Open Forum Infectious Diseases

MAJOR ARTICLE

A Novel Single-Nucleotide Polymorphism Loop Mediated Isothermal Amplification Assay for Detection of Artemisinin-Resistant *Plasmodium falciparum* Malaria

Abu Naser Mohon,[1,2] Didier Menard,[5] Mohammad Shafiul Alam,[6] Kevin Perera,[2,3] and Dylan R. Pillai[1,2,4]

Departments of [1]Microbiology, Immunology and Infectious Disease, [2]Pathology and Laboratory Medicine, [3]Biological Sciences, and [4]Medicine, University of Calgary, Alberta, Canada; [5]Unité Biologie des Interactions Hôte-Parasite, Institut Pasteur, Paris, France; [6]Parasitology Laboratory, Centre for Vaccine Sciences, International Center for Diarrheal Disease Research, Mohakhali, Bangladesh

Background. Artemisinin-resistant malaria (ARM) remains a significant threat to malaria elimination. In the Greater Mekong subregion, the prevalence of ARM in certain regions has reached greater than 90%. Artemisinin-resistant malaria is clinically identified by delayed parasite clearance and has been associated with mutations in the propeller domain of the *kelch 13* gene. C580Y is the most prevalent mutation. The detection of ARM currently relies on labor-intensive and time-consuming methods such as clinical phenotyping or in vitro susceptibility testing.

Methods. We developed a novel single-nucleotide polymorphism loop mediated isothermal amplification (SNP-LAMP) test method for the detection of the C580Y mutation using a novel primer design strategy.

Results. The SNP-LAMP was 90.0% sensitive (95% confidence interval [CI], 66.9–98.3) and 91.9% specific (95% CI, 82.6–96.7) without knowledge of the parasite load and was 100% sensitive (95% CI, 79.9–100) and 97.3% specific (95% CI, 89.7–99.5) when the parasitemia was within the assay dynamic range. Tests with potential application near-to-patient such as SNP-LAMP may be deployed in low- and middle-income and developed countries.

Conclusions. Single-nucleotide polymorphism LAMP can serve as a surveillance tool and guide treatment algorithms for ARM in a clinically relevant time frame, prevent unnecessary use of additional drugs that may drive additional resistance, and avoid longer treatment regimens that cause toxicity for the patient.

Keywords. artemisinin resistance; loop mediated isothermal amplification (LAMP); malaria diagnosis; molecular; *Plasmodium*.

Since 2001, the World Health Organization has recommended artemisinin combination therapy (ACT) for the treatment of uncomplicated falciparum malaria. However, over the last decade, artemisinin-resistant malaria (ARM) emerged and spread rapidly in Southeast Asia [1–3]. At this time, there are no alternative candidates capable of reaching patients in the next 5 years. The mechanism of artemisinin resistance is partially understood [4, 5]. Artemisinin resistance is clinically defined by the delay of the parasite clearance rate after artemisinin monotherapy or a 3-day regimen of ACT. More specifically, a parasite clearance half-life of more than 5 hours or detection of the circulating parasites by microscopy after 3 days of ACT treatment (day 3 positivity) are considered proxies of resistance [1, 6, 7]. Slow parasite clearance was confirmed for the first time in the Thai-Cambodian border in 2008–2009 [6, 8]. Subsequently, ARM spread throughout the greater Mekong region and as far west as the India-Myanmar border [1, 9]. Multiple single-nucleotide polymorphisms (SNPs) in the propeller domain of a *kelch* gene located on the chromosome 13 (*kelch 13*, Pf 3D7_1343700) have been associated with artemisinin resistance [10].

C580Y and R539T mutations constitute 85% of ARM in Southeast Asia. *Kelch* 13 mutants associated with ARM are confined to Southeast Asia, and their prevalence ranges from 10% to 90% [11]. Currently, the efficacy of alternative therapeutic options including longer treatment periods [12], substituting drugs [3, 13], or increasing the numbers of the partner drugs (triple combination) are being evaluated for ARM [13, 14]. However, overuse of these drugs can increase the risk of toxicity [15] and emergence of multidrug-resistant malaria. First screening for the resistant clones and then providing treatment regimens will provide greater stewardship of existing antimalarials. This is particularly relevant because ARM may eventually spread to or develop de novo in sub-Saharan Africa where over 90% of the malaria cases occur, thereby markedly increasing

Received 20 November 2017; editorial decision 28 December 2017; accepted 8 January 2018.
Correspondence: D. R. Pillai, MD, PhD, Departments of Pathology and Laboratory Medicine, Medicine, and MIID, University of Calgary, 9-3535 Research Road NW, 1W-416, Calgary, AB, Canada, T2L2K8(drpillai@ucalgary.ca).

Open Forum Infectious Diseases®

DOI: 10.1093/ofid/ofy011

morbidity and mortality [16]. Other protein-altering SNPs in the *kelch 13* propeller gene exists in Africa, and therefore risk of treatment failure in Africa also exists [17].

