Biosensors and Bioelectronics 62 (2014) 295–301

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Silver quartz crystal microbalance for differential diagnosis of *Plasmodium falciparum* and *Plasmodium vivax* in single and mixed infection

Nantawan Wangmaung [a], Sirinart Chomean [b], Chamras Promptmas [c], Sumana Mas-oodi [a], Dalina Tanyong [a], Wanida Ittarat [a,*]

[a] Clinical Microscopy, Faculty of Medical Technology, Mahidol University, 999 Putthamonthon 4 Road, Salaya, Phutthamonthon, Nakhon Pathom 73170, Thailand
[b] Medical Technology, Faculty of Allied Health Science, Thammasat University, Thailand
[c] Clinical Chemistry, Faculty of Medical Technology, Mahidol University, Thailand

ARTICLE INFO

Article history:
Received 3 April 2014
Received in revised form
23 June 2014
Accepted 24 June 2014
Available online 30 June 2014

Keywords:
Malaria
Biosensor
Quartz crystal microbalance
Mixed malaria infection
Malaria cryptic species

ABSTRACT

The most severe form of malaria is cerebral malaria caused by *Plasmodium falciparum*. Standard malaria diagnosis is Giemsa stained peripheral blood smear but false negative findings are always reported. Moreover, mixed infections are underestimated by routine microscopy. Many methods have been developed to overcome these disadvantages and the most specific and sensitive is molecular diagnosis. Specific malaria genes are amplified by polymerase chain reaction (PCR) and many post-PCR methods have been created. We developed a gold fabricated quartz crystal microbalance (QCM) as a post-PCR method of malaria diagnosis. In this work a cheaper silver fabricated QCM was developed to identify both single and mixed infection of *P. falciparum* and *Plasmodium vivax*. The biotinylated malaria probe was immobilized on silver surface via specific avidin–biotin interaction. The target DNA fragment of 18s rRNA gene was amplified and hybridized with a QCM immobilized probe. Mass changes due to DNA hybridization were indicated by changes of quartz resonance frequencies. Validation showed that malaria silver QCM had high diagnostic potency. Evaluation of suspected 67 febrile blood samples from malaria endemic area demonstrated that the malaria silver QCM could identify both false negative and misdiagnosis cases of routine microscopy. The analysis cost of malaria silver QCM was $1/sample and analysis time was 30 min after blood collection. The malaria silver QCM is stable at tropical temperature for up to 6 months. Thus, it can be transported to be used in a remote endemic area. Thus, the malaria silver QCM is accurate, precise, rapid, cheap, and field applicable.

1. Introduction

Malaria is an infectious disease caused by *Plasmodium* spp. which is transmitted by mosquitoes of *Anopheles* spp. (Bueno-Marí and Jiménez-Peydró, 2013). There are four human *Plasmodium* spp., *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. But the more commonly found are *P. vivax* and *P. falciparum*. Malaria is widely spread in tropical and subtropical regions of the world (Rantala et al., 2010). It was recently estimated that there were 216 million cases of malaria worldwide and 660,000 cases died with severe infection (WHO, 2012). The gold standard of malaria diagnosis is microscopic examination of either thin or thick blood film. However, false negative findings are often reported especially in cases of low parasitemia and mixed infections (Rougemont et al., 2004; Gupta et al., 2010). A more sensitive rapid diagnostic test (RDT) was developed to detect malarial antigen by lateral flow immunochromatographic assay (Murray and Bennett, 2009). Nonetheless, low parasitemia still decreased test sensitivity and the RDTs identified falciparum from only non-falciparum malaria. The most sensitive and specific malaria diagnosis is molecular methods based on malarial DNA amplification by polymerase chain reaction (PCR) (Mangold et al., 2005). Mixed malaria infection can be identified by gene amplifications with appropriate primer designs (Shokoples et al., 2009) and post-PCR analysis. The most widely used post-PCR methods are conventional agarose gel electrophoresis and Southern blot (Manca et al., 2000; Kho et al., 2003). New tools based on gold fabricated quartz crystal microbalance (QCM) were created and applied in genotyping of *P. falciparum* infection (Potipitak et al., 2011). It was also used in differential diagnoses of

* Corresponding author. Tel.: +66 2 441 4370 9x2726; fax: +66 2 441 4380.
E-mail address: wanida.itt@mahidol.ac.th (W. Ittarat).

http://dx.doi.org/10.1016/j.bios.2014.06.052
0956-5663/

P. falciparum and *P. vivax* (Ittarat et al., 2013). In the present study, we developed a silver fabricated QCM to differentially identify single and mixed infection of *P. falciparum* and *P. vivax*. The malaria silver QCM was validated and evaluated in clinical diagnosis. Results were confirmed by RDTs and agarose gel electrophoresis.

2. Materials and methods

This study was approved by the Mahidol Ethical Committee on Institutional Review Broad (MU-IRB 2011/012.0707).

2.1. Blood collection and DNA extraction

The EDTA anticoagulant blood samples requested for malaria diagnosis were collected from malaria endemic area of Thailand. Diagnosis was performed by standard routine microscopy of Giemsa stained blood smear. Suspected samples were collected from febrile cases while controls were from healthy volunteers with no history of malaria infection. Three hundred microliters of each blood sample was subjected to DNA extraction using a PUREGENE Blood core kit (Qaigen, USA) according to the manufacturer's protocols. The extracted DNA was kept at −20 °C until use. The malaria DNA was amplified and analyzed by the new malaria silver QCM and compared to that of agarose gel electrophoresis. Blood samples infected with *P. falciparum* were confirmed by Malaria Ag P.f/Pan Rapid Diagnostic Tests (RDTs) (Standard Diagnostics, INC, Germany).

