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Two-stage sample-to-answer system based on nucleic acid amplification approach for detection of malaria parasites

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ABSTRACT

Rapid, early, and accurate diagnosis of malaria is essential for effective disease management and surveillance, and can reduce morbidity and mortality associated with the disease. Although significant advances have been achieved for the diagnosis of malaria, these technologies are still far from ideal, being time consuming, complex and poorly sensitive as well as requiring separate assays for sample processing and detection. Therefore, the development of a fast and sensitive method that can integrate sample processing with detection of malarial infection is desirable. Here, we report a two-stage sample-to-answer system based on nucleic acid amplification approach for detection of malaria parasites. It combines the Dimethyl adipimidate (DMA)/Thin film Sample processing (DTS) technique as a first stage and the Mach-Zehnder Interferometer-Isothermal solid-phase DNA Amplification (MZI-IDA) sensing technique as a second stage. The system can extract DNA from malarial parasites using DTS technique in a closed system, not only reducing sample loss and contamination, but also facilitating the multiplexed malarial DNA detection using the fast and accurate MZI-IDA technique. Here, we demonstrated that this system can deliver results within 60 min (including sample processing, amplification and detection) with high sensitivity (<1 parasite μL^{-1}) in a label-free and real-time manner. The developed system would be of great potential for better diagnosis of malaria in low-resource settings.

1. Introduction

Malaria is a mosquito-borne infectious disease with high mortality caused primarily by several *Plasmodium (P.)* species, such as *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*, in tropical and subtropical regions (White et al., 2014; WHO, 2014). Malaria continues to be a major global health problem as over 40% of the world's population is at risk of malarial infection, and it frequently occurs in children below 5 years old with high risk of leading to death (Tangpukdee et al., 2009; WHO, 2014). In particular, humans are mostly infected by *P. falciparum* via the Anopheles mosquitoes. *P. falciparum* infection can be life threatening because it causes morphological changes in red blood cells, thereby disrupting microcirculation and subsequently leading to organ failure within 48 h (Peng et al., 2014; Maier et al., 2009). Nevertheless, malaria is treatable if detected early and before the infection disrupts other normal cells. However, early diagnosis remains a challenge owing to the difficulty in detecting low-abundance parasites from blood at an early stage of infection. Therefore, early and accurate diagnosis is essential for effective management and surveillance of malaria.

Current gold standard assays for malaria diagnosis are the routine optical microscopy of Giemsa-stained thick and thin blood films and quantitative buffy coat (QBC), which have been used as the main method of detection for decades in many countries (Bailey et al., 2013; Hopkins et al., 2013; Newman et al., 2008; Tangpukdee et al., 2009; WHO, 2014). However, microscope-based diagnostic assays require well-trained technicians and well-equipped facilities and entail complicated steps such as multiple incubation, staining, and drying. Moreover, it is labor-intensive, often unfeasible in remote rural settings, and has poor sensitivity at low parasitemia (Bailey et al., 2013; Hopkins et al., 2013;

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Tangpukdee et al., 2009; WHO, 2014). There is an increasing unmet need for the development of a fast, accurate, and cost-effective diagnostic method for the detection of malaria parasites that can overcome the limitations of microscopy, as recommended by the World Health Organization (WHO) (WHO, 2014). Recently, WHO has supported a rapid antigen/antibody test, which can detect an antigen from the malaria parasites in blood within 15–60 min depending on the concentration of the parasites, as a rapid diagnostic test (RDT) (Hopkins et al., 2013; WHO, 2012). Most of RDTs are based on hybridization assay between antigen and antibody that have been implemented in many diagnostic studies (Chou et al., 2012; Drakeley and Reyburn, 2009; Kim et al., 2015; Ndao, 2009). Therefore, RDTs are rapid (15 min) and easy to use as compared with the gold standard microscopic assay method. Although the reading time of RDT is within 15 min for high concentration of malaria parasites (sensitivity of 60–94.2% at levels of >500 parasites μL^{-1}), it still required much longer time (60 min) to detect malaria in low concentration of malaria parasites (sensitivity of 37–89.7% at levels of 200–500 parasites μL^{-1}) (Chou et al., 2012; Ndao, 2009). Moreover, RDTs do not allow specific, quantitative, and sensitive detection of malaria species in small volumes.

Alternatively, malaria detection techniques based on nucleic acid amplification testing approaches (NAAT) can be considered for the initial diagnosis of malaria because of its comparable sensitivity. NAAT can not only detect malarial DNA at relatively low levels of parasitemia, but also identify it to the species level (Moody, 2002; Tangpukdee et al., 2009; Warkiani et al., 2015). However, NAAT based techniques require sophisticated infrastructure including large thermal cyclers for DNA amplification. Alternatively, loop-mediated isothermal amplification (LAMP) and nucleic acid sequence based amplification (NASBA) are emerging as isothermal DNA amplification techniques that amplify the malarial DNA by using relatively simple equipment and is less time-consuming (60–90 min) (Aonuma et al., 2008; Han et al., 2007; Hopkins et al., 2013; Mens et al., 2006; Njiru, 2012; Omar et al., 2005; Paris et al., 2007; Poon et al., 2006; Schoone et al., 2000; Yamamura et al., 2009). Unfortunately, neither of these approaches is very sensitive (>200 parasites μL^{-1}), rapid (>1 h) and their accuracy can be affected by many factors such as non-specificity of primers and presence of PCR inhibitors (Aonuma et al., 2008; Cook et al., 2015; Han et al., 2007; Hopkins et al., 2013; Mens et al., 2006; Njiru, 2012; Omar et al., 2005; Paris et al., 2007; Poon et al., 2006; Schoone et al., 2000; Yamamura et al., 2009).

