Acta Tropica 182 (2018) 4–11

Contents lists available at ScienceDirect

Acta Tropica

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

Molecular diagnosis of protozoan parasites by Recombinase Polymerase Amplification

A. Castellanos-Gonzalez[a,b,*], A.C. White Jr[a,b], P. Melby[a,b], B. Travi[a,b]

[a] Department of Internal Medicine, Division of Infectious Diseases, University of Texas Medical Branch, United States
[b] Center for Tropical Diseases, University of Texas Medical Branch, United States

ARTICLE INFO

Keywords:
Isothermal amplification
RPA
Recombinase amplification
Molecular diagnostic
Protozoan

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.

1. Introduction

1.1. 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 humans (Fletcher et al., 2012). 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 *Plasmodium* (*P. falciparum*, *P. ovale*, *P. malariae*, and *P. vivax*) (Levinson, 2014). Despite the huge burden of these diseases, research into their diagnosis, treatment, and prevention has been relatively neglected (Liese et al., 2010). 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) (Piepenburg et al., 2006).

1.2. 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 countries (WHO, 2017). 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,

* Corresponding author at: Infectious Disease Division, Department of Internal Medicine, University of Texas Medical Branch, 301 University Boulevard, Route 0435, Galveston, TX 77555-0435, United States.

https://doi.org/10.1016/j.actatropica.2018.02.002
Received 11 July 2017; Received in revised form 8 January 2018; Accepted 11 February 2018
Available online 13 February 2018
0001-706X/

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 DNA (Notomi et al., 2000). 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 method (Piepenburg et al., 2006). 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 double-stranded DNA without the need for thermal or chemical melting of the DNA (Fig. 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.

1.3. Enteric infections by protozoan parasites

Diarrheal diseases constitute the second leading cause of death in children worldwide (Kotloff et al., 2013). 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 protozoa (Kotloff et al., 2013). 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 morbidity (Lima and Guerrant, 1992). 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.

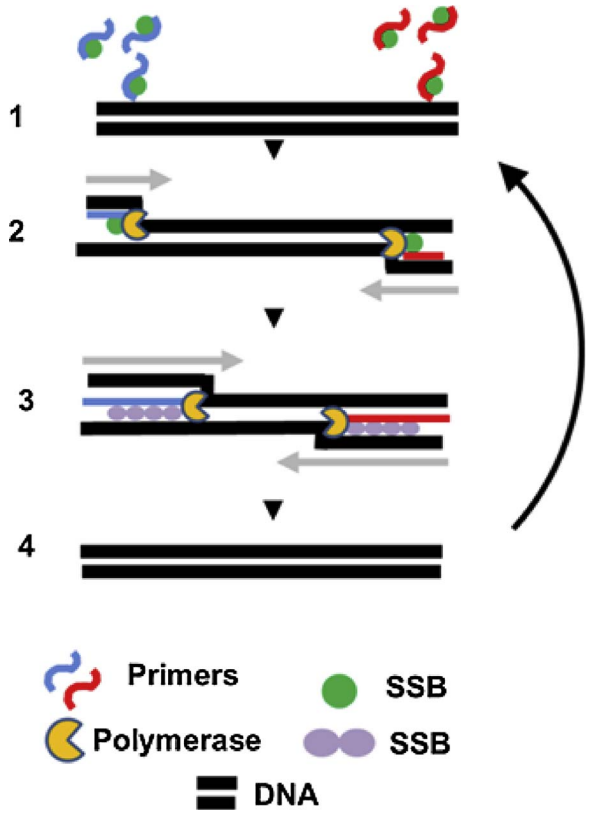

Fig. 1. Isothermal amplification by recombinase polymerase amplification (RPA). In RPA, two oligonucleotides primers (blue and red lines) form a complex with the recombinase proteins (green) (Fletcher et al., 2012). This complex is able invade the target DNA and directs the primer to homologous sequences (Levinson, 2014). 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 (Liese et al., 2010). The new double strand of DNA is copied multiple times (Piepenburg et al., 2006). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1.4. 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 (Kotloff et al., 2013; Sow et al., 2016). 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% (Sow et al., 2016). The gold standard for *Cryptosporidium* detection is widely considered to be PCR, with a limit of detection of $\leq 10^3$ organisms/gram. Molecular methods, including multiplex detection, are being increasingly used to diagnose intestinal pathogens in some hospitals of wealthy countries (Reddington et al., 2014). 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 samples (Gallas-Lindemann et al., 2016). An evaluation of LAMP to detect *Cryptosporidium* in animal stool showed a limit of detection of 0.6 parasites and 70% specificity in