structural transitions occur) [19]. Pattern similarity scores (PaSS) were calculated using MicroTGGE analyser software and the PaSS values were used to plot the dendrograms for subtype level identification.

4.5. Results and Discussion

4.5.1. RPA Assay optimization:

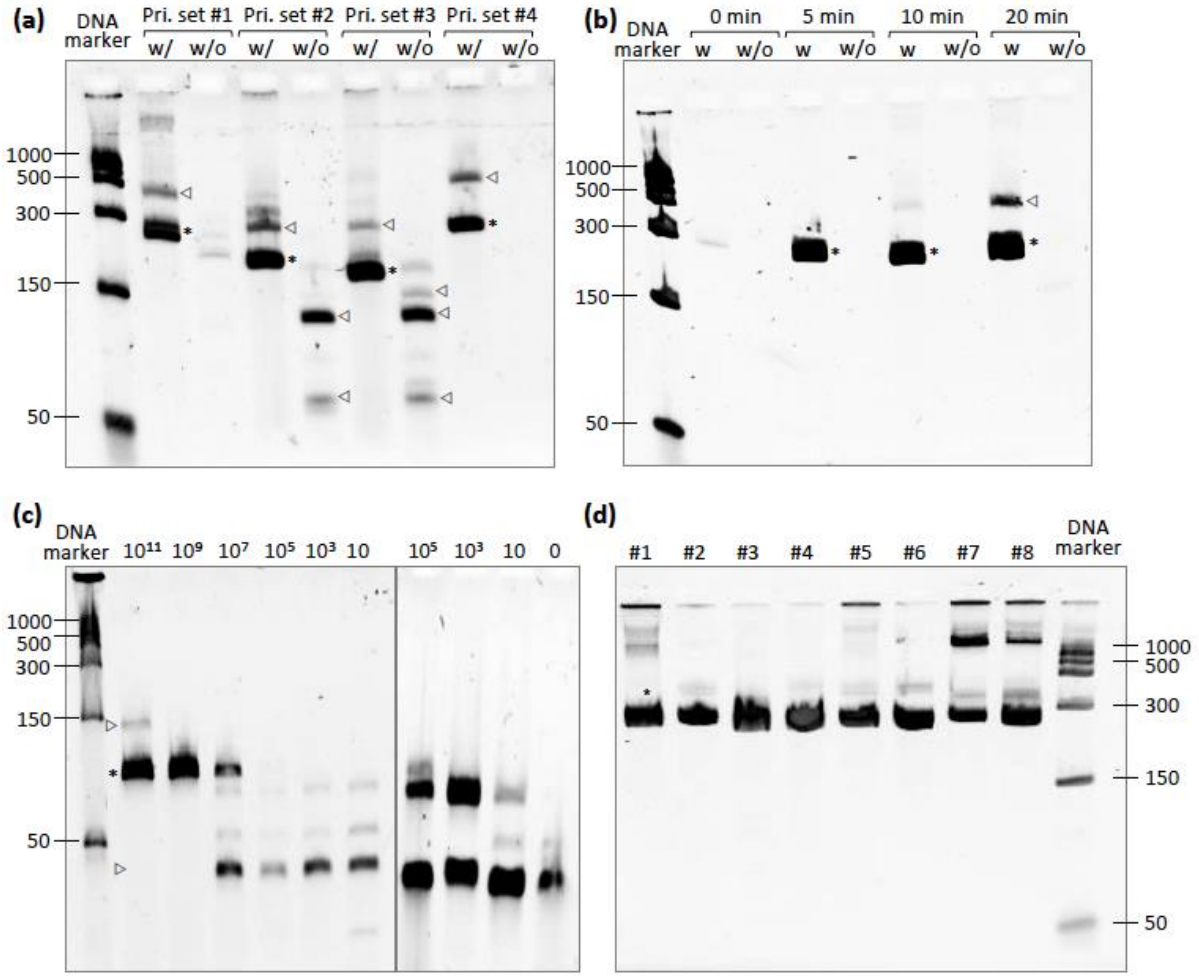

Figure 16: Primer/target directed non-specific products; *- Target band, RPA reaction performed for: (a) Primer length dependence- Set 1: RL_1, FS_1; Set 2: RL_2, FS_1; Set 3: FL_1, RL_1; Set 4: RS_1, FS_1. (b) Reaction incubation time dependence- for 0, 5, 10 and 20 minutes at 37°C. (c) Target copy dependence: ranging from 10^{11}-10 copies with 10 minutes incubation for 10^{11}-10^7 copies and 20 minutes for 10^5-10 copies. (d) RPA performed using the optimized protocol for: 1- *L.braziliensis* INH03, *L.mexicana* PT103, *L.donovani*, *L.guyanensis*, *L.major* 5ASKH, *L.lainsoni* M6426, *L.peruviana* LC26, *L.braziliensis* LC53.

RPA, being highly sensitive to non-specific amplification, needs to be optimized using various factors to avoid false positives and to amplify the DNA rapidly. Therefore, the effect of primer length, reaction time and target concentration was evaluated to demonstrate a protocol to amplify *hsp70* gene fragment from 8 *Leishmania* species specifically. Figure 16a shows the gel electrophoretic analysis of the RPA reactions performed using primer sets of different lengths: longer primers that target to amplify a fragment of 191 bp and shorter primers to amplify a fragment of 286 bp. For optimization, 4 sets are used with a combination of each

longer and shorter primers: set #1, a long reverse primer (LR, 30bp) and a short forward primer (SF, 19bp); set #2, a long forward primer (LF, 33bp) is used with a short reverse primer (SR, 19bp); set #3, LR and LF; set #4, SR and SF. An intense band was observed in all positive reactions using all combination of primer set. However, non-desired band was observed in negative reactions in the case of set #1, #2, and #3. Based on these observations, it can be concluded that, non-specific amplification