Rapid Point-of-Care Diagnostic for Malaria

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Abstract

Our laboratory has utilized nucleic acid amplification to detect malarial DNA at levels down to 40 parasites/mL in less than 30 min. The technique utilizes Loop-Mediated Isothermal Amplification (LAMP) with primers that target the mitochondrial cytochrome oxidase subunit 1 gene capable of distinguishing *P. falciparum* from *P. vivax*. Malaria diagnosis by the "Gold Standard" microscopic examination of thick and thin blood smears is generally carried out only after symptoms of the disease have become moderate to serious. Microscopy is a labor-intensive process that requires trained personnel. Several companies have introduced rapid diagnostic tests (RDTs) targeting a malarial antigen that is typically histidine-rich protein-2. These RDTs require an infection level in the range of ~500-1,000 parasites/µL, which is a relatively low level of sensitivity that can result in false negative results, but can also lead to a false positive after the disease has been cleared since the target antigen persists. The project is being carried out in collaboration with OptiGene, Ltd (Horsham, England). OptiGene has commercialized two battery-operated units that can simultaneously measure malarial DNA in 8 or 16 samples. We have demonstrated proof-of-principle for a portable point-of-care device using an existing platform that can provide ultra-sensitive diagnostics for both major forms of malaria. The goal of this work is to provided a robust point-of-care analysis platform that is easy to use, can operate on battery power, and can provide results in <30 min for use in endemic infection areas as part of a treatment plan.

Introduction

An alternative approach to a vaccine to alleviate the burden of malaria involves the "Test and Treat" paradigm currently being evaluated for eradication of HIV [1]. This approach predicts that if all symptomatic and asymptomatic individuals in a region are tested, and all of those who test positive are immediately treated, the burden of disease in that area will decrease. We believe that this approach is feasible for malaria with an inexpensive point-of-care diagnostic test for *Plasmodium* DNA, followed by appropriate therapeutic intervention.

Currently, malaria is detected by (1) microscopic examination of thick and thin blood smears, a process that is both laborious and lengthy and requiring trained personnel; (2) laboratory-based PCR tests, which are expensive, time-consuming, and require specialized equipment and trained personnel; or (3) rapid antigen-based tests, which are generally insensitive. Commercial rapid-diagnostic tests (RDT) for malarial antigens were first introduced in 1994, and currently there are many such products available. The WHO has been evaluating these tests since 2009, and the third report assessing available RDTs was published in December 2012 [2]. The shortcomings with the available RDTs include: (1) Poor quality of some products; (2) Inability to detect mutant forms of Plasmodia that have deleted the histidine-rich protein 2 (HRP2) target gene; (3) Low sensitivity (500-1,000 parasites/µl); and (4) False positive reactions after the infection is cleared due to residual antigen in the bloodstream

Isothermal amplification methods make use of polymerases with strand displacement capabilities that do not require a high temperature denaturation step. The entire amplification reaction occurs under isothermal conditions (65˚C). The reaction is extremely efficient and yields an enormous amount of amplified DNA. Reaction progress is typically visualized using an intercalating dye. Our group designed a Point-of-Care (POC) Loop-mediated Isothermal Amplification (LAMP) nucleic acid detection test using either blood or oral fluid that has a sensitivity 10-100 fold greater than conventional PCR (6,7), 10-25 times more sensitive than antigen detection, requires no sample purification, and takes less than 30 min. Traditional LAMP utilizes two or three sets of primers that target the conserved 18S rRNA gene, however, we use primers that target the mitochondrial cytochrome C oxidase gene, which is 100 times more sensitive than other isothermal amplification methods currently available for *Plasmodium falciparum* (8) and is unlikely to be mutated or deleted.

Methods

LAMP Reagents and Primers Design: Isothermal master mix containing Geobacillus sp. enzyme and the LAMP real time processer were obtained from OptiGene Ltd, Horsham, West Sussex, UK. The primers, F3 (forward outer primer), B3 (back outer primer), FIP (forward inner primer), and BIP (back inner primer), were previously described (9,10) and verified using PrimerExplorerV4 (available from http://primerexplorer.jp/e/). Currently samples are pipetted into the reaction tube manually, but for the final test we need to develop a simple module for introducing the blood or oral fluid sample. A potential device is shown in Figure 1.

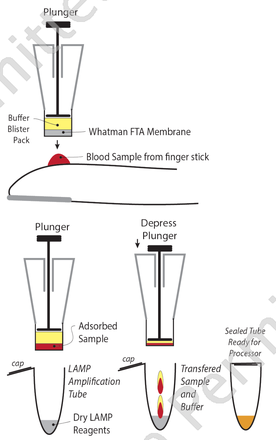

Figure 1. Potential design for transferring sample to LAMP processing tube.

Results

We have carried out a series of experiments utilizing the OptiGene processor (Genie II, Figure 2) and have been able to utilize fresh samples of blood or saliva as well as dried blood (Figure 3) and saliva spots on filter paper. The amplification reaction is completed in less than 30 min and can provide either a YES/NO result or an estimate of the parasite load (Figure 3).

Figure 2. OptiGene® amplification deivces. Left) Genie II can simultaneously analyze 16 samples and Right) Genie III can analyze 8 samples.

The amplification reaction progress can be monitored in real-time as shown with isolated genomic DNA in plasma (Figure 3) or whole parasites. Real-time LAMP analysis is a valuable strategy the simplification and shortening of the reaction conditions and it utilizes stable reagents that can be prepared in a dry format compatible with a POC device.