2.2. Amplification of malaria DNA by multiplex PCR

Primers and a probe specific to 18s rRNA gene of both *P. falciparum* and *P. vivax* were designed using Primer 3 software (GeneBank accession number M19172 and U03079, respectively) and ordered for synthesis (1st BASE, Singapore). The nucleotide sequences of primers and probe are demonstrated in Table 1.

Gene amplifications of both *P. falciparum* and *P. vivax* were performed by multiplex PCR as described previously (Ittarat et al., 2013). The amplification mixture in a total volume of 20 μL consisted of 10 μL of 2× PCR master mix solution (*i*-Taq, 2×) (iNtRON Biotechnology, Korea), 1 μL of each 1 μM primer, 2 μL of extracted DNA and 5 μL of H_2O. The amplification was performed for 35 cycles composed of 1 min of 95 °C denaturation, 1 min of 54 °C annealing, 1 min of 72 °C extension, and 5 min of 72 °C final extension. The amplified products were analyzed by 1% agarose gel electrophoresis at 100 V for 45 min and examined under UV illumination. The amplified target DNA was kept at −20 °C until use.

2.3. Preparation of malaria silver QCM

The QCM used was fabricated out of silver, with 12 MHz specification and 4 mm diameter (Metal Inter Supply Ordinary Partnership, Thailand). The silver surface of QCM was gently cleaned by soaking in 1:10 diluted Piranha solution (Wangmaung et al., 2013). The baseline quartz resonance frequency (f_0) was measured by an in-house oscillation counter (Chomean et al., 2010). Then the clean silver surface was chemically modified by sequentially soaking in 10 mM 3-mercaptopropionic acid (MPA), 20 mM N-(3-diethylaminopropyl)-N′-ethylcarbodiimine (EDC) (Sigma, USA) and 50 mM N-hydroxysuccinimide (NHS) (Sigma, USA). Avidin (Sigma, USA) solution at 0.1 μg/mL was immobilized and the resonance frequency after avidin coating (f_1) was recorded to estimate the immobilized avidin by the Sauerbrey equation (Sauerbrey, 1959).

Probe immobilization was then sequentially performed via the strongest non-covalent bonding of avidin–biotin interaction (Goodwin et al., 1998). The synthesized biotinylated malaria probe at 0.5 μM was coated on avidin-immobilized QCM silver surface (Ittarat et al., 2013). The resonance frequency after biotinylated probe immobilization (f_2) was recorded and its deposited number was also estimated by the Sauerbrey equation.

The preparation of malaria silver QCM was performed by a modified method as previously described (Wangmaung et al., 2013). Drying of QCM silver surface after immobilization and water washing was done by an air-pump at 2.5 psi for 30 min.

To characterize the nature of resonance frequency shifts of each immobilization, the frequency was continuously measured every 10 s. The frequency changes were followed for 5 min after chemical modification as a baseline and after immobilization of avidin and biotinylated probe, respectively.

The QCM immobilized with biotinylated probe was ready to be validated as a tool of malaria molecular diagnosis. The malaria silver QCM was kept in a sealed cap at room temperature until use.

2.4. Validation of malaria silver QCM

Samples of uninfected as well as *P. falciparum* and *P. vivax*-infected blood ($n=3$) were collected to validate the malaria silver QCM. Each genomic DNA was extracted and amplified using three primers in a single PCR reaction. The amplified target DNA was heated at 95 °C for 10 min. The single stranded target DNA (10 μl of 100 μg/ml) was then hybridized with malaria silver QCM at room temperature for 30 min (Ittarat et al., 2013). The silver surface was gently washed with distilled water and dried in a closed box connected to an air pump at 2.5 psi for 30 min (Wangmaung et al., 2013). The resonance frequency (f_3) after hybridization was recorded. The difference of frequency shifts ($\Delta f=f_2-f_3$) of each sample was calculated and analyzed for indication of hybridization reaction. The typical silver QCM frequency after hybridization (f_3) was characterized by continuous measurement every 10 s for 1 h.

The malaria silver QCM was then validated for its potency to detect cryptic *P. falciparum* in mixed species infection of *P. vivax* and *P. falciparum*. The amplified target DNAs of *P. vivax* and *P. falciparum* were mixed at various ratios from 1:1 to 20:1. Then the DNA mixture was hybridized to QCM-immobilized biotinylated probe as described above. The frequency shifts ($\Delta f=f_2-f_3$) of each mixture were determined.

Storage stability of malaria silver QCM was tested by keeping in a sealed cap at room temperature for 0–180 days. During these time intervals, malaria silver QCM was tested for its diagnostic potency using target DNA of uninfected as well as *P. falciparum* and *P. vivax*-infected blood.

2.5. Clinical application of malaria silver QCM

Suspected febrile blood samples ($n=67$) were collected from three endemic areas of malaria in Thailand. Genomic DNA of each sample was extracted and amplified by multiplex PCR. The

Table 1
Sequences of three primers and probes.