A rapid detection method for ARM in endemic areas could enable clinicians to treat patients with alternative regimens. Current methods to detect ARM such as parasite clearance half-life determination, day 3 positivity, and sequencing of the *kelch 13* propeller gene do not provide clinically actionable results. Detection of ARM SNPs such as C580Y from a fingerprick of blood with a simple molecular test would guide the therapeutic choice for clinicians. Loop mediated isothermal amplification (LAMP)-based methods have been proven to be user-friendly, cost-effective, and deployable near-to-patients [18, 19]. LAMP can be directly visualized by the naked eye because massive deoxyribonucleic acid (DNA) amplification in LAMP leads to accumulation of magnesium pyrophosphate, the byproduct of DNA synthesis, which makes the reaction mixture turbid [20]. Pre- or postaddition of different colorimetric or fluorescent dyes in the reaction mixture further enables the user to observe a color change or fluorescence after the amplification [21, 22]. *Bst* DNA polymerase, a strand-displacing DNA-synthesizing enzyme used in LAMP, is able to tolerate crude, simplified DNA template extraction methods from human specimens and can be prepared as a dry reagent. Simple heat treatment of the blood specimen can provide suitable templates for isothermal amplification [23]. Single-nucleotide polymorphisms (SNPs) associated with chloroquine and sulfadoxine resistance have been successfully detected by SNP-LAMP relying on a special primer designing scheme [24, 25]. We sought to investigate whether SNP-LAMP could be applied to the detection of the predominant ARM SNP, C580Y.

MATERIAL AND METHODS

Samples

Twenty Cambodian *Plasmodium falciparum* positive samples containing the *kelch 13* Y580 propeller mutation and 74 *P. falciparum* samples harboring a *kelch 13* wild-type allele (C580) were obtained from the Institut Pasteur in Cambodia and Matiranga Upazila Health Complex of Khagrachari district in Bangladesh, respectively [26-27]. Clinical sample collections were approved by the institutional Ethics Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b, Ethics number PR-2008-049) and by the Ethics Review Boards of the National Ethics Committee at the National Institute of Public Health, Phnom Penh, Cambodia (Australian New Zealand Clinical Trials Registry, ACTRN12615000793516). The two laboratory-adapted Cambodian strains MRA-1236 (C580) and MRA-1240 (Y580) were provided by the Malaria Research and Reference Reagent Resource Center (MR4) for distribution by BEI Resources, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Deoxyribonucleic Acid Extraction

Deoxyribonucleic acid was extracted from 200 µL whole blood or from 80 µL blood spotted on Whatman 3MM filter papers (GE Healthcare Life Sciences, Marlborough, MA) by QIAamp Blood DNA mini kit (QIAGEN, Valencia, CA). We also used a simple boil-spin DNA extraction method [28]. We mixed 20 µL whole blood with an equal volume of lysis buffer (40 mM Tris-HCl of pH 6.5, 400 mM NaCl, and 0.4% SDS). The mixture was then boiled at 95°C for 5 minutes and subsequently centrifuged at 10 000 ×*g* for 10 minutes after cooling. After centrifugation, 20 µL supernatant was separated for SNP-LAMP application.

Real-Time Polymearse Chain Reaction

Quantitative real-time polymearse chain reaction (PCR) was carried out on DNA extracted from the dried blood spots and *P. falciparum* culture-spiked malaria blood to determine the parasitemia of the positive samples as described previously [27].

Sequencing of *kelch 13* Propeller Gene

The *P. falciparum kelch 13* propeller gene was amplified from the samples and Sanger sequenced bidirectionally by ABI 3730 DNA Analyser (Applied Biosystems, Foster City, CA) as described previously [10].

Loop Mediated Isothermal Amplification Primer Designing

Primers for the SNP-LAMP assay were designed initially by using the Primer Explorer v4 (https://primerexplorer.jp/lamp4.0.0/index.html). Each primer set contains 5 primers, namely F3, B3, F1P, B1P, and LPB. It is notable that our design does not rely on the use of a LPF primer as observed in other LAMP strategies. Although F3, B3, and F1P remain constant in all primer sets, the 3′ end of the B1P and 3′ end of the LPB primer were modified. The 3′ end of the B1P and 3′ end of the LPB primer were designed to simultaneously overlap codon 580 of *kelch 13* (Figure 1). If the mutated nucleotide of the codon is designated "0," mismatches were introduced at position -1, -2, and -3 of both complementary B1P and LPB primers in relation to the wild-type sequence. B1P and LPB primers in Set 30 are a perfect match with the mutant type sequence, Set 31 has one mismatch at position -1, Set 32 has two mismatches at position -1 and -2, and Set 33 has three mismatches at positions -1, -2, and -3 (Table 2). The addition of mismatched nucleotides in these 2 primers were predicted to preferentially amplify mutant over wild type because more mismatches were present when compared with the wild-type sequence. LAMP conditions were optimized as follows: 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, and 0.1% Tween 20 pH 8.8 (Isothermal Buffer, New England Biolabs, Whitby, ON), 8 mM $MgSO_4$, 0.8 M Betaine, and 8 units of *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs, Whitby, ON).

