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Molecular diagnosis of protozoan parasites by Recombinase Polymerase Amplification

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Abstract

Infections caused by protozoan parasites affect millions of people around the world. Traditionally, diagnosis was made by microscopy, which is insensitive and in some cases not specific. Molecular methods are highly sensitive and specific, but equipment costs and personnel training limit its availability only to specialized centers, usually far from populations with the highest risk of infection. Inexpensive methods that can be applied at the point of care (POC), especially in places with limited health infrastructure, would be a major advantage. Isothermal amplification of nucleic acids does not require thermocyclers and is relatively inexpensive and easy to implement. Among isothermal methods, recombinase polymerase amplification (RPA) is sensitive and potentially applicable at POC. We and others have developed RPA diagnostic tests to detect protozoan parasites of medical importance. Overall, our results have shown high specificity with limits of detection similar to PCR. Currently, the optimization of RPA for use at the POC is under development, and in the near future the tests should become available to detect protozoan infections in the field. In this review we discuss the current status, challenges, and future of RPA in the field of molecular diagnosis of protozoan parasites.

Keywords

Isothermal amplification; RPA; recombinase amplification; Molecular diagnostic; Protozoan

Introduction

Diagnosis of protozoan parasites

Infections by protozoan parasites affect hundreds of millions of individuals around the world. Intestinal parasites of the genus *Cryptosporidium* (*C. hominis* and *C. parvum*), *Giardia* (*G. duodenalis*), and *Entamoeba histolytica* cause diarrhea in humans¹. Blood and tissue protozoa of major clinical significance include members of genera *Trypanosoma* (*T. brucei* and *T. cruzi*), *Leishmania* (*L. donovani*, *L. major*, *L. braziliensis*, and others), and

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Plasmodium (*P. falciparum*, *P. ovale*, *P. malariae*, and *P. vivax*)². Despite the huge burden of these diseases, research into their diagnosis, treatment, and prevention has been relatively neglected³. Despite its initial development in the 19th century, microscopy remains the most widely used diagnostic approach for protozoan infections. It requires relatively inexpensive equipment and reagents, however, considerable technical training is required. Even in the best-qualified hands, microscopy is insensitive for detecting many organisms. Furthermore, some pathogens cannot be readily distinguished from common commensal organisms (e.g. *Entamoeba histolytica* and *Entamoeba dispar*). Therefore, in the last years several new diagnostic methods have been developed. This review is focused in molecular detection of parasites by using a novel method based on recombinase polymerase amplification (RPA)⁴.

Molecular diagnosis by recombinase polymerase amplification (RPA)

Infectious diseases caused by protozoa can affect individuals from all countries, however the populations at the highest risk are in low-income countries. For example, World Health Organization (WHO) data indicate that diarrheal diseases and malaria are in the top 10 most frequent causes of death in low income countries⁵. However, the clinical syndromes of diarrhea and febrile illness have numerous causes, which differ in optimal treatment. Faced with limitations in performance and availability of diagnostic tests, practitioners frequently give treatments based on the empirical diagnosis. This leads to both over use of drugs when not needed and under treatment when needed. In the case of malaria, improved diagnosis is one of the pillars of current efforts at disease control, since it will target appropriate patients for therapy and will decrease pressure for drug resistance. Therefore, in order to decrease this empiricism and reduce the impact of these diseases in low-income countries, low-cost diagnostic tests are needed at the point-of-care (POC). For many infectious diseases, polymerase chain reaction (PCR) tests are considered the gold standard for diagnosis. However, PCR requires specialized equipment including thermal cyclers, technical expertise of personnel, and a cold chain to preserve heat-labile reagents. Even so, the reagents are relatively expensive. For these reasons, PCR assays are typically only available in reference or research laboratories and are seldom used for initial diagnosis.