Since existing malaria diagnostic methods remain problematic, many new malaria diagnostic techniques based on various biosensors are being developed for rapid and effective malaria diagnosis, alleviating suffering and decreasing community transmission (Sin et al., 2014; Jain et al., 2014; Moody, 2002). Biosensors are promising for the development of rapid detection devices that can be portable, label-free, real-time, multiplex, sensitive, and low-cost. Several studies have reported electrochemical immunosensors using antibodies against horseradish peroxidase (HRP) II, *P. vivax* lactate dehydrogenase (PvLDH), and *P. falciparum* lactate dehydrogenase (PfLDH). The detection limit of these sensors was 8 ng mL^{-1}, 108.5 fM, and 120.1 fM, for HRP II, PvLDH, and PfLDH, respectively (Lee et al., 2012; Sharma et al., 2008). Other studies have also reported malarial detection using spectrophotometric immunosensors and magnetic resonance relaxometry with relatively low sensitivity (>50 parasites μL^{-1}, 88%) (Peng et al., 2014; Piper et al., 1999; Juul et al., 2012). Although most studies have used biosensors as detection tools, the sensitivity of these approaches is not optimal because of limitations of the immunoassay itself, such as false positives and low sensitivity due to inhibitors in human body fluids including blood.

In addition, sample processing is increasingly recognized as the critical bottleneck for enhancing detection sensitivity in clinical application because it is directly involved in the enrichment of the target molecule, removal of inhibitors for specific detection, and reduction of sample volume (Boom et al., 1990; Breadmore et al., 2003; Ritzi-Lehnert, 2012; Sin et al., 2014). Despite the necessity of sample processing in clinical applications, conventional methods for sample processing are conducted on a filter/membrane with organic solvent. However, these methods lack standardization and reproducibility for DNA yield. They are also tedious, time-consuming, labor-intensive and require complex instruments such as centrifuges and vortexers (Sambrook and Russell, 2006; Wright et al., 2009; Gauch et al., 1998; Tan and Yiap, 2009; Cao et al., 2006; Kim et al., 2009). Therefore, an ideal sample processing method that would provide high quality DNA and be adaptable for subsequent downstream analysis to enhance detection sensitivity is needed.

Here, we report a two-stage sample-to-answer system for the detection of malaria. We first combined a dimethyl adipimidate (DMA)/thin film sample processing (DTS) technique as a sample-processing module with the portable Mach-Zehnder interferometer-isothermal solid-phase DNA amplification (MZI-IDA) platform as a detection module to create a sample-to-answer system for malaria detection. DTS without centrifuges, vortexers and complicated steps allows the extraction of high quality DNA with simplicity, rapidity, and low cost (Shin et al., 2015a). In addition, DTS is a closed system, in which lysis, washing and elution steps are completed in a single microfluidic chamber without any transfer, therefore reducing sample loss and contamination. The MZI-IDA consists of a portable MZI sensing platform and the isothermal recombinase polymerase amplification (RPA) technique that has emerged as an alternative to PCR in molecular diagnostics bypassing the use of thermal cycling (Liu et al., 2015a). The MZI-IDA can detect epidermal growth factor receptor (*EGFR*)-DNA biomarkers in clinical samples of non-small cell lung cancer patients within 30 min with high sensitivity (Liu et al., 2015a). Based on the combination of these two recently developed techniques, we demonstrate the malarial detection in spiked blood with this two-stage sample-to-answer system. It is verified that this system can not only deliver results within 60 min with high sensitivity of up to <1 parasite μL^{-1}, but also enables multiple target detection at three different concentrations simultaneously.

2. Experimental

2.1. Malaria sample preparation using DTS system

A DTS system is prepared for the DNA extraction from malaria parasites spiked in human whole blood following the procedures as reported recently (Shin et al., 2015a). Briefly, the microfluidic chamber for DNA extraction in the DTS system was simply fabricated by a laser cutting machine (Universal Laser Systems, Scottsdale, USA). The extraction chamber design [8.4 cm × 3.7 cm] was cut into a 300-μm thick film formed by sandwiching a 100 μm thick polyester film between two 100-μm thick double-sided tapes. The extraction chamber design consisted of 36 slot-type micro-wells connected to each other with a flow path as shown in Fig. 1A and its total volume was 300 μL. To create amine group on the surface of cover and bottom thin films, a solution of 2% 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich) in H_2O was flowed into the chamber and baked at 65 °C for 40 min, followed by thorough rinsing with DI (de-ionized) water. Finally, to extract malaria DNA from the parasites using the DTS system, a modified protocol and optimized assay solution were used as reported recently (Shin et al., 2015a). For optimized assay solution, lysis buffer containing 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, and