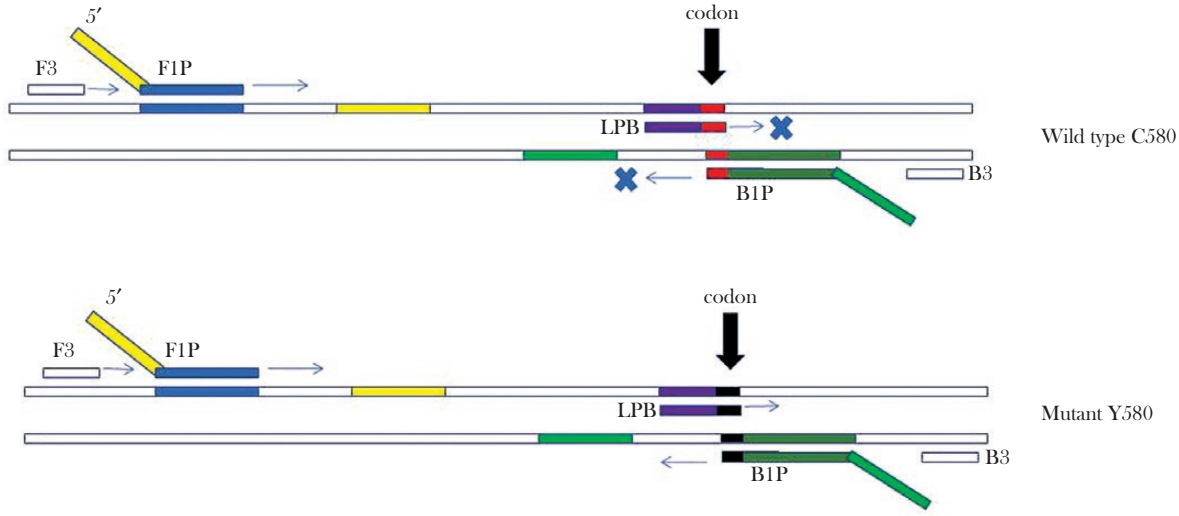

Figure 1. The diagram outlines the primer designing scheme used in this study. Five primers in total are used: F3, F1P, LPB, B1P, and B3. Although F3, F1P, and B3 remain constant in the various primer sets, LPB and B1P are varied. For the wild-type C580, introduction of mismatches in primers overlapping primers LPB and B1P results in failure to amplify (top panel). For the mutant Y580, fewer mismatches are present at the codon, resulting in amplification. Primer sets are highlighted in Table 2. Set 31 provided optimal results with introduction of 1 mismatch nucleotide at position -1 relative to the mutated nucleotide C580Y (TGT→TAT).

Loop Mediated Isothermal Amplification Condition Optimization

To validate the assay, the *kelch* motif of the 2 laboratory-adapted Cambodian strains MRA-1236 (C580) and MRA-1240 (Y580) was amplified by PCR [10]. Purified PCR product concentrations were measured by NanoDrop microvolume spectrophotometer (ThermoFisher Scientific, Waltham, MA) to determine the amplicon copy number. Subsequently, the amplicons were serially diluted within a range of 100–1 000 000 copies per µL. Concentrations of F1P, B1P (0.8–2.4 µM), and LPB (0.2–0.8 µM) primers and reaction temperatures (59–65°C) were altered to find the optimum conditions for *Bst* 2.0 WarmStart DNA polymerase. Final primer concentrations were as follows: 1.6 µM F1P and B1P, 0.4 µM LPB, 0.2 µM F3, and B3 primer concentration in a 25-µL reaction volume. After initial optimization with the PCR amplicons, we spiked uninfected blood with laboratory cultures of MRA-1236 and MRA-1240 and determined the parasite count per microliter. The spiked blood specimens were serially diluted with uninfected blood to obtain a parasite density ranged from 1 to 1 000 000 parasites per µL. DNA was extracted (as described earlier) directly from whole blood and from filter paper dried blood spots. We have used these extracts to optimize the reaction time. Approximately 6 µL of filter paper-extracted DNA was found to be equivalent to 2 µL of whole blood-extracted DNA (data not shown). The SNP-LAMP was initially validated on the CFX-96 real-time system (Bio-Rad, Mississauga, ON). Amplification by *Bst* 2.0 WarmStart DNA polymerase was detected with addition of GelGreen (Biotium Inc., Fremont, CA) after the reaction. Detection of fluorescence was performed using a standard Blue Light Transilluminator (New England Biogroup, Atkinson, NH) and turbidity measurements at 600 nm by NanoDrop at different time points (50, 55, 60, 65, 70, and 75 minutes).

Table 1. Analytical Sensitivity and Specificity of the SNP-LAMP Assay for Detecting C580Y Mutation (n = 94)

Test Method	Sensitivity (%)	95% CI	Specificity (%)	95% CI
SNP-LAMP[a]	90	66.9–98.3	91.9	82.6–96.7
SNP-LAMP[b]	100	79.9–100	97.3	89.7–99.5

Abbreviations: CI, confidence interval; SNP-LAMP, single-nucleotide polymorphism loop mediated isothermal amplifications.

[a]Results obtained without a priori knowledge of parasitemia.

[b]Parasitemia known and optimized to fall within dynamic range of assay.

Loop Mediated Isothermal Amplification Assay on Clinical Specimens

We used between 2 µL of the whole blood extracted DNA and 6-µL filter paper-extracted DNA to perform the SNP-LAMP assay. Assay results were determined by measurement of turbidity by NanoDrop. Cut off value for turbidity was set at optical density = 0.01 because at this turbidity level, three independent readers agreed for a positive call (data not shown). In the second round of testing, to ensure the samples were within the dynamic range of the assay, extracted DNA was either diluted with diethyl pyrocarbonate-treated water (VWR, Edmonton, AB) or input template volume was increased where required to fall within a parasite density ranging from 500 to 100 000 per µL. Low parasitemia specimens containing 370 and 381 parasites per µL were repeated with an increment in the template volume from 6 µL to 10 µL to increase template concentration and fall within the assay range.

Table 2. List of Primers Designed Based on the SNP-LAMP Assay[a]

Primer Name	Sequence
F3 _30(C580Y)	GGTGTTACGTCAAATGGTAGA
B3_30(C580Y)	TTTAATCTCTCACCATTAGTTCC
F1P_30(C580Y)	GCTTTCATACGATGATCATATGCTTCTA-TGTATTGGGGGATATGATGG
B1P_30(C580Y)	ATGGGTAGAGGTGGCACCTT-GACATAAATTTTATTATCAAAAGCAACATA
LPB_30(C580Y)	GAATACCCCTAGATCATCAGCTATGTAT
B1P_31(C580Y)	ATGGGTAGAGGTGGCACCTT-GACATAAATTTTATTATCAAAAGCAAC**T**TA
LPB_31(C580Y)	GAATACCCCTAGATCATCAGCTATG**A**AT
B1P_32(C580Y)	ATGGGTAGAGGTGGCACCTT-GACATAAATTTTATTATCAAAAGCAA**AC**TA
LPB_32(C580Y)	GAATACCCCTAGATCATCAGCTAT**TG**AT
B1P_33(C580Y)	ATGGGTAGAGGTGGCACCTT-GACATAAATTTTATTATCAAAAGCA**CAC**TA
LPB_33(C580Y)	GAATACCCCTAGATCATCAGCTA**ATG**AT

Abbreviations: SNP-LAMP, single-nucleotide polymorphism loop mediated isothermal amplification.