in RPA is highly dependent upon the primer length. For instance, in the case where both primers were long, non-specific products are found to increase as compared to the case where one of the primers is long. In the other case, where both the primers are short, the non-specific amplification is negligible in both positive and negative reactions. This can be due to the decreased tendency of shorter primers to form primer-primer interactions and result in primer-dimer formation. Therefore, further experiments were performed using primer set #4. Figure 16b shows the electrophoretic analysis of RPA reactions performed for different reaction incubation time. It is shown that amplification in RPA starts as soon as the reaction is incubated at 37°C and detectable target concentration is obtained in as short as 5 min. Increasing the reaction incubation time to more than 10 min can give rise to non-specific amplification in addition to the target band. Figure 16c depicts the sensitivity of the RPA reaction. Different template copies were used to perform the RPA reaction and it was found that RPA can amplify as low as 10^3 copies of the target. Although, it was found that sufficient target concentration is needed to amplify the target rapidly and without non-specific amplification. For 10 min incubation time, 10^9 copies of DNA are required to amplify the target fragment without non-specific amplification. Figure 16d shows the electrophoretic analysis of RPA performed using the optimized conditions of 8 *Leishmania* species. The positive reactions (with template) show a clear band of the same length (280bp) in all the cases. This rapid electrophoretic analysis using hsp70 as target can also be used to discriminate between true positives and false positives and/or negative results, although, the copies of hsp70 genes in the parasite are low which results in false negative results sometimes. Therefore, it is advised that conventional PCR be used as the first step to get enough copies of the target and then RPA assay can be used to amplify highly mismatched sequences.