	DNA sequences (5′→3′)	Expected size of target DNA (bp)
Malaria forward (FU)	TTTCTTGGATGGTGATGCAT	–
Reverse primer of *P. falciparum* (RF)	ATACTTTTCCTCCGCCGAAAG	445
Reverse primer of *P. vivax* reverse (RV)	TACGTGGGACTGAATTCGG	134
Malaria specific probe	Biotin—AAAAAGGCCGTTTTTAG TTCGTGAA	–

amplified target DNA was analyzed by either agarose gel electrophoresis or by malaria silver QCM. Malaria diagnosis was compared to those of microscopy and commercial RDTs.

2.6. Statistical analysis

All experiments in this study were done in triplicate. The difference among sample groups was analyzed by one way ANOVA. A significant difference was reported when p-value was less than 0.05.

3. Results and discussion

3.1. Amplification of malaria DNA by multiplex PCR

Blood samples were collected from the leftovers after routine microscopic examination. Genomic DNA was extracted and specific target DNA of each *Plasmodium species* was amplified using three primers as shown in Table 1. The PCR products were then analyzed by agarose gel electrophoresis.

As shown in Fig. 1, the amplified product of *P. vivax* was 134 bp (lane 2) while that of *P. falciparum* was 445 bp (lane 3) as expected when primers were designed (Table 1). The sample that contained mixed species infection of *P. vivax* and *P. falciparum* showed two bands at both positions (lane 4) while the uninfected blood sample showed no amplified band of malaria gene (lane 1). The amplified DNA products suggest that the designed malaria primers have no cross-reaction with any of human genes. The product sizes of both *P. vivax* (134 bp) and *P. falciparum* (445 bp) were $\leq$500 bp. This was suitable for development of QCM (Tombelli et al., 2005) whose hybridization occurred on silver surface of tiny reaction area (0.126 mm^2). The optimal size of PCR products would reduce effect of steric hindrance and increase hybridization sensitivity (Tombelli et al., 2005). These amplified products were then used to validate diagnostic potency of the malaria silver QCM developed in this study.

3.2. Preparation of malaria silver QCM

The silver reaction area of QCM was chemically modified and coated with avidin. Then the designed malaria probe (Table 1) was biotinylated and immobilized on silver surface via avidin–biotin interaction. This interaction is the strongest non-covalent bonding suitable for QCM preparation, for which surface cleaning and drying was repeatedly performed many times. The principle of QCM (Fig. 2) use in medical diagnosis is based on correlation between quartz frequency shifts and surface deposited mass changes. Increased surface deposited mass will decrease quartz resonance frequency.

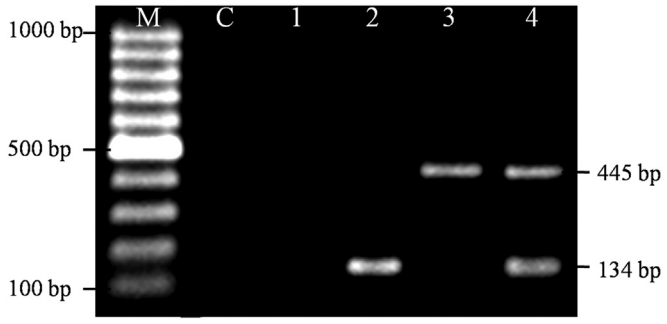

Fig. 1. Amplified target DNA by three malaria primers analyzed by 1% agarose gel electrophoresis in TBE buffer at 100 V for 45 min. Genomic DNA was extracted from uninfected blood sample (lane 1) and infected samples of *P. vivax* (lane 2), *P. falciparum* (lane 3) and mixed species infection (lane 4). C is control with no DNA template and M is molecular weight marker.

The typical biosensor signals of each sequential immobilization were continuously measured every 10 s for 5 min–1 h (Fig. 3). After chemical modification, the baseline frequency of QCM was measured (f_0 = 11986299 ± 83.39 Hz). The frequency was also continuously measured after immobilization of avidin (f_1 = 11986132 ± 84.55 Hz; Δf_1 = 161.5 ± 26.96 Hz) and biotinylated probe (f_2 = 11986077 ± 86.28 Hz; Δf_2 = 208.5 ± 12.96 Hz).

It has long been observed that AT-cut quartz crystal is sensitive to deposited mass changes (Sauerbrey, 1959). The basic equation explaining relationship between frequency change (Δf) and mass change (Δm) was derived by Sauerbrey:

$$\Delta f = \frac{-2f02\Delta m}{A\sqrt{\mu q \rho q}}$$

where Δf is the frequency change, f_0 is baselines frequency in Hz, Δm is the mass change due to the surface deposition (g), A is the reaction area of the quartz crystal (0.126 cm^2 since radius is 0.2 cm), μq is a shear modulus of AT-cut quartz (2.947×10^{11} $g/cm/s^2$) and ρq is the density of quartz (2.648 g/cm^3).

In the present work, frequency shift after avidin immobilization (Δf_1) was 161.5 ± 26.96 Hz (Fig. 3). Therefore, the deposited avidin mass was estimated as follows:

$$\Delta m = -\frac{0.126(-161.5 \pm 26.96)}{2.3 \times 10^{-6}(12^2 \times 10^6 \times 10^6)}$$

$$\Delta m = 61.37 \pm 10.25 \text{ ng}$$

This mass change was used to estimate the number of deposited avidin molecules using the molecular weight of avidin (66 kDa) and Avogadro's number (1.66×10^{24}). There were $5.6 \pm 0.94 \times 10^{11}$ molecules of deposited avidin on the reaction area.