[a]The underlined nucleotide represents the mutated nucleotide (C580Y [TGT→TAT], position "0") in Y580 within the codon. Bold letters denote mismatches introduced at position -1 (Set 31), -2 (Set 32), and -3 (Set 33).

Data Analysis

GraphPad Prism 6.0 software (Graphpad Softare Inc., San Diego, CA) was used to calculate mean, standard error of mean (SEM), and to generate the figures. Mean and SEM were calculated from 2 separate experiments.

RESULTS

Loop Mediated Isothermal Amplification Optimization: Selection of Primer Set and Enzyme

Using the primer design scheme in Figure 1, all primers listed in Table 2 were evaluated. In screening experiments, primer Set 31 was able to distinguish mutant Y580 and C580 across the broadest dynamic range and was chosen for further evaluation. In particular, primer Set 31 could specifically detect mutant templates starting at 50 000 to 1 000 000 copies of the PCR product to distinguish wild-type from mutant in *P. falciparum* culture-spiked blood specimens between 5000 to 100 000 parasites per µL (data not shown). *Bst* 2.0 WarmStart DNA polymerase, *Bst* 3.0 DNA polymerase, and GspSSD2 polymerase enzymes were assessed on the CFX96 real-time detection system. *Bst* 2.0 WarmStart DNA polymerase achieved the broadest dynamic range for specific amplification of mutant Y580 between 100 and 10 000 parasites per µL (Figure 2b). Amplification by GspSSD2 was specific for the mutant and was restricted to 10–1000 parasites per µL (Figure 2a), whereas *Bst* 3.0 DNA polymerase was unable to achieve specific amplification of the mutant at any parasitemia (data not shown). A reaction temperature of 63°C was empirically chosen for these experiments.

Loop Mediated Isothermal Amplification Optimization: Reaction Temperature

To confirm or refute whether 63°C was the optimal temperature for SNP-LAMP, we performed SNP-LAMP on *kelch 13* PCR product templates at various reaction temperatures from 59°C to 65°C with 2°C increments (Figure 3). Amplification specificity of mutant Y580 compared with wild-type C580 was greatest at 63°C with the broadest dynamic range of 10 000–1 000 000 template copy numbers observed. Amplification products were confirmed by gel electrophoresis. A ladder-like pattern typical of LAMP products was observed with a 150-base pair amplicons as predicted from primer designing (data not shown).

Loop Mediated Isothermal Amplification Optimization: Reaction Time

Single-nucleotide polymorphism LAMP was performed using *Bst* 2.0 WarmStart DNA polymerase at 63°C with primer Set 31 on culture-spiked blood specimens (mutant Y580 *kelch13* from laboratory strain MRA 1240 and wild-type MRA 1236

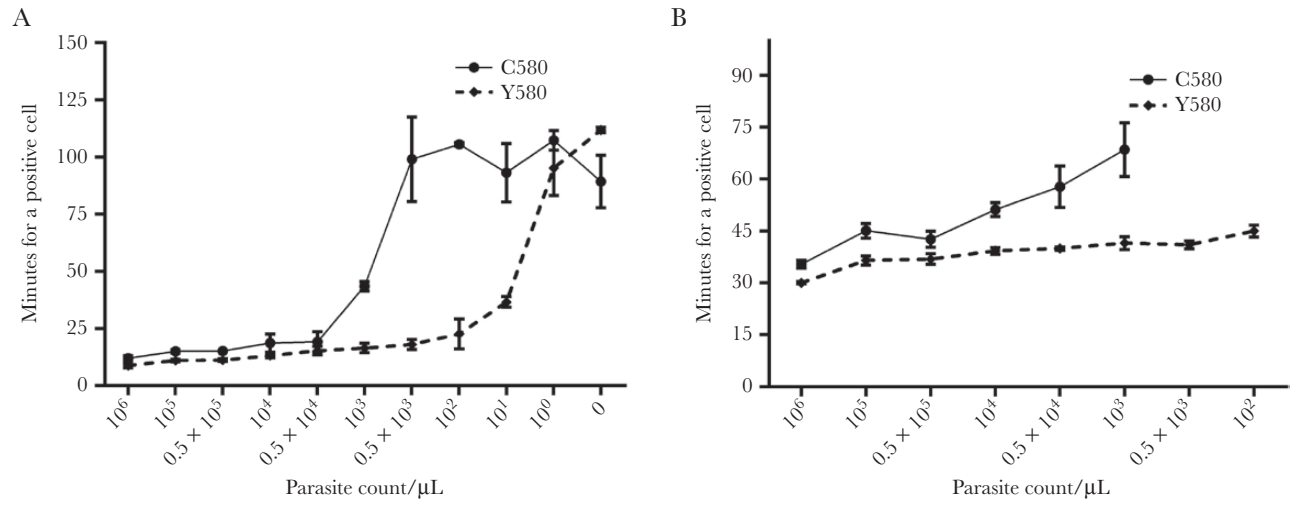

Figure 2. Amplification time (minutes) required for a positive call by 3 different enzymes using single-nucleotide polymorphism loop mediated isothermal amplification for Plasmodium *falciparum*-culture spiked whole blood: (a) GspSSD2 enzyme (63°C); (b) ***Bst*** 2.0 WarmStart DNA polymerase (63°C). These studies were performed on the CFX-96 detection system.