Alternative nucleic acid amplification methods have been developed recently to bypass the requirement for a thermal cycler. These platforms use a fixed temperature heater instead of a thermal cycler, which costs an order of magnitude less, potentially allowing broader access to diagnostics based on isothermal amplification. Loop mediated isothermal amplification (LAMP) was the first isothermal method used for diagnostic purposes. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA⁶. The primers produce a stem-loop DNA that allows DNA synthesis by displacement. The main limitation of this method is the complexity of primer design, which requires the use of specific software. In addition, primers need extensive optimization in the laboratory and can be expensive. RPA is a more recently developed isothermal amplification method⁴. In the RPA reaction, a recombinase enzyme forms a nucleoprotein complex with oligonucleotide primers and scans for homologous sequences in a DNA template. Recognition of a specific homologous sequence leads to the initiation of strand invasion and the opposing oligonucleotides are then extended by isothermal strand displacement amplification via *Sau* polymerase. This results in

amplification of the doublestranded DNA without the need for thermal or chemical melting of the DNA (Figure 1). The main advantage of RPA over LAMP is that this technique only requires a single set of primers to produce specific amplicons that can easily observed by electrophoresis. Also, RPA amplicons can be detected in real time if the reverse primer and a probe are labeled with fluorophores. Double-labeled amplicons can then be detected using portable fluorimeters. Alternatively, RPA amplicons can be detected in paper strips by for lateral flow (LF). Both RPA-Lateral flow and RPA-Real time can be adapted for multiplex detection. RPA can also be used for RNA by adding reverse transcriptase enzyme (RT-RPA). RPA is also relatively resistant to inhibitors commonly found in clinical samples. Therefore, the consensus is that RPA offers significant advantages for POC diagnostics, which can be exploited to detect infections caused by protozoan at POC. Recently, we and others, demonstrated the feasibility of using this technique to detect enteric and blood and tissue infections caused by protozoa. Here, we will discuss the current status of each developed RPA.

Enteric infections by protozoan parasites

Diarrheal diseases constitute the second leading cause of death in children worldwide.⁷ Despite the importance of this illness, the etiology of diarrhea remains undiagnosed in most patients. A variety of enteric pathogens can cause diarrhea, including viruses, bacteria, and protozoa⁷. While viral causes dominate among cases of acute diarrhea, parasites are more common causes of persistent and chronic diarrhea, which are increasingly recognized as major causes of morbidity⁸. Among the parasites, *Cryptosporidium* species, *Giardia intestinalis*, and *Entamoeba histolytica* are major contributors for diarrhea and childhood malnutrition in poor countries. These organisms cause a similar clinical picture, but differ in optimal diagnostic methods, treatment, and prevention. For example cryptosporidiosis is treated with nitazoxanide, while giardiasis and amoebiasis are treated with metronidazole. Therefore, it would be very useful to establish a method that would allow practitioners in remote areas to quickly decide the appropriate treatment. The following sections discuss the limitations of conventional diagnostic methods and RPA for three of the most important diarrhea-causing parasites.

Cryptosporidiosis

Cryptosporidiosis, caused by parasites of the genus *Cryptosporidium*, is acquired by oral ingestion. A recent multicenter study conducted in sub Saharan Africa and Southern Asia showed *Cryptosporidium* as second to rotavirus as a cause of morbidity and mortality from childhood diarrhea^{7,9}. The typical approach to diagnosis of cryptosporidiosis is based on the identification of *Cryptosporidium* oocysts in stools by microscopic analysis of stool smears. Microscopy requires acid fast stains, which are often not used even in high risk populations. Even with a trained microscopist and acid-fast staining, the sensitivity is poor. Enzyme-linked immunosorbent-assays (ELISA) and lateral flow tests that rely on antibodies have been developed to detect parasite antigens; however, their reported sensitivity in the field varies widely. In a multicenter, blinded study the four leading commercial assays demonstrated clinical sensitivities between 47.2% and 68.8%⁹. The gold standard for *Cryptosporidium* detection is widely considered to be PCR, with a limit of detection of 10³ organisms/gram. Molecular methods, including multiplex detection, are being increasingly

used to diagnose intestinal pathogens in some hospitals of wealthy countries¹⁰. These panels have shown a high sensitivity and specificity. However, cost is prohibitive, especially for resource-limited settings. Isothermal amplification by LAMP has been mainly tested with water, animal, or spiked samples¹¹. An evaluation of LAMP to detect *Cryptosporidium* in animal stool showed a limit of detection of 0.6 parasites and 70% specificity in spiked samples in stool¹². Therefore, to date there is no optimal method for molecular detection of *Cryptosporidium* in clinical samples at POC.