The frequency shift (Δf_2) after probe immobilization was 208.75 ± 12.96 Hz (Fig. 3). Based on the molecular weight of biotinylated probe (86.4 kDa) and by a similar approach, the number of biotinylated probe molecules deposited on the reaction area was estimated to be $1.25 \pm 0.37 \times 10^{12}$.

According to the number of avidin and biotinylated probe molecules immobilized on silver surface, the binding ratio of avidin:biotinylated probe ($5.6 \pm 0.94 \times 10^{11}$:$1.25 \pm 0.37 \times 10^{12}$) is 1:2.2. In general, one molecule of avidin binds 4 molecules of biotin (Gitlin et al., 1988). In this study, avidin was immobilized on the silver surface and biotin was attached with the malaria probes. Therefore, the binding ratio less than 1:4 (1:2.2) as demonstrated is reasonable.

In QCM development, it has been shown that higher quartz resonance frequency would increase test sensitivity by reducing the system noise (Furusawa et al., 2009). We used 12 MHz AT-cut quartz crystal with diameter 0.4 mm to develop the malaria QCM (Wangmaung et al., 2013; Ittarat et al., 2013). Many inert metals have been fabricated on the quartz surface to function as electrodes. These are gold, platinum, and silver (Ram et al., 2001). In this study, we used silver fabricated QCM which is 10 times cheaper than gold fabricated QCM (Wangmaung et al., 2013). Cost effective medical devices are more suitable since malaria endemic areas are mostly in developing countries of Southeast Asia and Africa. However, silver fabricated QCM was easily oxidized by strong acid which is a component of Piranha cleaning solution. Hence 10 times diluted Piranha solution was used to avoid the acid oxidation (Wangmaung et al., 2013).

The development of QCM under vacuum (gas sensor) was first described by King (1964). The use of QCM in liquid environments was then developed (Auge et al., 1994). In liquid environments, the

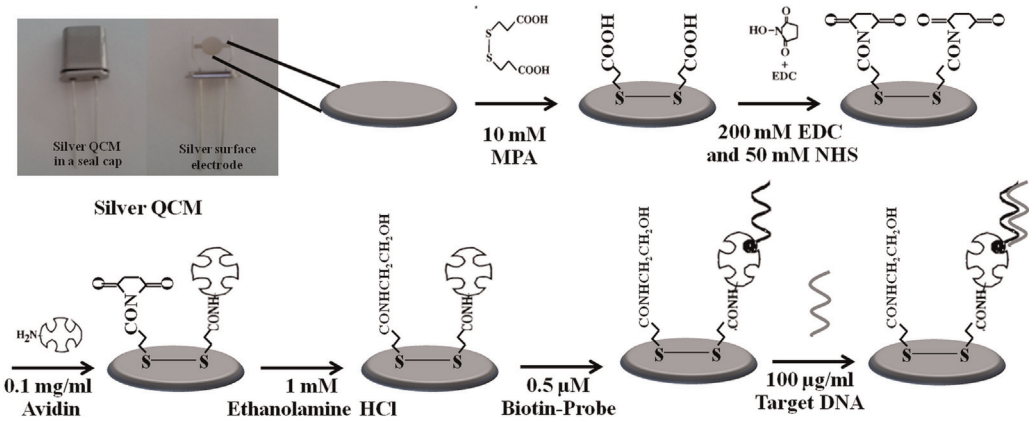

Fig. 2. The principle of QCM preparation. The silver surface was chemically modified by MPA, EDC, and NHS. The modification added carboxyl group to bind with amine group of avidin. Then ethanolamine HCl was added to block the free space area before the biotinylated probe was immobilized via avidin–biotin interaction. The complementary target DNA was hybridized to the specific probe.

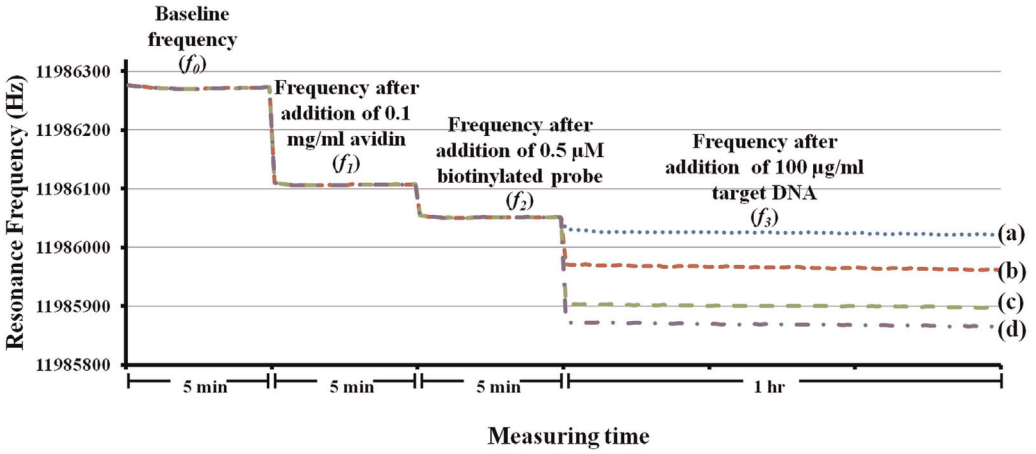

Fig. 3. Typical silver QCM signals after each sequential immobilization of avidin and biotinylated probe. The baseline of quartz frequency and frequencies after immobilization of avidin and biotinylated malaria probes were continuously measured for 5 min. The frequencies after hybridization of target DNA of uninfected samples (a), and samples infected with *P. vivax* (b), mixed infection (c) and *P. falciparum* (d) were also measured every 10 s for 1 h. Each immobilization and continuous measurement were done in triplicate.

