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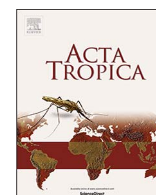

A multiplex microfluidic loop-mediated isothermal amplification array for detection of malaria-related parasites and vectors

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ABSTRACT

Malaria infection poses a great threaten to public health even nowadays. The conventional diagnosis tools of malaria parasites and vectors require systematic training for the observers accompanied by the low throughput. In this study, a new detection system, i.e., multiplex microfluidic loop-mediated isothermal amplification (mμLAMP) array, was developed to provide a convenient, rapid and economical detection system for malaria diagnosis. A microfluidic-based detection chip was designed and developed, targeting the conserved gene of four *Anopheles* and two *Plasmodium* species responsible for most of the malaria cases occurred in China. The DNA preparation of *Anopheles* and *Plasmodium* samples was realized by using a newly-developed DNA extraction method. For this mμLAMP array system, the detection limit was determined to be 1 pg of targeting DNA with high sensitivity (> 95%) and specificity (100%). Further, the accuracy of such mμLAMP analysis was evaluated by the analysis of 48 *Anopheles* mosquito samples, of which 30 were termed to be target *Anopheles*, displaying high consistency with that by morphological analysis. In conclusion, the mμLAMP detection system was proved to be a visible, sensitive, specific and high-throughput diagnostic tool. Considering the portable manipulation of such detection system, our studies shed light on its potential application of malaria surveillance on the spot.

1. Introduction

Malaria, a female *Anopheles* mosquitoes-borne infectious disease of human and other animals, is caused by parasitic protozoans belonging to the genus *Plasmodium*, and the typical symptoms include fever, fatigue, vomiting, and headaches (World Health Organization, 2015). Although great efforts have been made to control the malaria breakout worldwide, it still poses a great threat to public health in some underdeveloped areas such as Latin America and Africa (Ferreira and Castro, 2016; World Health Organization, 2015). Until now, about 3.3 billion people are at risk of malaria infection every year in the world (White et al., 2014). According to the statistics, there were 214 million malaria cases and 438,000 deaths occurred in 2015 (Tham and Kennedy, 2015). Since *Plasmodium* is transmitted exclusively through the bites of female *Anopheles* mosquitoes, the vector surveillance has become vital to malaria prevention. Especially in those areas with limited detection conditions, a simple and high-throughput screening technique for *Anopheles* and *Plasmodium* will be particularly valuable to warn against the potential outbreak of malaria.

Currently, there have been several methods developed for malaria monitoring. Among them morphological analysis by using the microscope is the most commonly used and is regarded as golden standard. However, this method requires systematic training for the observers as well as a big sample amount (Agianian et al., 2007; Wilson et al., 1998). To overcome the above deficiency, polymerase chain reaction (PCR) analysis targeting the ribosomal deoxyribonucleic acid (rDNA) (Rider et al., 2012) and enzyme-linked immunosorbent assay (ELISA) (Doderer et al., 2007; Park et al., 2008) were applied to the diagnosis on *Anopheles* and *Plasmodium* samples. Regretfully, the relatively low sensitivity, high cost and demanding of large-scale instruments for both methods have brought great inconvenience to those undeveloped regions with sudden malaria outbreak, where usually lack enough funding and necessary instrument platform such as RT-PCR equipment and microplate reader.

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Loop-mediated isothermal amplification (LAMP), a simple, rapid and specific DNA amplification method, is characterized by the isothermal reaction and point-of-care test (Notomi et al., 2000; Tomita et al., 2008). The LAMP provided a one-step nucleic acid amplification that is dependent on DNA synthesis using a DNA polymerase with strand displacement activity. To initiate such amplification, four primers are needed, i.e., one forward outer primer (F3), one backward outer primer (B3), one forward internal primer (FIP) and one backward internal primer (BIP). Only if all of the four primers interacted with the gene biomarker, positive signals would be generated, and this makes LAMP a more specific diagnosis tool than any other detection methods. So far, LAMP has already been used for *Plasmodium* and *Anopheles* mosquito identification (Abdul-Ghani et al., 2012; Aonuma et al., 2008; Bonizzoni et al., 2009; Britton et al., 2016; Buates et al., 2010; Han et al., 2007; Lucchi et al., 2010; Mohon et al., 2014). Though accompanied by high sensitivity, LAMP displayed inherent false positive results, low throughput and time-consuming in multiplex targets typing, which have greatly limited the application of LAMP to nucleic acid detection (Zhang et al., 2014b).

The microfluidic systems, also called lab-on-a-chip, can integrate all analysis steps onto a chip including sample collection, nucleic acid preparation and molecular detection (Foudeh et al., 2012). This system offers automatic operation with high throughput pathogen detection and has proved to be more suitable for point-of-care test even under extreme circumstances with limited equipment platform (Yager et al., 2006). So far, the *Plasmodium* detection has been performed on microfluidic platform by using immunoassay, microscopy or enzymatic activity measurement (Horning et al., 2014; Juul et al., 2012; Lafleur et al., 2012).

In this study, we developed a visible multiplex microfluidic LAMP (mμLAMP) array system, which integrated LAMP and lab-on-chip, and made it possible to simultaneously realize the multiple malaria-related targets detection on a single chip. By using this detection system, we specifically identified four malarial vectors including *Anopheles sinensis*, *An. lesteri*, *An. dirus* and *An. minimus*, by which most of the *Plasmodium* were spread in China (Huang et al., 2015; Makhawi et al., 2013). In addition, *Plasmodium falciparum* and *P. vivax*, posing the greatest public health challenge to human malaria in China (World Health Organization, 2015), were also successfully differentiated from other species. The accuracy of such mμLAMP analysis was evaluated by the analysis of 48 *Anopheles* mosquito samples, of which 30 were termed to be target *Anopheles*, displaying high consistency with that by morphological analysis, showed potential to identify the malaria infected mosquitoes from a large number of field-collected mosquitoes.