RPA for cryptosporidiosis

We developed an RPA test to detect *Cryptosporidium* oocysts in stool samples¹³. Pathogen DNA extracted from stool was amplified to detectable levels in 30 min and visualized on simple lateral flow strips. Initially, our RPA-based *Cryptosporidium* assay was developed and optimized using DNA from human stool samples spiked with the pathogen. The RPA was then tested using DNA extracted from the stool of infected mice and human clinical samples. The results showed that the RPA correctly identified the presence or absence of 27 out of 28 mouse stool samples previously tested by PCR. In stool samples from infected patients, the RPA correctly identified the presence or absence of *Cryptosporidium* in all 21 stool samples, showing 100% correlation with PCR. Recently, others developed a lateral flow RPA to detect *Cryptosporidium* in cattle stool¹⁴, which confirms the feasibility of using RPA for stool samples. Despite its excellent correlation with PCR, so far *Cryptosporidium* RPA has been tested only with a small number of clinical samples. These studies must be expanded in order to validate its potential use as diagnostic test in the field.

Giardiasis

The gastrointestinal parasite *Giardia duodenalis* causes 200–300 million cases of disease (giardiasis) each year¹⁵. Infection may be acute or chronic, and symptoms include nausea, vomiting, diarrhea, and dehydration^{16,17}. While most infections in resource-limited settings are subclinical, infection is associated with weight loss and malnutrition^{18,19}. Diagnosis of *Giardia* infection is usually based on identification of the cyst or trophozoite forms of the parasite by stool microscopy¹. Although highly specific, microscopic identification of *Giardia* tends to have poor sensitivity²⁰. A number of nucleic acid-based and antigen-based diagnostic assays for stool sample detection of *Giardia* at the point-of-care are available and have demonstrated reliability^{21–23}. Traditional nucleic acid diagnostics such as (PCR) require the use of expensive thermal cycling equipment, limiting their use to central laboratories. Isothermal amplification LAMP assays have been developed for *Giardia*; the LAMP assay was able to detect both *Giardia* assemblages A and B and showed a limit of detection of 4–6 cysts with serial dilutions of parasites. The assay showed a high correlation with PCR and Immunofluorescence tests (IFT) when 35 clinical and environmental samples were tested²⁴. To date *Giardia* LAMP has not yet been implemented for clinical diagnostics in the field.

RPA for giardiasis

Our group has developed an RPA assay for *Giardia*²⁵. We designed primer sets and screened each for amplification of a specific DNA target. The RPA assay utilizes DNA primers that target a unique 183 base pair sequence on the beta giardin gene of *Giardia intestinalis*. We

found that we could detect as few as 10 *Giardia* cysts/ml spiked into PBS, which is similar to the analytical sensitivity of qPCR. The RPA assay detected both the A and B assemblages of *Giardia*, and reliably detected as few as 10^3 – $10^{3.5}$ cysts per milliliter stool, or about 50 cysts per reaction. When the RPA assay was tested using DNA extracted from clinical stool samples and benchmarked against a composite gold standard (where either a positive PCR or a positive microscopy result yields a gold standard positive result), the RPA assay yielded 73% sensitivity and 95% specificity. Lateral flow RPA has demonstrated a limit of detection of as few as 3,000 *Giardia* cysts per gram of stool, slightly better than PCR-based assays ²⁵.

Amoebiasis

Globally, as many as 500 million people may harbor *Entamoeba* species, including the pathogenic species *Entamoeba histolytica*, as well as *Entamoeba dispar*, *Entamoeba moshkovskii*, and *Entamoeba bangladeshi*, which are generally considered non-pathogenic. *E. histolytica* causes intestinal infection, which can range for asymptomatic carriage to fulminant colitis, as well as extra-intestinal disease including liver disease. Diagnosis of amoebiasis remains challenging. *E. histolytica* is morphologically indistinguishable from other *Entamoeba* species. Microscopic stool examination is also not sensitive for diagnosis ²⁶. Detection of antigen by ELISA and immunochromatic test has proven useful with good sensitivity and specificity for *E. histolytica* ^{26,27}. However, fixation of the stool samples denatures the antigen, thus limiting testing to fresh or frozen samples ²⁶. Some studies have identified problems with the specificity of antigen detection due to cross-reactions ^{26,28}. Molecular methods, such as PCR, have improved sensitivity compared with antigen detection ²⁹. Furthermore, real-time PCR is quantitative, which can help separate disease from colonization ³⁰. The sensitivity is comparable to standard PCR, with an ability to detect 0.1 cell per gram of feces ^{31,32}. Despite the obvious advantage of molecular techniques, they are not widely used. As a result, many practitioners resort to empiric therapy. The current practice of empiricism results in both over treatment and under treatment ³³. Over treatment with an antiprotozoal agent for all patients with cysts in feces is leading to increasing minimum inhibitory concentrations (MICs) against *E. histolytica* ³⁴. A LAMP assay for *E. histolytica* demonstrated a specificity of 100% and a limit of detection of 5 parasites per reaction, however, it was not tested with clinical samples ³⁵.