QCM resonance frequency is known to be affected by both mass and liquid viscous loads (Ward and Buttry, 1990). In the present study, we prepared malaria silver QCM by the dip-and-dry method. Measurements of frequency shifts were performed after drying QCM under controlled humidity using a vacuum pump at 2.5 psi for 30 min (Wangmaung et al., 2013). Therefore, the mass sensitivity was unaffected by the liquid viscosity. Moreover, the frequency shift of each immobilization step was continuously recorded and compared to control without target DNA and to malaria uninfected samples.

3.3. Validation of malaria silver QCM

The diagnostic potency of malaria silver QCM was validated using blood samples diagnosed for malaria infection. Samples of both malaria-infected and uninfected blood were collected and genomic DNA was extracted. The target DNA sequences were amplified and analyzed by agarose gel electrophoresis and malaria silver QCM. Each 10 µL of 100 µg/mL of target DNA ($n=3$) was hybridized with malaria probes on the silver surface. The frequency shifts after hybridization were recorded and compared (Fig. 4a).

The frequency shifts in Fig. 4a are consistent with size of amplified target DNA (Fig. 1). The longer target DNA of *P. falciparum* (445 bp) generated higher frequency shifts (187.6 ± 2.7 Hz) than that of *P. vivax* (134 bp in Fig. 1 and 79.83 ± 8.89 Hz in Fig. 4a). The mixed species infection showed amplified products of both *P. vivax* and *P. falciparum* (134 and 445 bp) and showed frequency shifts between that of *P. vivax* and *P. falciparum* (151.33 ± 5.86 Hz). Control uninfected blood showed no amplified DNA band and baseline frequency shifts (23.5 ± 4.33 Hz). Statistical analysis indicated significant differences among all frequencies ($p < 0.05$).

Results in Fig. 4a indicate high potency in differential diagnosis of malaria infection by malaria silver QCM developed in this study. Either malaria uninfected or infected blood was clearly differentiated. Either single or mixed infection of *P. vivax* and *P. falciparum* could also be clearly diagnosed.

However, the major clinical significance in cases of mixed infection is potency to detect the cryptic *P. falciparum*. Cryptic infection is mixed infection in which one species cannot be identified at the time of diagnosis because of undetectable low parasitemia (Mayxay et al., 2001). The cryptic *P. falciparum* is important since it can lead to lethal cerebral malaria. It should be noted that 13% of diagnosed *P. vivax* single infection co-existed with untreated cryptic *P. falciparum* infections (Luxemburger et al., 1997; Putaporntip et al., 2009). Therefore, malaria silver QCM was further validated for potency to identify cryptic *P. falciparum*.

The amplified DNAs of *P. vivax* and *P. falciparum* were mixed to mimic mixed infection with cryptic *P. falciparum*. The DNAs were

