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Sensitivity of Loop-Mediated Isothermal Amplification Increases 100-Fold after Triton™ X-100 Treatment of *Plasmodium*-Infected Erythrocytes

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Abstract

Highly sensitive and rapid diagnostic methods that should also be appropriate for Point-of-Care (POC) testing are required for Mass Screening and Treatment (MSAT) programs to be effective in blocking transmission of malaria, and eventually eradicating the disease. Loop-Mediated Isothermal Amplification (LAMP) is an accurate, sensitive, rapid, and easy-to-perform method for gene amplification. Preparation of *Plasmodium*-Infected Red Blood Cell (IRBC) samples for the LAMP assay normally includes boiling and/or centrifugation. To increase the chances of success for MSAT programs in endemic countries where resources might be limited, it should become possible to perform the preparation in a short time and without having to resort to expensive equipment. Herein, we report that treating IRBCs with Triton™ X-100 prior to LAMP assay results in 100-fold increase in the test sensitivity. Our findings may contribute to improve the use and efficacy of LAMP assay as a malaria diagnostic test within MSAT programs in endemic countries.

Keywords: Malaria; Diagnosis; LAMP; Triton™ X-100

Introduction

At present, malaria diagnosis is carried out mainly through microscopic examination of thin blood smears and/or antigen-based Rapid Diagnostic Test (RDT) [1]. The establishment of diagnostic methods to be performed easily and effectively in the context of Mass Screening and Treatment (MSAT) programs is a prerequisite for eventual eradication of malaria [2]. In fact, prompt detection and treatment of asymptomatic *Plasmodium*-infected individuals with low parasitemia who might contribute to spread the disease in an endemic area, would reduce malaria transmission and contribute to keep the disease under control. Therefore, it is essential to develop highly sensitive malaria diagnostic methods that should be able to detect sub-microscopic parasitemia [3]. Further, asymptomatic parasite-infected individuals should be screened through Point of Care (POC) testing and treated at once, to increase the success rate of MSAT programs [4].

Loop-Mediated Isothermal Amplification (LAMP) is widely studied as a diagnostic method that might be implemented within MSAT programs [5,6]. LAMP assay can be completed within 1h, and its sensitivity is 10 to 100-fold higher than that of conventional Polymerase Chain Reaction (PCR) when purified DNA is used for the template [4]. As LAMP is performed using three pairs of specific primers for DNA amplification, its specificity is higher than that of PCR [7]. Moreover, as LAMP is performed at around 65˚C, expensive equipment requiring a considerable amount of electricity, such as a thermal cycler, is not required. Finally, the amplification reaction by LAMP can be visually detected using Ultraviolet (UV) lamp or by the naked eye. Therefore, LAMP appears to be a promising method for POC testing.

Recently, Leu, et al. described a parasite species-specific LAMP assay targeting the *18S rRNA* gene [8]. When sensitive differential diagnosis need to be achieved by LAMP assay, researchers normally use as template either genome DNA purified from blood or samples that have been boiled and roughly cleaned up by centrifugation [9,10]. Recently, Modak, et al. reported that high-sensitive LAMP can be performed using blood samples that have been heated at 90˚C for 5 min to lyse blood cells and parasites, releasing target DNA as templates [4]. Because at least two (one to be set at 90˚C, another at 65˚C) heating blocks are necessary for high-throughput analysis by LAMP assay a considerable amount of electricity is required, which might be not abundant or easily available in rather deprived areas or basic health care environments where the test need to be performed. In conclusion, in order to make the LAMP assay more suitable for POC testing, it is very important to establish a method for preparing blood samples without boiling. In this study, we demonstrate that treatment of *Plasmodium*-infected red blood cells (iRBCs) with TritonTM X-100 prior to LAMP assay results in lysis of the cells and a 100-fold increase in sensitivity of LAMP.

Methods

Malaria culture

P. falciparum strain 3D7 was cultured as previously described [11].

LAMP assay

LAMP reactions were performed with Loopamp DNA Amplification Kit and Loopamp Fluorescent Detection Reagent (Eiken Chemical Co., Ltd., Tochigi, Japan) as described in the manufacturer's manual. LAMP primer sets were used to detect the *P. falciparum 18S rRNA* [8]. The LAMP primer sets included a forward inner primer (5'-AGTAGTCCGTCTCCAGAAAATCTTACTTTGGGGGCATTCGTATT-3'), backward inner primer (5'-GCGAAAGCATTTGCCTAATCTATTTAAGATTACGACGGTATCTGATC-3'), loop forward primer (5'- TCACCTCTGACATCTG-3'), loop backward primer (5'-GTTAAGGGAGTGAAGACG-3'), forward outer primer (5'-GCTTAGTTACGATTAATAGGAGTA-3'), and backward outer primer (5'-AGTCGGCATAGTTTATGGT-3').The resulting fluorescence was visualized using Ultra Slim Trans illuminator (Maestrogen Inc., Hsinchu City, Taiwan) and recorded with a digital camera (Stylus TG-4, Olympus Corporation, Tokyo, Japan).

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Results

LAMP assay displays low sensitivity to detect malaria when performed without previous DNA purification

Red blood cells (RBCs) with 50 % hematocrit (Ht) were infected with in vitro cultivated *P. falciparum* at parasitemia of 1 %. The iRBCs were serially diluted from 10-fold to 1,000-fold with RBCs (50 % Ht) from a healthy patient to determine the detection limit of LAMP assay. Each diluted iRBC sample was further diluted 100-fold in distilled water to release the parasite DNA by rupturing the iRBCs through osmotic shock. Then, each sample (1 µL) was added to the LAMP reaction mixture (10.5 µL) prepared as described in the instruction manual, and incubated at 63 °C for 1 h. LAMP was also performed with uninfected RBCs (Ht of 50%) as a negative control. Primers specific to *18S rRNA* gene of P. falciparum were as described previously [8]. LAMP reaction was detected with Fluorescent Detection Reagent by using a UV trans-illuminator. The detection limit of this method was parasitemia of 0.1% (Figure 1A, 1B) .