RPA for amoebiasis

We identified RPA primers that efficiently detect the 18S rDNA gene of *E. histolytica* in as little as 20 min ³⁴. These primers enabled differentiation of *E. histolytica* from the nonpathogenic species *E. dispar*. In addition, we adapted the *E. histolytica* RPA for lateral flow detection and tested the feasibility of RPA in remote areas by testing a portable DNA mini-extractor to isolate DNA from parasites spiked in stool samples. The results showed a limit of detection down to 2.5 fg of parasite DNA. This limit of detection was the same as when we used SYBR Green realtime PCR. We analyzed the sensitivity of RPA-LF with parasites spiked in stools and observed a limit of detection of 40 parasites. To validate our method, we analyzed 32 samples of DNA extracted from clinical samples obtained from ongoing studies in Colombia. In the first experiment, using 2.5 µL of sample, we obtained an 86% correlation with real-time PCR (data not shown). In a second experiment, using 5 µL of sample resulted in 100% correlation with PCR and ELISA results. To verify our ability to

detect *E. histolytica* DNA in clinical samples, we evaluated the efficacy of the *E. histolytica* RPA with samples previously evaluated by ELISA and PCR. Using 2.5 µL of template, we noted 86% and 98% correlation, respectively ^{34,36}.

RPA and multiplex detection of enteric protozoan parasites

While individual RPA assays may be helpful, a test to detect multiple pathogens simultaneously would be more clinically useful. Most multiplexed RPA assays require the use of a fluorescence reader ^{37,38}. Since fluorescence detectors may cost thousands of dollars, LF detection may represent an affordable alternative to conduct diagnostic testing in low resource settings. The ability to detect multiple targets using a low-cost platform, such as a lateral flow strip that could be interpreted visually or with a simple reader, could facilitate the implementation of DNA-based diagnostics for syndromic panels. We integrated separate RPA assays for *Giardia*, *Cryptosporidium*, and *E. histolytica* into a multiplex assay ³⁹. When the multiplex assay was tested using DNA extracted from live parasites spiked into stool at various concentrations, the respective limits of detection were 444, 6, and 9 parasites per reaction. However, further optimization is still needed for the multiplex RPA assays for detection of *Cryptosporidium*, *Giardia*, and *E. histolytica*.

Blood and tissue infections by protozoan parasites

Cutaneous leishmaniasis—Dermal and mucosal leishmaniasis are widely distributed in Central and South America, the Middle East, and North Africa affecting an estimated 190,000–300,000 people annually ⁴⁰. Many different *Leishmania* species grouped under the subgenera *Leishmania* or *Viannia* can produce dermal leishmaniasis ^{41,42}. Microscopy is still the most common diagnostic method used in endemic regions but its sensitivity is low and markedly affected by the experience of the microscopist ⁴³. Furthermore, the sensitivity of this method tends to decrease in chronic disease, which is characterized by a low number of amastigotes in the lesions ⁴³. Although in the past serological tests were used for diagnosing American cutaneous leishmaniasis and new antigens are still being considered, the tests are of limited value ^{44,45}. Conventional or quantitative PCR from dermal or mucosal samples have high diagnostic sensitivity (≈87–98%) and specificity (84%) ⁴⁶. This molecular method is currently the gold standard in leishmaniasis reference centers or tertiary care facilities. However, the need for expensive equipment, trained personnel, and relatively complex laboratory facilities are beyond the capability of the typical health infrastructure in endemic areas. Isothermal amplification by LAMP methods can detect as low as 0.1–0.01 parasites ⁴⁷. A direct boil LAMP method was recently reported, however, only two clinical samples were tested and this assay exhibited a limit of detection of 1 parasite/µL.