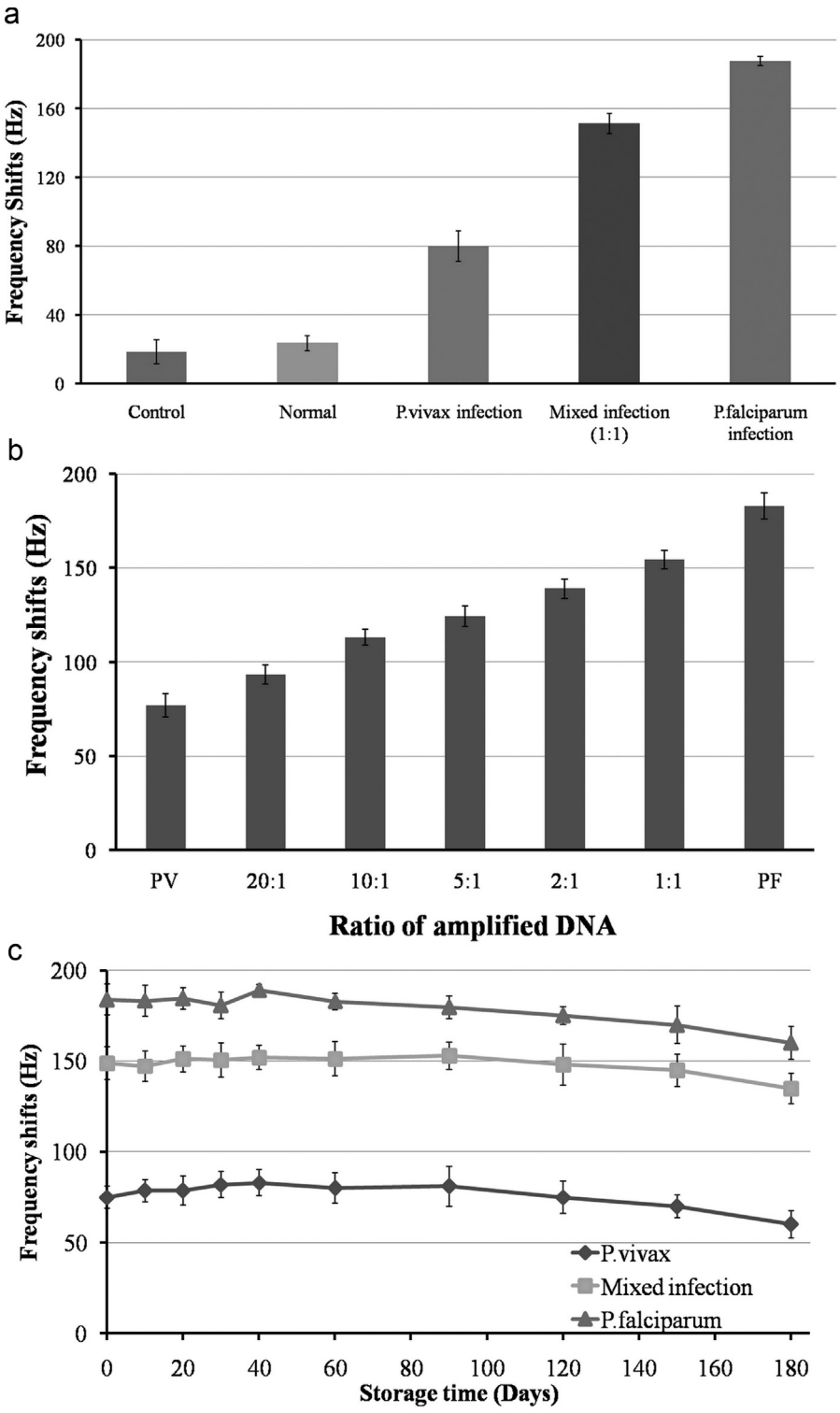

Fig. 4. Validation of the malaria silver QCM. Genomic DNA of malaria-diagnosed blood samples was extracted, amplified and hybridized with malaria silver QCM. The frequency shift after hybridization was recorded (a). Identification of the cryptic *P. falciparum* in mixed species infection was also validated by various ratios of target DNA of *P. vivax* to that of *P. falciparum* (b). Storage stability of malaria silver QCM after keeping at tropical room temperature was validated up to 6 months (c).

mixed at various *P. vivax*:*P. falciparum* ratios (1:1–20:1) and then each sample was analyzed by malaria silver QCM (Fig. 4b). The lowest ratio that could be significantly differentiated was 20:1. This implies that in mixed infection with cryptic *P. falciparum* present less than *P. vivax* for 20 times can still be identified by the malaria silver QCM.

In clinical report, it was demonstrated that in cases of mixed infection the parasitemia of *P. vivax* was 2516 ± 900 parasites/μl blood while that of cryptic *P. falciparum* was 235 ± 100 parasites/μl blood (Mayxay et al., 2001). It could be roughly estimated that in clinical cryptic *P. falciparum* the ratio was around 11:1. Therefore, the ratio of 20:1 as shown in Fig. 4b covered the detectable ranges of real clinical cases. Results indicated potency of the malaria silver QCM to identify cryptic *P. falciparum* in real clinical situations.

The present work is different from our previous work (Ittarat et al., 2013) by novel design of primers' and the corresponding

probe's sequences. This work focused on identification of mixed infection of *P. falciparum* and *P. vivax* in cases where *P. falciparum* was the cryptic species. The amplified DNA target of *P. falciparum* (445 bp) was designed to be longer than that of *P. vivax* (134 bp). Therefore, single infection of *P. vivax* would be diagnosed from co-infection with cryptic *P. falciparum*. The amplified DNA products in previous study (Ittarat et al., 2013) could not be differentially identified in cases of the cryptic *P. falciparum* in mixed infection.

For application in field analysis, storage stability of malaria silver QCM was investigated. The prepared malaria silver QCM was kept at tropical room temperature for 0–180 days. During these time intervals, the QCM was tested for its diagnostic potency using amplified target DNA from samples of uninfected as well as *P. vivax* and *P. falciparum* infected blood. Fig. 4c demonstrates stability of malaria silver QCM after keeping at tropical room temperature for up to 180 days. The frequency shifts of each time interval slowly declined but there was no statistical difference from that of freshly prepared QCM (day 0). When kept for 6 months, the potency of malaria differential diagnosis of QCM was still high. Fig. 4c suggests field applicability of the malaria silver QCM. It could be prepared and transported to be used in remote areas of endemic malaria where gene amplification is available.

All results in Fig. 4 demonstrate high potency of malaria silver QCM in differential diagnosis of both single and mixed infection of *P. vivax* and *P. falciparum*. It showed ability to detect the cryptic *P. falciparum* at clinical level. It could be transported to be used at any malaria endemic area away from production site.

3.4. Clinical application of malaria silver QCM

Suspected malaria febrile blood samples ($n=67$) were analyzed by malaria silver QCM after amplification of malaria target DNA. All of these samples were diagnosed by standard Giemsa stained blood smear and commercial RDTs. The amplified DNA products of all 67 samples were also investigated by agarose gel electrophoresis.