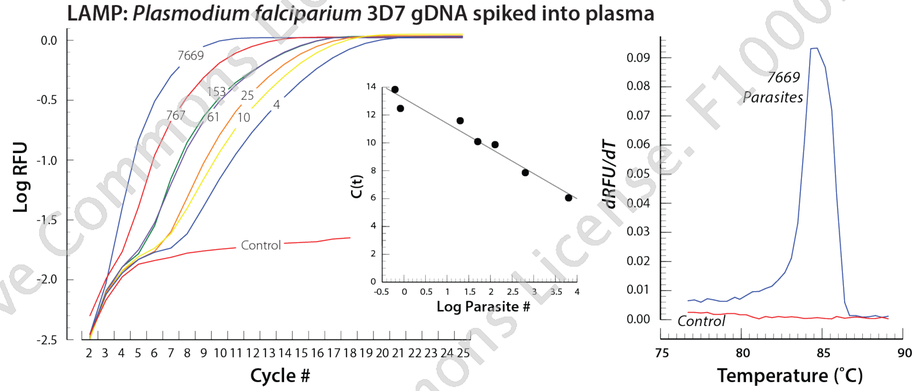

Figure 3. Real time curves for a titer of Loop-mediated isothermal Amplification (OptiGene® Isothermal Master Mix) of 3D7 genomic DNA spiked into plasma from a healthy subject using primers for the mitochondrial cytochrome C oxidase gene. Units represent the equivalent-parasites per 25 µl total reaction volume. The thermal melting curve shows a single peak that is characteristic of a single product associated with the targeted gene.

P. vivax can be detected by LAMP amplification of dried blood spots as shown in Figure 4. The higher the parasitemia level, the shorter the amplification time. Parasite DNA was also detectable in an oral fluid sample (Data not shown).

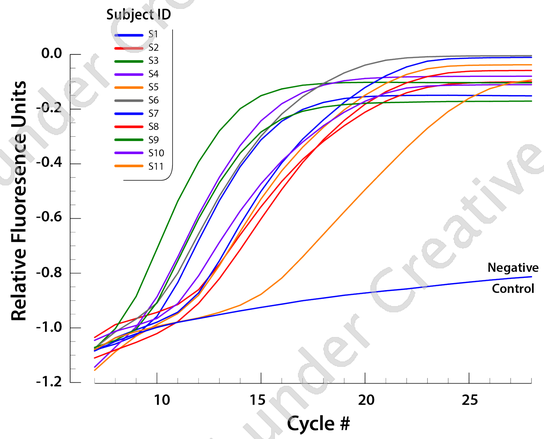

Figure 4. Clinical samples. The real time curves for Loop-mediated isothermal Amplification (OptiGene® Isothermal Master Mix ISO-001) is shown for a series of dried blood spots taken from human subjects infected with unknown levels of parasitemia due to *Plasmodium vivax*. Parasite DNA was extracted and eluted using the QIAamp DNA Mini Kit.

Figure 5 shows the ability to detect *P. vivax* in samples obtained from different geographical regions.

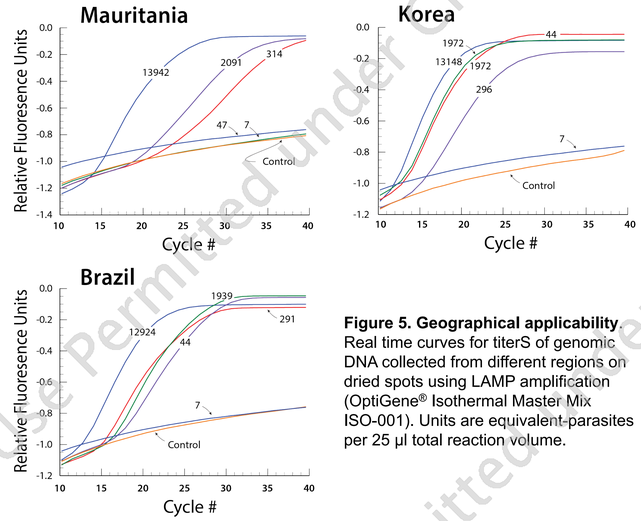

Figure 5. Geographical applicability. Real time curves for titerS of genomic DNA collected from different regions on dried spots using LAMP amplification (OptiGene® Isothermal Master Mix ISO-001). Units are equivalent-parasites per 25 µl total reaction volume.

Summary

Our goal is the commercialization of a robust point-of-care analysis platform that is easy to use, can operate on battery power, and provide results in <30 min.

We have demonstrated proof-of-principle for a portable point-of-care device using an existing platform that can provide ultra-sensitive diagnostics for both major forms of malaria.

Plasmodium falciparum gDNA (3D7), *P. vivax* gDNA, and intact malaria parasites in plasma were successfully amplified and detected.

The limit of detection (LOD) is ~1 parasite-equivalent per 25 µl reaction or 40 parasites-equivalent/ml when parasite DNA is spiked into buffer. Parasite-equivalent refers to the conversion between *Plasmodium falciparum* DNA to the equivalent number of parasites (~0.0238 pg DNA/parasite).

Detection was possible when using oral fluid or dried blood spots as samples, suggesting that we can optimize an isothermal based diagnostic assay for point-of-care detection of both *P. falciparum* and *P. vivax* DNA.

The final device will be based on either the OptiGene® Genie II or III and will incorporate the Loop mediated isothermal Amplification assay for the detection of Plasmodium DNA with a limit of detection of ~40 parasites/ml of blood or saliva in less than 30 minutes.

Advantages of this system includes: 1) confirmation of malarial infection prior to treatment, which will save on drug expenses since there is no need to treat if the infection is controlled; 2) the test can be used to monitor inadequate treatment if the parasite is resistant to the therapy prescribed; and will allow a "Test & Treat" protocol to be carried out, particularly in low transmission areas, to decrease the burden of disease in a community.

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