RPA for cutaneous leishmaniasis—We developed an RPA assay for *Leishmania Viannia* spp. ⁴⁸. Our primers and probes were designed to target the kinetoplast DNA minicircles due to the high copy number (~10,000) of this circular network of genomic mitochondrial DNA. The amplification product was detected with the naked eye in a lateral flow immunochromatographic strip (LF). The capacity of RPA-LF to detect the most relevant species of the subgenus *Viannia* was initially determined by the amplification of a small number of banked strains of *Leishmania Viannia* spp: *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. lainsoni*, *L. shawi*, and *L. naiffi*. The test amplified the principal *L. Viannia*

species isolated from multiple countries: *L. (V.) braziliensis* (n = 33), *L. (V.) guyanensis* (n = 17), and *L. (V.) panamensis* (n = 9). The less common *L. (V.) lainsoni*, *L. (V.) shawi*, and *L. (V.) naiffi* were also amplified. In a small number of clinical samples (n = 13) we found 100% agreement between PCR and RPA-LF. The RPA-LF amplified *Leishmania* DNA with an analytical sensitivity equivalent to 0.1 parasite per reaction, which corresponded to a Ct value of 28 using real-time PCR as the gold standard. The specificity was confirmed by the lack of amplification of *L. donovani*, *L. chagasi*, *L. mexicana*, *L. amazonensis*, *L. major*, *Trypanosoma cruzi*, and human DNA.

Visceral leishmaniasis—Visceral leishmaniasis (VL), which is caused by *L. infantum* (both in the New and Old World) or *L. donovani* (only in the Old World), accounts for 200–400,000 new cases each year. There are an additional undetermined number of subclinical infections and millions of people at risk in endemic areas⁴⁰. The parasite is transmitted by the bite of infected sand flies of several different species. VL is characterized by a progressive increase in visceral parasite burden, massive splenomegaly, anemia, cachexia, pancytopenia, and ultimately death if left untreated. Humans are considered to be both victims and reservoir hosts of *L. donovani* and *L. infantum*^{49,50}. Thus, early diagnosis and treatment of patients may constitute an efficacious strategy to decrease morbidity and interrupt transmission^{51,52}. Current parasitological diagnosis requires invasive, potentially dangerous procedures (bone marrow or spleen aspirates), which must be performed in hospital settings not accessible to many patients. Rapid antibody-based diagnostic tests such as the Direct Agglutination Test or the K39® serologic test are frequently negative until the infection is well established and parasite burden is high. Consequently, the sensitive detection of parasite DNA, when parasite loads are still low, should be the preferred diagnostic approach. A major constraint in diagnosing visceral leishmaniasis is that the disease occurs in remote or resource-limited areas where basic health infrastructure and/or access to care is limited. Standard PCR and real-time PCR have demonstrated greater sensitivity to detect *Leishmania* infections compared with serology^{53,54}. However, the need for sophisticated, expensive equipment, infrastructure, and trained personnel makes this approach impossible in most of the endemic areas⁵⁵. The LAMP assay for *Leishmania* detection was found to be positive in 68 of 75 confirmed VL cases, yielding 90.7% (95.84–81.14, 95% CI). All controls were negative by LAMP assay, indicating a specificity of 100% (100–95.43, 95% CI). The Ln-PCR yielded a sensitivity of 96% (98.96–87.97, 95% CI) and a specificity of 100% (100–95.43, 95% CI). High diagnostic sensitivity and excellent specificity were observed in this first report of a LAMP diagnostic evaluation in Bangladesh⁵⁶.

RPA for visceral leishmaniasis—We developed an RPA test coupled with LF reading with the naked eye with potential to be adapted as a POC test⁵⁷. The *L. infantum* RPA-LF had an analytical sensitivity comparable to real-time PCR, detecting the DNA of 0.1 parasites spiked in blood, which was equivalent to 40 parasites/mL. There was no cross amplification with dog or human DNA or with *Leishmania braziliensis*, *Leishmania amazonensis*, or *Trypanosoma cruzi*. The test also amplified *Leishmania donovani* strains (N = 7). In a group of clinically normal dogs (N = 30), RPA-LF detected more subclinical infections than the rK39 serological strip test (50% versus 13.3% positivity, respectively; p =