Results of both malaria silver QCM (Fig. 5a) and agarose gel electrophoresis (Fig. 5b) are consistent. They both diagnosed 26 cases of *P. vivax* infection, 25 cases of *P. falciparum* infection, 2 cases of mixed infection and 14 cases of no malaria infection. It should be emphasized that 3 cases (samples 31, 39 and 44) are diagnosed *P. vivax* infection by agarose gel electrophoresis and the malaria silver QCM but are reported as *P .falciparum* infection by microscopic examination. Moreover, 2 cases (samples 62 and 65) were diagnosed as no malaria infection by microscopic examination but the gene analysis reported *P. falciparum* infection. More misdiagnosis of microscopy was found in another 2 cases (samples 24 and 54). No malaria infection was consistently diagnosed by molecular methods while microscopy reported *P. malariae* infection.

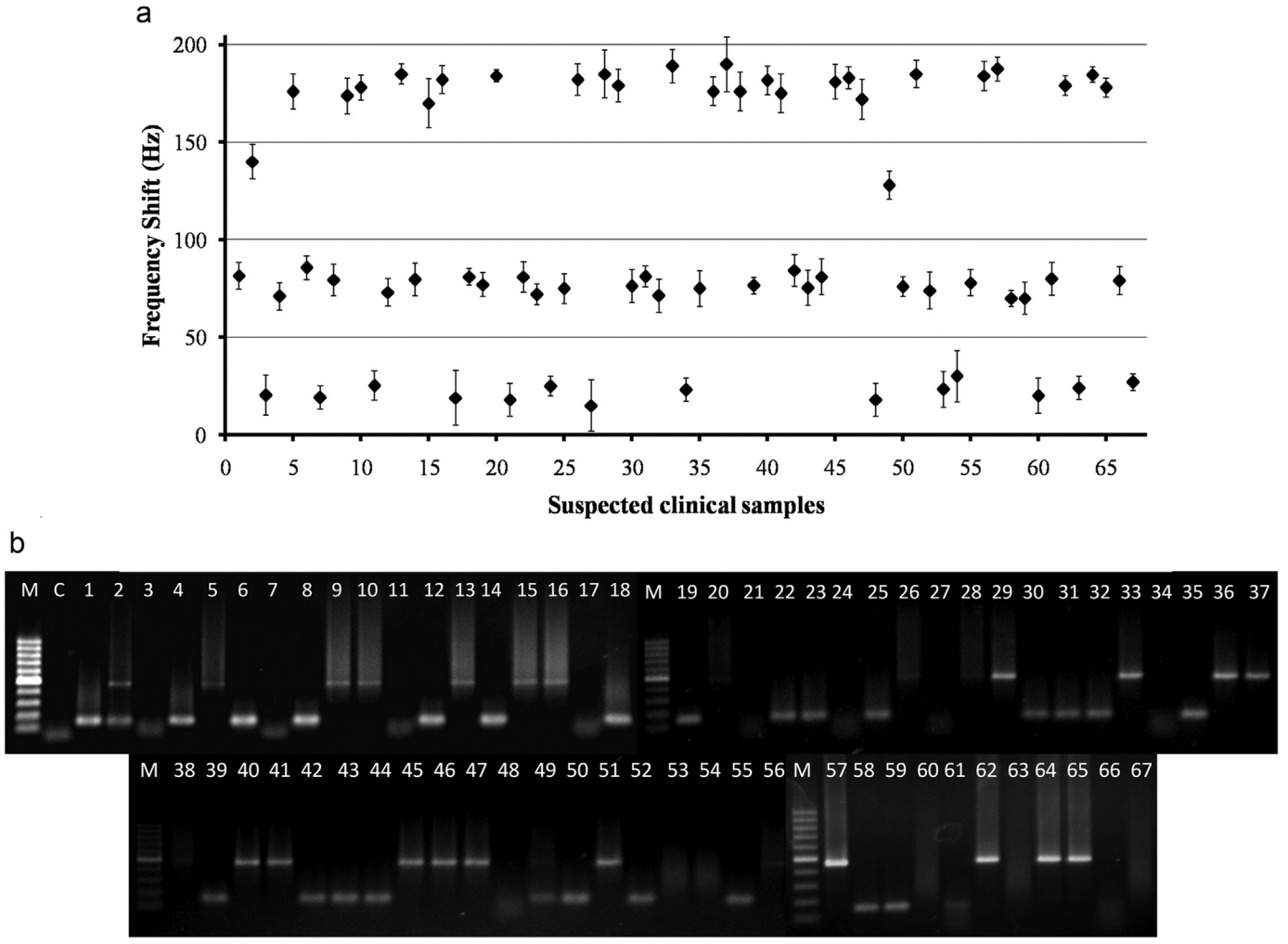

Fig. 5. Evaluation of clinical diagnostic potency of malaria silver QCM. Febrile samples ($n=67$) were collected from malaria endemic area. Malaria target DNA of each sample was amplified and hybridized with malaria silver QCM (a). Analysis of malaria target DNA by agarose gel electrophoresis was performed to confirm the presence of correct DNA target (b).

Results in Fig. 5 demonstrate high false reports of malaria by routine microscopy. This might be related to low parasitemias and inexperienced examiners (Swan et al., 2005). In addition, mixed infections are always undetectable by microscopy.

The RDTs based on identification of the histidine-rich protein 2 (HRP2) antigen was used to confirm the presence of *P. falciparum*, either single or mixed infection. But the RDTs can differentiate only falciparum from non-falciparum malaria by examined number of chromatographic bands (Wongsrichanalai et al., 2007). However, it was reported that cryptic *P. falciparum* infections can be revealed in approximately 75% of cases by HRP2-based RDTs (Koita et al., 2012). Identification of cryptic *P. falciparum* in mixed species infection cannot avoid confirmation at the gene level.