4.5.2. Subtype identification:

RPA products obtained from all the eight species was subjected to μTGGE analysis. In micro-TGGE, DNA is analysed based on two dimensions: x-axis, i.e., electrophoretic migration axis for the size-based separtion of DNA and y-axis, i.e., temperature gradient axis for the sequence-

based separation of DNA. Structural changes of partially melted DNA under a temperature gradient (from T_L= 20°C to T_H= 65°C) are based on the sequence composition of the analysed DNA as all the four bases have different melting points. This can be used to detect single base sequence mismatches and can act as a tool for species level detection. This structural change can be characterized by the electrophoretic mobility of DNA in the gel which is based on the size of DNA. The DNA is electrophoresed under denaturing condition and temperature gradient T_L to T_H where it passes through the polyacrylamide gel sieves in vertical direction based on size and in horizontal direction based on the melting temperature. The melting profiles obtained are normalized using internal references (R_1 and R_2) and species identification dots are assigned to calculate pattern similarity scores (*PaSS*) which can facilitate as a measure of the genomic distance between two species [19]. The *PaSS* values obtained through computer-aided normalization can then be used to plot the dendrograms for the phylogenetic analysis of the target species. The melting profiles generated from all the species are shown in Fig. 17. The featuring points (I_m, I_{sd}, sd) correspond to the structural transitions of DNA with temperature and mobility. These spiddos were normalized by using common internal references, the coordinates of which were set to be equal to work out the *PaSS* values based on initial melting of the dsDNA (I_m), pre-strand dissociation (I_{sd}) and strand dissociation (sd) using uTGGE analyser software. For instance, I_m in the case of *L.braziliensis* INH03 was reached earlier (at lower temperature) as compared to all the other species. I_{sd} for *L. mexicana* PT103 and *L. guyanensis* is higher as compared to the other species which sheds light onto the higher stability of DNA obtained from these species. The stability of DNA is based on the sequence composition, thus sequence mismatches can be used to differentiate between species. Using the normalized coordinates, *PaSS* were obtained. *PaSS* value of unity signifies the species is identical to the target species and less than 1 signifies the genome distance between the two species. For instance, *L. donovani, L. peruviana, L. guyanensis and L. lainsoni* having PaSS= 0.97, 0.98, 0.98, 0.99 respectively are closely related to *L. braziliensis* INH03 as compared to *L. mexicana* and *L. major* having PaSS= 0.93 and 0.95 respectively. Thus, using the melting profiles and the spiddos, the *PaSS* values were obtained and used to construct a phylogenetic tree as shown in Fig. 17. The phylogenetic tree produced by RPA/TGGE resulted in all the eight species being grouped in two clusters, one with *L. mexicana* PT103 and *L. major* 5ASKH, and the rest are in another.