0.005). Also, RPA-LF detected *L. infantum* in noninvasive mucosal samples of symptomatic dogs with a sensitivity comparable to blood samples. In addition to *L. infantum*, the RPA-LF assay detected *L. donovani* and other species within the *Leishmania* subgenus (*L. major*, *L. aethiopica*). Recently, another RPA assay for the detection of VL was developed⁵⁸. The assay amplified both *L. donovani* and *L. infantum*. It deployed a lateral flow system to read the results with naked eye, which decreases the assay run costs. The Limit of Detection (LD) RPA assay detected equivalent to one LD genomic DNA, and was performed at constant temperature (42°C) in 15 min. The RPA assay also detected other *Leishmania* species (*L. major*, *L. aethiopica*, and *L. infantum*), but did not identify nucleic acid of other pathogens. Forty-eight blood samples (buffy coat) from VL, asymptomatic, and post-kala-azar dermal leishmaniasis subjects were detected positive and 48 LD-negative samples were negative by both LD RPA and real-time PCR assays, which indicates 100% agreement. Nevertheless, this assay required transferring the amplified product to another tube for dilution and DNA detection, which increased the possibility of cross contamination and assay run time. In contrast, our approach utilized a probe system that allowed detection of the amplification product in a single closed tube.

Plasmodium falciparum—Malaria remains an important cause of morbidity and mortality worldwide⁵⁹. The WHO estimates that 212 million cases occurred during 2015 (range 148–304 million), with 429,000 deaths (range 235,000 – 639,000)⁶⁰. About 3.2 billion people, almost half of the world's population, are living in areas at risk of malaria transmission. Sub-Saharan Africa (15 countries) continues to carry a high share of the global malaria burden, accounting for 88% of malaria cases *Plasmodium falciparum* is the principal species, which is responsible for 90% of all malaria cases⁶¹. Effective treatment and surveillance of malaria is only possible with a reliable and sensitive diagnostic tool. At present, the parasite is routinely detected by microscopy, requiring instrumentation and a trained eye. Rapid diagnostic tests, usually based on antigen detection, are becoming more popular as point-of-care tests. However, like microscopy, they often lack high sensitivity or provide unclear results. Molecular tests, such as PCR, have the advantage of detecting low levels of parasitaemia⁶² and determining the species of *Plasmodium* in a single assay^{63,64}. Nevertheless, PCR still remains a laboratory-based method due to the high complexity of the assay and the unmet true portability of devices designed for POC testing. Furthermore, the dependency on reliable electricity for the thermocycling process and the rather expensive PCR equipment makes it difficult to implement it in resource-limited settings. Thus, in developing countries the method is restricted to centralized laboratories and only used as a confirmatory test⁶⁵. There are LAMP assays for the detection of *P. falciparum*⁶⁶, *P. vivax*⁶⁷, or the amplification of four species of human malaria parasites⁶⁸. A similar concept has been used for developing the real-time quantitative nucleic-acid-sequence-based amplification (realtime QT-NASBA) for the quantification of rRNA samples of three different *Plasmodium* species⁶⁹. These methods simplified the assay set-up and improved the speed of nucleic acid amplification in comparison to standard PCR.

RPA for Plasmodium—An RPA for *Plasmodium falciparum* coupled with lateral flow (RPA-LF) exhibited high sensitivity and specificity⁷⁰. It detected 100 fg of genomic parasite DNA, corresponding to a sensitivity of approximately four parasites per reaction. The RAP-

LF amplified all *P. falciparum* strains tested (n = 77) while all 11 *non-Plasmodium* samples, were negative. The authors stated that the enzymatic reaction could be conducted under a broad range of temperatures (30–45°C) and in the presence of contaminants that usually act as inhibitory substances for PCR. A time to result of 15 min from start of the reaction to read-out was determined. Additionally, the RPA reaction was tested for sensitivity without lateral flow probe by subsequent visualization by means of agarose gel electrophoresis. The detection limit was 500 fg which corresponded to approximately 20 parasites/reaction. This RPA-LF test was also tested by spiking 1 ng of gDNA of *P. falciparum* in human serum and human whole blood ⁷⁰.