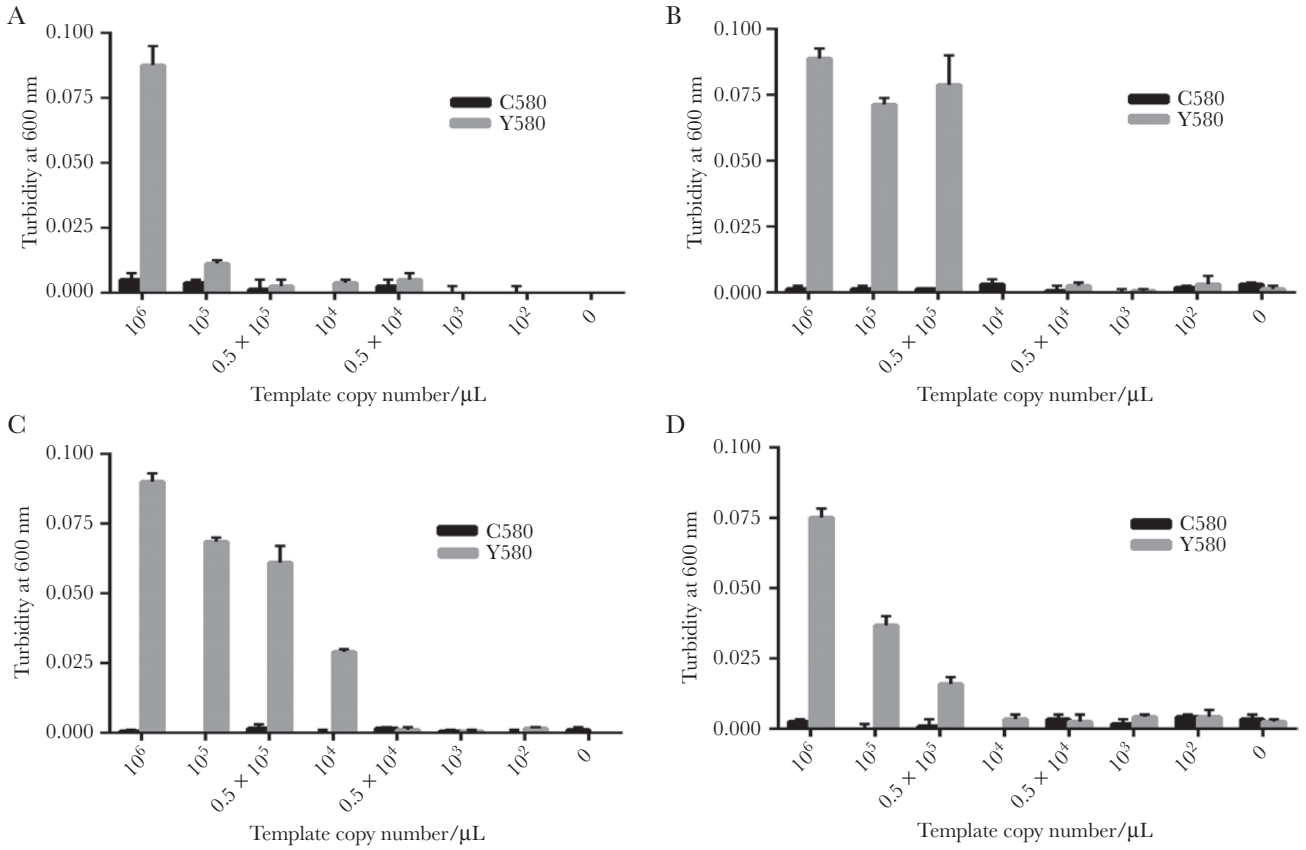

Figure 3. Single-nucleotide polymorphism loop mediated isothermal amplification of mutant Y580 *kelch13* from laboratory strain MRA 1240 (Y580, gray bars) versus wild-type MRA 1236 (C580, black bars) using *Bst* 2.0 WarmStart DNA polymerase at (a) 59°C, (b) 61°C, (c) 63°C, and (d) 65°C for 60 minutes. The template used was a polymerase chain reaction product of the *kelch13* propellor domain with primer set 31.

harboring C580). Turbidity was measured at 600-nm wavelength by NanoDrop measured at different reaction time points ranging from 50 to 75 minutes at 5-minutes increments (Figure 4). Superior discrimination of mutant from wild type was achieved between 500 and 100 000 parasites per μL at 65 minutes. In serial dilution experiments, a limit of detection of 100 parasites per μL was observed (Figure 4). Similar discrimination (dynamic range: 500–100 000 parasites/μL) was achieved by using 3 μL of boil spin extract at 90 minutes (Supplementary Figure 1).

Loop Mediated Isothermal Amplification Optimization: Fluorescence Detection

In an effort to design a naked-eye detection method for SNP-LAMP, 2.0 μL 1000× GelGreen was added after 55 minutes of amplification with *Bst* 2.0 WarmStart DNA polymerase at 63°C. Culture-spiked blood specimens (mutant Y580 *kelch13* from laboratory strain MRA 1240 and wild-type MRA 1236 harboring C580) were used as template. The shorter reaction time was based on validation studies using fluorescence (Supplementary Figure 2). Detection of the Y580 mutant could be specifically achieved by naked-eye detection of fluorescence between 500 and 100 000 parasites per μL for the Y580 mutant (Figure 5).