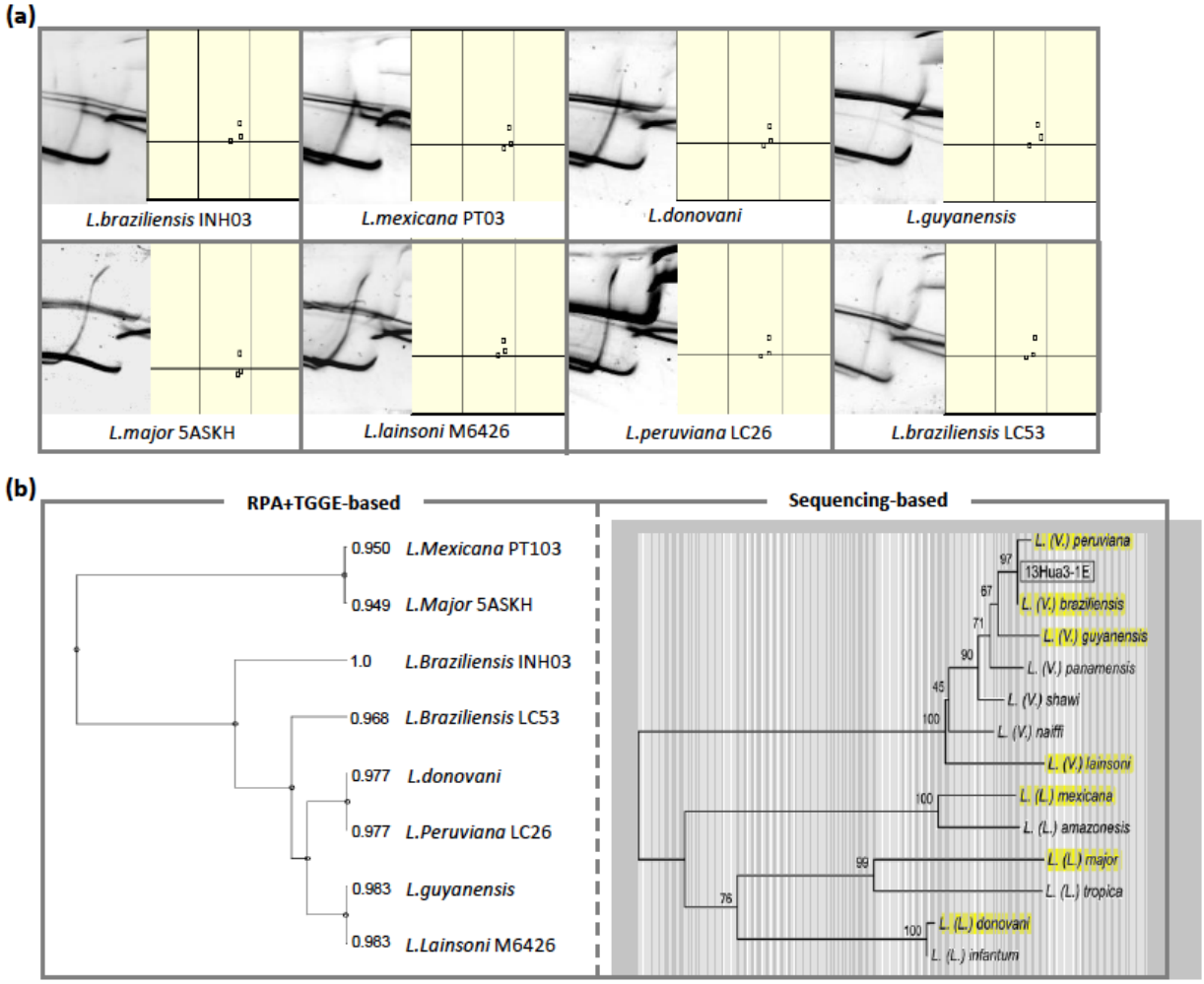

Figure 17: Micro-TGGE analysis of all the eight species of leishmania using RPA products obtained by amplifying hsp70 gene fragment: (a) Melting profiles generated from all the eight species and the assigned spiddos after computer-aided normalization. (b) Pattern Similarity scores generated for all the eight species and the dendrogram plotted using the PaSS values, inset: previously published data for species analysis of leishmania using cytochrome B gene.

4.6. Conclusion

This chapter proposes a method to identify and differentiate the different subtypes of *Leishmania* parasite using hsp70 as the target sequence. It presents an advanced method to differentiate between the clinical forms of leishmaniasis. Hsp70 is one of the non-conserved region in the parasite and has been used for species level identification in previous researches. Therefore, first, the RPA assay for hsp70 is optimized according to various parameters such as primer length, primer concentration, incubation time and template concentration. Based on the above observations, it can be concluded that, non-specific amplification in RPA is highly dependent upon the primer length. For instance, in the case where both primers were long, non-specific products are found to increase as compared to the case where one of the primers is long. In the other case, where both the primers are short, the non-specific amplification is negligible in both positive and negative reactions. This can be due to the decreased tendency of shorter primers to form primer-primer interactions and result in primer-dimer formation. Therefore, further experiments were performed using primer set #4. The background amplification was found to increase with increasing primer concentration and time. However, in cases where, sufficient template is not available, longer incubation times are advised. Moreover, the stability of DNA is based on the sequence composition, thus sequence mismatches can be used to differentiate between species. Using the normalized coordinates, *PaSS* were obtained. *PaSS* value of unity signifies the species is identical to the target species and less than 1 signifies the genome distance between the two species. For instance, *L. donovani, L. peruviana, L. guyanensis and L. lainsoni* having PaSS= 0.97, 0.98, 0.98, 0.99 respectively are closely related to *L. braziliensis* INH03 as compared to *L. mexicana* and *L. major* having PaSS= 0.93 and 0.95 respectively. Thus, using the melting profiles and the spiddos, the *PaSS* values were obtained and used to construct a phylogenetic tree. The phylogenetic tree produced by RPA/TGGE resulted in all the eight species being grouped in two clusters, one with *L. mexicana* PT103 and *L. major* 5ASKH, and the rest are in another. These results encouraged us to use a combination of RPA and TGGE analsis for rapid typing of *Leishmania* species in field for point-of-care testing.