2. Materials and methods

2.1. Reagents

The *Bst* DNA polymerase, proteinase K and deoxynucleotide triphosphates (dNTPs) were purchased from New England BioLabs (Ipswich, MA). Eva Green was obtained from Biotium (Hayward, CA). Wizard Genomic DNA purification kit was purchased from Promega (Madison, WI) and universal real time LAMP kits from Guangzhou HF biotech Co. Ltd (Guangzhou, Guangdong province, China). Deionized water with the resistivity 18.2 MΩ cm at 25 °C was prepared by using Millipore-D 24^{uv} system (Billerica, MA) and further sterilized. Other reagents, unless specified, were obtained from Sigma-Aldrich (Saint Louis, MO).

2.2. DNA preparation of mosquitoes and Plasmodium samples

By indoor light traps at livestock corrals or laboratory-rearing, the mosquitoes and *Plasmodium* samples were collected and stored at −20 °C. The genomic DNA of mosquitoes and *Plasmodium* parasites, including *Anopheles sinensis*, *An. lesteri*, *An. dirus*, *An. minimus*, *Plasmodium falciparum* and *P. vivax*, were extracted using a DNA purification kit (Promega, Madison, WI) with the indicated protocol.

To meet the experimental demands under extreme conditions, an economic and simple DNA extraction procedure was developed as referenced with some modification (Bonizzoni et al., 2009). In brief, the samples were ground in a freshly prepared extraction buffer (1% SDS, 0.5 M NaCl, 25 mM EDTA, 0.1 M Tris-HCl, pH 8.0) for 5 min. Then, proteinase K was added and incubated at 45 °C for 55 min. After centrifugation at 13,000*g* for 10 min, the supernatant was collected and precipitated in cold 100% ethanol. Finally, the genomic DNA was washed with 70% ethanol, dried and rehydrated in deionized water. By using this method, the genomic DNA of above-mentioned mosquito and *Plasmodium* samples was extracted for next experiments.

2.3. Primer design for LAMP

The LAMP primers were designed using the Primer Explorer V4 program (http://primerexplorer.jp/e/) and synthesized by Sangon Co. Ltd (Shanghai, China). The primers target the conserved sequences of *An. sinensis* (GenBank accession number: AY316156.1), *An. lesteri* (AJ004941.3), *An. dirus* (U60410.1), *An. minimus* (AY737081.1), *P. falciparum* (M19173.1) and *P. vivax* (U03080.1). For each *Anopheles* and *Plasmodium* species, a set of primers was designed as reported by Tomita N et al. (2008) comprising two outer ones (F3 and B3) and two inner ones (FIP and BIP) (Tomita et al., 2008).

2.4. Microfluidic chip design and fabrication

The microfluidic chip with 8 reaction wells was designed by using the AutoCAD software as shown in Fig. 1a, composed of the injection hole, flow channels, reaction wells and vent holes. The chip made of polymethylmethacrylate (PMMA) was fabricated utilizing an injection molding method (Katoh et al., 2008; Volpatti and Yetisen, 2014).

2.5. LAMP assay for primer selection

The LAMP reactions were carried out using the universal real time LAMP kit. In brief, 1 μl of genomic DNA sample was mixed in a reaction tube with 12.5 μl of reaction buffer, 5 pmol of each outer primer (F3 and B3), 40 pmol of each inner primer (BIP and FIP), 0.5 μl of *Bst* DNA polymerase and 0.25 μl of fluorescent reagent. Then, molecular grade water was added to a final volume of 25 μl. The reaction was performed by a real-time fluorescence isothermal amplification detector (Guangzhou HF biotech Co. Ltd., Guangzhou, China) at 62 °C for 1 h, followed by 5 min at 85 °C to inactivate the enzyme.

2.6. Visual mμLAMP array analysis

For the LAMP amplification with low cost, it is necessary to make the mμLAMP test easily manipulated and visually read. In present study, a type of multiplex microfluidic chip with primers immobilized was prepared: 1 pmol of each outer primer and 8 pmol of each inner primer, targeting the specific *Anopheles* and *Plasmodium* species, were fixed in the reaction wells utilizing mixture of 0.1% agarose (Fig. 1b), allowed to dry and completely sealed by pressure membrane (Fig. 1c) and the prepared chip can be preserved for 3 months at room temperature in the primary studies. The left six wells were immobilized with individual primers in sequence, i.e., *An. sinensis*, *An. lesteri*, *An. dirus*, *An. minimus*, *P. falciparum* and *P. vivax*, and the right two blank wells were set as negative controls. Secondly, a 2 × reaction mixture was pre-mixed as followed: 40 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 20 mM KCl, 12 mM $MgSO_4$, 1 M Betain, 2.5 mM dNTPs and 0.01% Tween 20. Thirdly, the cocktail for amplification was prepared comprising 1 μl of target DNA, 25 μl of 2 × reaction mixture, 3 nmol Hydroxynaphthol blue (HNB), 16 U *Bst* DNA polymercase and H_2O to make a total volume of 50 μl, which was then injected into the detection

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