Confirmation of Specificity and Limit of Detection

The SNP-LAMP assay was conducted on *Plasmodium vivax*, *Plasmodium ovale*, and human DNA. No cross-reactivity was observed (data not shown). Similarly, in the mixed population of wild-type and mutant type parasites, the assay dynamic range remains unchanged with a reliable positive detection between 500 and 100 000 parasites per μL (Supplementary Table 1).

Clinical Sensitivity and Specificity

Based on the series of validation experiments, *Bst* 2.0 WarmStart DNA polymerase with a reaction time of 65 minutes, temperature of 63°C, and turbidity measurement by NanoDrop was used to evaluate clinical specimens from patients. Clinical sensitivity of the SNP-LAMP method for detecting mutant Y580 was 90% (95% confidence interval [CI], 66.87–98.25) with a specificity of 91.89% (95% CI, 82.57–96.67) (Table 1). The SNP-LAMP assay detected six false positives, whereas two Y580 mutant specimens were falsely identified as wild type (false negatives).

The six falsely positive diagnosed samples contained 158 480, 210 000, 261 480, 126 000, 39 800, and 36 280 parasites per μL of whole blood. Because the dynamic range of SNP-LAMP assay is between 500 and 100 000 parasites per μL, four specimens were outside the range. After diluting the samples to fall within the assay dynamic

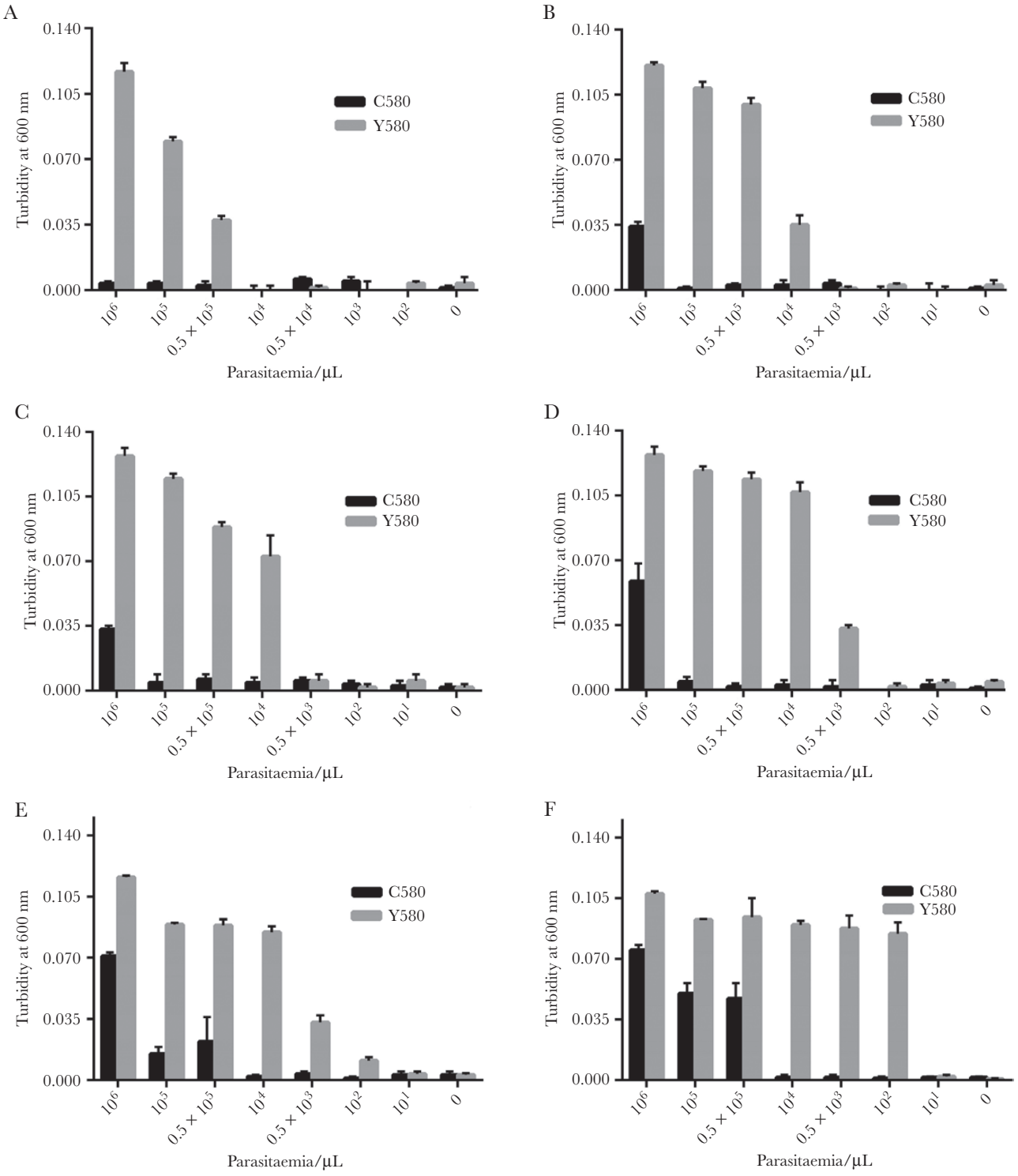

Figure 4. Single-nucleotide polymorphism loop mediated isothermal amplification (SNP-LAMP) of mutant Y580 *kelch13* from laboratory strain MRA 1240 (Y580, gray bars) versus wild-type MRA 1236 (C580, black bars). The SNP-LAMP was performed using *Bst* 2.0 WarmStart DNA polymerase at 63°C with primer set 31. Turbidity was measured at 600-nm wavelength by NanoDrop at different reaction time points: (a) 50 minutes, (b) 55 minutes, (c) 60 minutes, (d) 65 minutes, (e) 70 minutes, and (f) 75 minutes.

range, they became negative. Therefore, only two specimens with parasite density of 39 800 and 36 200 parasites per µL remained false positive. Both false-negative Y580 mutant samples were subsequently detected as positive after increasing input volume.