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5. Chapter 5: Summary and Future prospects

5.1. Summary

5.1.1. Chapter 1 highlights the general introduction of the terms and technical know-how for this research work. The available diagnostics such as microscopic examination, culture-based examination, immunologic tests, molecular diagnostic tests such as conventional polymerase chain reaction and isothermal amplification techniques have been summarised. The next section highlights the importance and wide applicability of RPA in point-of-care diagnostics, for instance in resource-poor settings. Then, the purpose of this research has been explained for the primary and secondary screening of leishmaniasis.

5.1.2. Chapter 2 highlights the epidemiology and the available diagnostic tools for leishmaniasis are discussed. Then, the protocol for the amplification of the target for the detection of leishmaniasis is optimized and various parameters of the RPA assay are evaluated accordingly. The RPA assay is depends on various parameters such as primer concentration, incubation time, template concentration and primer design. Therefore the optimum mix of these factors is needed to get maximum amplification from RPA. Template concentration and length is a crucial factor as low template amounts and longer templates may cause premature fuel burnout of the RPA reagents.

5.1.3. In Chapter 3, a rapid, portable and reliable RPA detection platform was designed for a sample-to-answer nucleic acid test in resource-poor settings and demonstrated the detection of *Leishmania* species. The FTA card was used as the direct sampling tool and the washing optimizations suggested that FTA cards as a source of parasite template DNA for the RPA reaction may be critical, since the parasite DNA is not evenly spread across the FTA matrix, and thus, the probability of finding parasite DNA in a punch from an FTA card can be very low or very high. This finding clearly indicated the need for a sensitive system to discriminate true-positive results from false-positive and/or negative results in case of a low template load in the reaction. The described approach can be readily used for nucleic acid testing outside the laboratory, with as little as 15 minutes to a yes/no answer, to guide treatment decisions in patients with cutaneous leishmaniasis.

5.1.4. Chapter 4 proposes a method to identify and differentiate the different subtypes of *Leishmania* parasite using hsp70 as the target sequence. It presents an advanced

method to differentiate between the clinical forms of leishmaniasis. The melting profiles from micro-TGGE, the spiddos and the *PaSS* values were obtained and used to construct a phylogenetic tree. The phylogenetic tree produced by RPA/TGGE resulted in all the eight species being grouped in two clusters, one with *L. mexicana* PT103 and *L. major* 5ASKH, and the rest are in another. These results encouraged us to use a combination of RPA and TGGE analsis for rapid typing of *Leishmania* species in field for point-of-care testing.

5.2. Future Prospects

In the future, the methods proposed in this research will be evaluated for clinical samples directly for near to patient diagnosis of leishmaniasis. Being a general method and just the need to change the primers for RPA are advantageous as this approach can easily be used for other targets. Moreover, the RPA process is still in development and in the future, various improvements in the reaction will be proposed and demonstrated. Subtype differentiation using RPA is in its initial stages, therefore a lot of work has to be done to collaborate for the development of an automated software for the assigning of species identification dots and the generation of pattern similarity scores. Although, this time the primers were designed using conserved regions in the target gene, in the future, primers will be designed from the non-conserved regions of the target which will help in separating the sequences specifically due to the mismatches. The micro-TGGE protocol still needs some improvement in buffer and gel considerations as well as parameters such as gel percentage, time and gradient temperature.