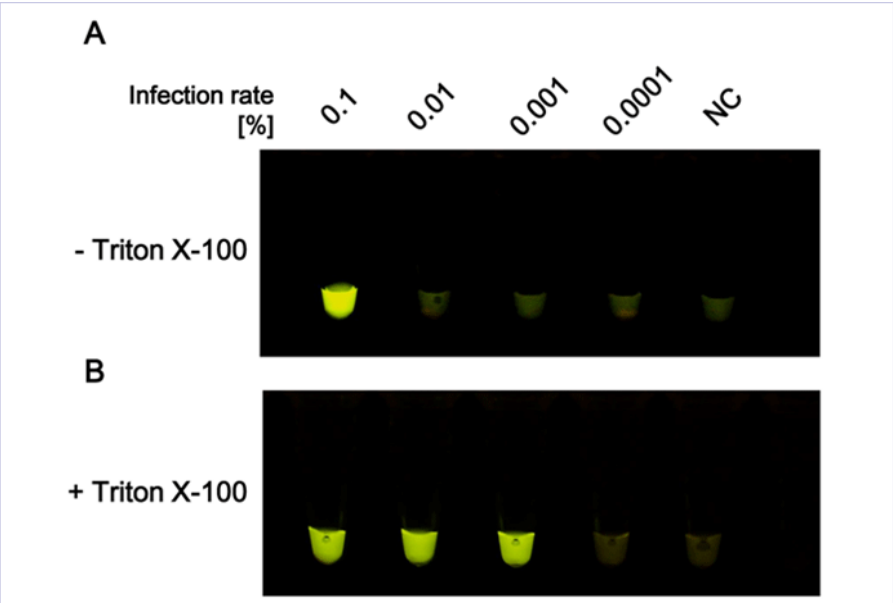

Figure 1: Sensitivity of LAMP assay. LAMP assay detection limit, without (A) or with (B) Triton™ X-100 treatment, was measured using serial dilutions of iRBCs

Triton™ X-100 treatment of *Plasmodium*-infected cells increases LAMP sensitivity in the absence of previous DNA purification

Plasmodium resides in Parasitophorous Vacuole (PV) within RBCs [12]. Although the IRBC membrane is broken by the increase in osmotic pressure caused by the dilution of the RBCs in water, the pressure might be insufficient to break the PV membrane and/or parasite plasma membrane. Therefore, we postulated that treatment of IRBC with a detergent would increase the sensitivity of LAMP assay [13]. This potential effect of a detergent has not been investigated yet. Serially diluted IRBCs (50% Ht) samples were diluted 100-fold in distilled water containing Triton™ X-100 at final concentration 3% (w/v). One microliter

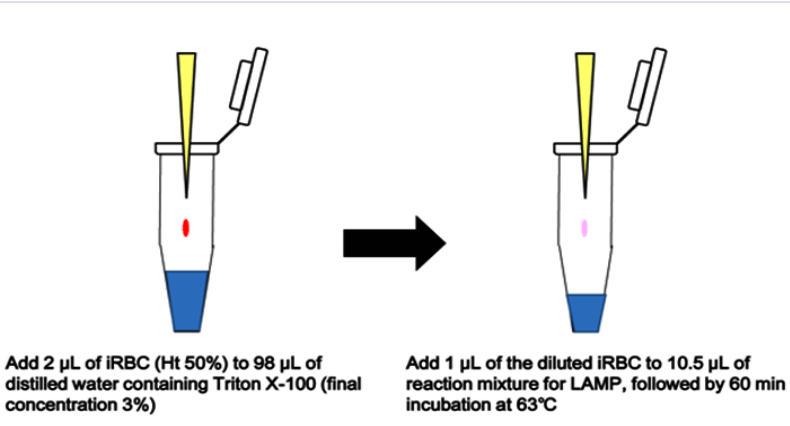

Figure 2: SProtocol for treatment of iRBCs with Triton™ X-100 followed by LAMP assay

of each diluted sample was added to 10.5 µL of LAMP reaction mixture followed by incubation at 63˚C for 1 h. Final concentration of Triton™ X-100 in the reaction mixture was 0.24% (Figure 2). As a result of adding Triton™ X-100 to the reaction mixture, the detection limit became 0.001%, and the sensitivity was found to be increased 100-fold, compared to the LAMP reaction without the detergent (Figure 1B).

Because asymptomatic people should also be screened during MSAT interventions, the place of screening is not limited to hospitals or laboratories with enough experimental instruments and electric energy resources. POC testing may become the key for MSAT programs to succeed, and eventually lead to the elimination of malaria. Therefore, it is very important to establish a LAMP method that does not require any instruments and power supply for centrifugation and/or boiling. Previously, Kemleu, et al. reported the until-date most sensitive method for a reverse transcriptase (RT)-LAMP in a field setting [13]. However, since that method requires reverse transcriptase, RT-LAMP is thus more expensive than LAMP. Furthermore, since RNA is less stable than DNA, the blood samples must be handled with care. Therefore, developing a method for highly sensitive LAMP without the use of reverse transcriptase would be ideal. Triton™ -X100 is one of the most widely used nonionic detergents for lysing cell membranes, a low-priced and relatively safe reagent [14]. Our results suggest that Triton™ X-100 treatment of IRBCs is the key for preparing templates for LAMP assay without the need for redundant equipment and long experimental time, and our findings may be important for establishing a diagnostic method suitable for MSAT.

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