DISCUSSION

The LAMP-based methods have been recognized for robustness, simplicity, rapidity, and cost effectiveness as a potential near-to-patient molecular test [19, 29, 30]. Recently, LAMP-based

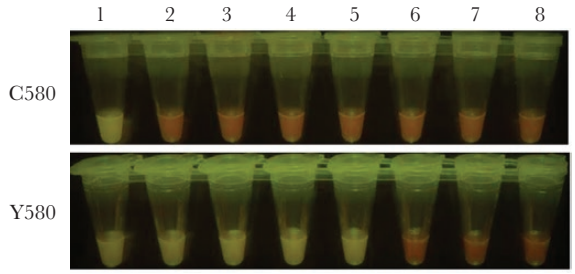

Figure 5. Visual detection of fluorescence (yellow) due to single-nucleotide polymorphism loop mediated isothermal amplification (SNP-LAMP) of mutant Y580 *kelch13* from laboratory strain MRA 1240 (bottom panel) versus wild-type MRA 1236 (top panel). Lack of amplification by SNP-LAMP in this assays results in an orange hue. The following parasitemia was used: (1) 10^6 parasites per µL, (2) 10^5 parasites per µL, (3) 0.5×10^5 parasites per µL, (4) 10^4 parasites per µL, (4) 500 parasites per µL, (6) 100 parasites per µL, (7) 10 parasites per µL, and (8) 0 parasites per µL (uninfected whole blood deoxyribonucleic acid).

methods have been adapted to detect SNPs for genotyping and drug resistance [24, 25, 31]. The SNP-LAMP assays have successfully detected mutations in *P. falciparum chloroquine resistance transporter* gene (*Pfcrt*) for chloroquine resistance [25] and *P. falciparum dihydrofolate reductase* gene (*Pfdhfr*) for pyrimethamine resistance [24]. Our novel SNP-LAMP detection strategy incorporates an overlap between the ends of an initial loop forming primer (B1P) with one of the boosting primers (LPB), introduction of artificial mismatches at the key nucleotides upstream of the mutation, and elimination of the LPF primer. Through a series of validation experiments, we used reaction conditions and enzymes that are geared to detect SNPs for a parasitemia range from 500 to 100 000 parasites per µL, which corresponds with most parasite counts for uncomplicated *P. falciparum*-malaria [27, 32, 33]. The SNP-LAMP assay in this study achieved a sensitivity of 100% and specificity 97.29% for the detection of Y580 associated with ARM.

Six false-positive and 2 false-negative specimens were found in clinical samples. Four of 6 false-positive and both false-negative specimens were resolved after adjusting the parasitemia to the range detectable by the SNP-LAMP assay. In case of the samples where parasite density is lower than 500 per µL, an increment of template volume or concentrating the samples by reducing DNA elution volume from a larger volume of whole blood can increase the chance of mutant detection. The minimum level of detection can be further improved by increasing the reaction time but may increase the likelihood of false positives. In contrast, high parasite density containing samples can be easily diluted to fit within the assay range. A similar primer designing strategy may be used to detect other common mutations in the *kelch 13* propeller gene. Detection of the *P. falciparum kelch 13* propeller gene mutation (SNPs) will be essential for the containment of ARM [34]. This SNP-LAMP assay can provide an indication of slow parasite clearance earlier than conventional methods. Therefore, clinicians may be able to adjust therapeutics based on early resistance diagnosis. The SNP-LAMP assays can be conducted within 2.5 hours from sample to result, in contrast to current in vitro and in vivo methods that may take days and are not provided in a clinically relevant timeframe.

Limitations of the study are severalfold. First, the study is retrospective in design, relying on field collected specimens. A prospective study in an endemic area for ARM would validate the feasibility of this approach in near-to-patient format. Although the assay has the potential to be implemented without significant training and capital equipment needs, it still requires basic knowledge in molecular techniques, centrifugation, and heat block usage. Of note, this type of equipment can be powered by lithium ion battery (our unpublished observations). Optimization of a simplified extraction method is required akin to the gravity flow columns used in certain commercial LAMP kits so that centrifugation can be avoided [35]. The best performance characteristics of the assay were achieved with a prior knowledge of parasite density. This will require reference microscopy facilities near-to-patient. Given these constraints, the assay remains best suited to a hospital setting in endemic areas where laboratory facilities exist or a more advanced health center with an adjoining laboratory. The two major equipment requirements are centrifuge and waterbath, together with training for the technologist.

CONCLUSIONS

The SNP-LAMP assay is highly sensitive and specific for the detection of ARM. This assay can also be used for surveillance of the Y580 mutation in the endemic areas as well. However, more work is required to further evaluate the clinical utility of this test prospectively in ARM-endemic areas such as Cambodia.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Financial support. Funding for this study was provided by Calgary Laboratory Services (Calgary, Canada). A provisional patent was filed on August 1, 2017 for the assay developed in this study (serial number US 62/539,902).

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

References

1. Ashley EA, Dhorda M, Fairhurst RM, et al. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med **2014**; 371:411–23.
2. Mohon AN, Alam MS, Bayih AG, et al. Mutations in *Plasmodium falciparum* K13 propeller gene from Bangladesh (2009–2013). Malar J **2014**; 13:431.
3. Fairhurst RM, Dondorp AM. Artemisinin-resistant *Plasmodium falciparum* malaria. Microbiology Spectr **2016** June; 4(3).