Challenges and future directions

DNA purification at POC—Generally, molecular methods show great sensitivity in the laboratory when the amplification capacity is evaluated using serial dilutions of purified DNA or parasites spiked in biological fluids. However, the sensitivity decreases when the tests are carried out with clinical samples. Sensitivity discrepancies are also commonly observed when tests are performed by different research groups. The main reason for this variability is the quality of the nucleic acids present in the sample, which heavily depends on the preservation of the biological material and extraction methods. Several substances are known to interfere with the enzymatic nucleic acid amplification by PCR. These substances are either present in the biological sample or can be introduced during preparation or sampling process. RPA has demonstrated tolerance to common PCR inhibitors and it was shown to operate with nucleic acids extracted from various sources. For instance, RPA functions well in the presence of 15%–25% milk (6.3–7.2 mmol/L), which is the maximum concentration tolerated by PCR. The RPA method amplified targets even in the presence of 50 g/L of hemoglobin, 4% vol/vol of ethanol, 0.5 U of heparin, or serum. However, RPA was inhibited by whole blood ⁷⁰. We have successfully used a portable mini extractor (QuickGene-Mini80) to extract DNA from spiked parasites or clinical samples. Using this mini extractor we obtained high quality DNA from 8 samples in 15 minutes, yielding RPA sensitivities comparable to PCR. However, despite its low cost the use of the mini extractor in the field requires basic training for its operation. In addition, if the samples are not used immediately then an alternative preservation procedure is required. Alternatively, Whatman FTA filter paper can be used to preserve and extract nucleic acids from clinical samples. For example this filter paper has been used to identify *Enterocytozoon bienuesi* in stool specimens by PCR ⁷¹. In these studies, three DNA extraction methods were evaluated: FTA filter paper, QIAamp stool mini kit, and a conventional phenol-chloroform method. The results showed that FTA filter paper and the QIAamp stool mini kit were the most sensitive methods ⁷¹. We have used DNA fixed in FTA paper to conduct RPA evaluations that yielded good results in terms of test sensitivity ³⁴. The utility of FTA filter paper to preserve DNA from stool samples still has to be determined. We also conducted RPA detection of canine visceral leishmaniasis (*Leishmania infantum*) directly from blood fixed in FTA paper. The results showed that based on this DNA preservation-extraction method the RPA-LF test detected 1 parasite per reaction. It is reasonable to assume that the same approach could be optimized for human samples ⁵⁷. Recently, we reported the successful use of tissue scrapings fixed on FTA filter paper in a RPA assay for cutaneous leishmaniasis. In these studies, the RPA showed high sensitivity, however, repeated washing steps were needed ⁴⁸.

Although the FTA paper is an excellent alternative for the collection and extraction of DNA, its main limitation is the requirement of additional steps to extract the DNA. During this process there is the potential risk of sample contamination, especially when working with large number of samples in remote areas.

Portability—Portability is an important factor for the implementation of diagnostic tests in the field. Although the detection of RPA products using lateral flow facilitates field diagnosis, further optimization of RPA for implementation in remote areas could broaden its application to a larger number of pathogens. Some researchers have proposed the use of portable briefcases that contain all the necessary tools to perform RPA in the field. The briefcase contains heat-blocks, minicentrifuges, pipettes, gloves, etc. By using this RPA-mobile suitcase investigators detected *Leishmania donovani* in the field ^{31,58}. However, this method is still suboptimal because the lyophilized RPA reagents need to be mixed with primers and cofactors by pipetting and transferring solutions. Thus, the transfer of reagents and the addition of the samples to open tubes represent a contamination risk, especially when simultaneously handling a large number of samples. Therefore, alternative methods should be considered to optimize RPA detection at POC. Richards-Kortum and co-investigators have reported a semi-integrated RPA method free of pipetting for RPA detection for *Plasmodium* ⁷². The system is based on the use of foldable paper and a plastic device to allow mixing and transfer of the RPA reaction to lateral flow detection by connecting pads which contains RPA reagents. The assay detected as few as 5 copies/ μ L of synthetic *Plasmodium* DNA (50 copies total) and was comparable with PCR sensitivity. In addition it had a low cost (~1 usd). However, this prototype is still in development and its performance has not been validated with clinical samples ⁷². Recently, we developed an RPA assay for *Leishmania* and integrated the assay into a completely closed device that enables DNA amplification and lateral flow detection without the use of pipets. The performance of the integrated assay is under optimization for several pathogens and is comparable to PCR, without requiring the use of thermal cycling (Castellanos, Melby, Saldarriaga, Travi; unpublished). Finally, field methods for heating samples are required to perform RPA reactions. Since this test works at temperatures between 37°C and 45°C, then utilizing body temperature (37°C) may represent an alternative. In fact, a recent study demonstrated that human body heat may be harnessed to incubate RPA reactions for isothermal amplification of HIV-1 DNA ⁷³.