4. Mok S, Ashley EA, Ferreira PE, et al. Drug resistance. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. Science **2015** Jan 23; 347(6220):431–5.
5. Mbengue A, Bhattacharjee S, Pandharkar T, et al. A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. Nature **2015**; 520:683–7.
6. Dondorp AM, Nosten F, Yi P, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med **2009**; 361:455–67.
7. World Health Organization. Global Report on Antimalarial Drug Efficacy and Drug Resistance: 2000–2010. Geneva: World Health Organization; **2010**.
8. Noedl H, Se Y, Schaecher K, et al. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med **2008**; 359:2619–20.
9. Tun KM, Imwong M, Lwin KM, et al. Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet Infect Dis **2015**; 15:415–21.
10. Ariey Fdr, Witkowski B, Amaratunga C, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature **2014**; 505:50–5.
11. Kamau E, Campino S, Amenga-Etego L, et al. K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. J Infect Dis **2015** Apr 15; 211(8):1352–5.
12. Dogovski C, Xie SC, Burgio G, et al. Targeting the cell stress response of *Plasmodium falciparum* to overcome artemisinin resistance. PLoS Biol **2015**; 13:e1002132.
13. Phyo AP, von Seidlein L. Challenges to replace ACT as first-line drug. Malar J **2017**; 16:296.
14. Schallig HD, Tinto H, Sawa P, et al. Randomised controlled trial of two sequential artemisinin-based combination therapy regimens to treat uncomplicated falciparum malaria in African children: a protocol to investigate safety, efficacy and adherence. BMJ Glob Health **2017**; 2:e000371.
15. Angus B. Novel anti-malarial combinations and their toxicity. Expert Rev Clin Pharmacol **2014**; 7:299–316.
16. Ménard D, Khim N, Beghain J, et al. A worldwide map of *Plasmodium falciparum* K13-propeller polymorphisms. N Engl J Med **2016**; 374:2453–64.
17. Bayih AG, Getnet G, Alemu A, et al. A unique *Plasmodium falciparum* K13 gene mutation in Northwest Ethiopia. Am J Trop Med Hyg **2016**; 94:132–5.
18. Mohon AN, Elahi R, Khan WA, et al. A new visually improved and sensitive loop mediated isothermal amplification (LAMP) for diagnosis of symptomatic falciparum malaria. Acta Trop **2014**; 134:52–7.
19. Mohon AN, Lee LD, Bayih AG, et al. NINA-LAMP compared to microscopy, RDT, and nested PCR for the detection of imported malaria. Diagn Microbiol Infect Dis **2016**; 85:149–53.
20. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res **2000**; 28:E63.
21. Tomita N, Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc **2008**; 3:877–82.
22. Tanner NA, Zhang Y, Evans TC Jr. Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. Biotechniques **2015**; 58:59–68.
23. Poon LL, Wong BW, Ma EH, et al. Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clin Chem **2006**; 52:303–6.
24. Yongkiettrakul S, Kampeera J, Chareanchim W, et al. Simple detection of single nucleotide polymorphism in *Plasmodium falciparum* by SNP-LAMP assay combined with lateral flow dipstick. Parasitol Int **2017**; 66:964–71.
25. Chahar M, Mishra N, Anvikar A, Dixit R, Valecha N. Establishment and application of a novel isothermal amplification assay for rapid detection of chloroquine resistance (K76T) in *Plasmodium falciparum*. Sci Rep **2017**; 7:41119.
26. Duru V, Khim N, Leang R, et al. *Plasmodium falciparum* dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations. BMC Med **2015**; 13:305.
27. Alam MS, Mohon AN, Mustafa S, et al. Real-time PCR assay and rapid diagnostic tests for the diagnosis of clinically suspected malaria patients in Bangladesh. Malar J **2011**; 10:175.
28. Polley SD, González IJ, Mohamed D, et al. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. J Infect Dis **2013**; 208:637–44.
29. Tegegne B, Getie S, Lemma W, et al. Performance of loop-mediated isothermal amplification (LAMP) for the diagnosis of malaria among malaria suspected pregnant women in Northwest Ethiopia. Malar J **2017**; 16:34.
30. Sema M, Alemu A, Bayih AG, et al. Evaluation of non-instrumented nucleic acid amplification by loop-mediated isothermal amplification (NINA-LAMP) for the diagnosis of malaria in Northwest Ethiopia. Malar J **2015**; 14:44.
31. Iwasaki M, Yonekawa T, Otsuka K, et al. Validation of the loop-mediated isothermal amplification method for single nucleotide polymorphism genotyping with whole blood. Genome letters **2003**; 2:119–26.
32. Wilairatana P, Tangpukdee N, Krudsood S. Definition of hyperparasitemia in severe falciparum malaria should be updated. Asian Pac J Trop Biomed **2013**; 3:586.
33. Cunnington AJ, Bretscher MT, Nogaro SI, et al. Comparison of parasite sequestration in uncomplicated and severe childhood *Plasmodium falciparum* malaria. J Infect **2013**; 67:220–30.
34. Burnham CD, Leeds J, Nordmann P, O'Grady J, Patel J. Diagnosing antimicrobial resistance. Nat Rev Microbiol **2017**; 15:697–703.
35. Rypien C, Chow B, Chan WW, et al. Detection of *Plasmodium* infection by the illumigene malaria assay compared to reference microscopy and real-time PCR. J Clin Microbiol **2017**; 55:3037–45.