RPA microfluidic microchip systems—Integrated systems that facilitate the conduction of RPA reactions without pipetting could be very useful in the diagnosis at POC. At present there are very few integrated systems for the diagnosis of protozoan parasites. The main advantage of these methods is the minimal manipulation required to carry out the tests. However, these systems are still not optimal as these devices operate with very small sample amounts; reduced volume can limit the sensitivity of the test especially when parasite loads are low. In the future, these systems will likely include concentration methods to increase the limit of detection. A research group reported the use of ring resonators in combination with solid-phase RPA ⁷⁴. As a proof of concept, they designed a model system using a *Francisella tularensis* sequence to describe the integration of the sensing chip and all the components to automate the assay, e.g. instruments, chip interfaces, devices, software,

microfluidics, heating system, and optical alignment. The integrated microfluidic diagnostic device is capable of on-site quantitative nucleic acid detection directly from the blood without any previous sample preparation. Furthermore, self-powered microfluidic pumping without any external pumps, controllers, or power sources is accomplished by an integrated vacuum battery on the chip. Another alternative developed for RPA detection is the Two-Stage Isothermal Enzymatic Amplification for Concurrent Multiplex Molecular Detection (RAMP) ⁷⁵. It is a two-step nested-like, rapid (40 min) isothermal amplification assay. This assay is implemented in a microfluidic chip. Multiple LAMP amplicons are detected *in situ* with colorimetric dye or with a fluorescent dye and a smartphone. This has already been used to detect *P. falciparum* ⁷⁵, however, this device has not been yet validated with clinical samples.

Towards the development of a commercial kit—In recent years many isothermal tests have been developed for the diagnosis of parasitic infections, but to date there is no FDA-approved isothermal amplification test for human use. In fact, the “ilumigene” based LAMP to detect *Clostridium difficile* is the only FDA-approved isothermal DNA amplification method for diagnosing an infectious disease ⁷⁶. It is likely that the first RPA-based commercial tests will be used in the veterinary and environmental fields due to somewhat less stringent regulations. Another consideration for commercialization of RPA-based diagnostics is that reagents are only sold by one company (TwistDx, UK) in kit configurations only for research use. Therefore, transition from the developmental stage to commercialization will not only depend on the performance of the test with clinical samples, but on the licensing of the technology for commercial purposes. POC tests for infectious diseases are of great benefit for people around the world, even more so for low income countries affected by multiple protozoan diseases. Thus, companies planning to develop RPA tests should consider the National Health Services of affected countries as target customers. Other important customers are the international humanitarian institutions that have adopted global strategies for the diagnosis, treatment, control, and elimination of these devastating infectious diseases. Due to the effect of globalization, diagnosis is also in high demand for travelers of industrialized countries and armed forces deployed to endemic areas.

Conclusions

Current RPA assays have demonstrated high sensitivity and specificity similar to PCR methods, therefore, the consensus is that RPA is a robust system that can be applied in the field. However, better systems of DNA preservation and extraction are necessary to optimize the performance of RPA. This method is currently under development for different pathogens and field validations are in progress. Consequently, we anticipate that in the next few years RPA-based tests will be available in the veterinary and medical fields. However, the development of integrated systems from nucleic acid extraction to amplification compatible with RPA assays will be essential to develop FDA-approved kits for detecting parasites at POC.

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Figure 1.

Isothermal amplification by recombinase polymerase amplification (RPA). In RPA, two oligonucleotides primers (blue and red lines) form a complex with the recombinase proteins (green) [1]. This complex is able to invade the target DNA and directs the primer to homologous sequences [2]. A continuous amplification at 37–42°C takes place by strand-displacement synthesis catalyzed by a DNA polymerase (yellow) while single-strand binding proteins (SSB) (purple) stabilize the displaced strand [3]. The new double strand of DNA is copied multiple times [4].