Agarose gel electrophoresis analyzed the amplified target DNA by illumination under UV light after staining with carcinogenic ethidium bromide. Ethidium bromide has disadvantages of being mutagenic and toxic although most uses in the laboratory are below the level required for toxicity (Kirsanov et al., 2010). Also, UV rays have been blamed for the high incidences of skin cancer (Ananthaswamy and Pierceall, 1990). These disadvantages are limitations of using agarose gel electrophoresis in clinical laboratory. However, no hazardous material or reagents are used in development of malaria silver QCM. To be used in malaria diagnosis, blood was collected and DNA was extracted, amplified and hybridized with the QCM. With an improved drying step, all of these processes took not more than 4 h (Wangmaung et al., 2013). The frequency counter can be locally created from general electronic components and costs around $30/unit (Chomean et al., 2010; Potipitak et al., 2011). As shown in our first silver QCM development (Wangmaung et al., 2013) we showed that the detection limit, specificity and stability of the immobilized DNA probes on silver QCM were not different from those of gold QCM. The analysis expense of silver malaria QCM is around $1/case which is 10 × cheaper than that of gold QCM (Wangmaung et al., 2013). The average expense of other conventional PCR based malaria diagnosis such as agarose nested-PCR and real-time PCR was $2.2 (Bass et al., 2008) and $3.3 (Khan et al., 2013), respectively. Moreover, most malaria molecular diagnoses are hard to access because of either requirement of sophisticated devices and personnel expertise (Kho et al., 2003) or of high analysis cost (Swan et al., 2005).

4. Conclusion

A malaria silver QCM was developed to diagnose malaria infection by either *P. falciparum* or *P. vivax*. Also, mixed species infection of both strains was clearly identified especially in cases of cryptic *P. falciparum*. Genomic DNA of tested blood was extracted. Target DNA was amplified by multiplex PCR containing one common forward and two reverse primers, one specific for *P. falciparum* and the other for *P. vivax*. The amplified target DNA was then hybridized with specific probe immobilized on silver QCM. Difference of deposited mass after hybridization induced different quartz frequency shifts which were measured by a locally produced counter. Validation and clinical evaluation demonstrated high potency of molecular diagnosis of malaria silver QCM. It is accurate, precise, simple, rapid and cheap. The malaria silver QCM is stable at tropical temperature for up to 180 days, indicating its field applicability. The malaria silver QCM is suitable for malaria endemic area where sophisticated and expensive medical care is hardly accessible.

Acknowledgment

This work was supported by the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative. It was also partially supported by 2V Research Program 2010 from National Research Council of Thailand (88/2554).

References

Ananthaswamy, H.N., Pierceall, W.E., 1990. Photochem. Photobiol. 52, 1119–1136.
Auge, J., Hauptmann, P., Eichelbaum, F., Rösler, S., 1994. Sens. Actuators B Chem. 18–19, 518–522.
Bass, C., Nikou, D., Blagborough, A.M., Vontas, J., Sinden, R.E., Williamson, M.S., Field, L.M., 2008. Malar. J. 7, 177.
Bueno-Marí, R., Jiménez-Peydró, R., 2013. J. Arthropod Borne Dis 7, 147–153.
Chomean, S., Potipitak, T., Promptmas, C., Ittarat, W., 2010. Clin. Chem. Lab. Med. 48, 1247–1254.
Furusawa, H., Ozeki, T., Morita, M., Okahata, Y., 2009. Anal. Chem. 81, 2268–2273.
Gitlin, G., Bayer, E.A., Wilchek, M., 1988. Biochem. J. 250, 291–294.
Goodwin, D.A., Meares, C.F., Osen, M., 1998. J. Nucl. Med 39, 1813–1818.
Gupta, B., Gupta, P., Sharma, A., Singh, V., Dash, A.P., Das, A., 2010. Trop. Med. Int. Health 15, 819–824.
Ittarat, W., Chomean, S., Sanchomphu, C., Wangmaung, N., Promptmas, C., Ngrenngarmlert, W., 2013. Clin. Chim. Acta 419, 47–51.
Khan, S.A., Ahmed, S., Mushahid, N., Anwer, M., Saeed, S., Khan, F.A., Shamshad, G. U., Joyia, Z., 2013. J. Coll. Phys. Surg. Pak 23, 787–792.
Kho, W.G., Chung, J.Y., Sim, E.J., Kim, M.Y., Kim, D.W., Jongwutiwes, S., Tanabe, K., 2003. Parasitol. Int. 52, 229–236.
King, W.H., 1964. Anal. Chem. 36, 1735–1739.
Kirsanov, K.I., Lesovaya, E.A., Yakubovskaya, M.G., Belitsky, G.A., 2010. Mutat. Res. 699, 1–4.
Koita, O.A., Doumbo, O.K., Ouattara, A., Tall, L.K., Konaré, A., Diakité, M., Diallo, M., Sagara, I., Masinde, G.L., Doumbo, S.N., Dolo, A., Tounkara, A., Traoré, I., Krogstad, D.J., 2012. Am. J. Trop. Med. Hyg. 86, 194–198.
Luxemburger, C., Ricci, F., Nosten, F., Raimond, D., Bathet, S., White, N.J., 1997. Trans. R. Soc. Trop. Med. Hyg. 91, 256–262.
Manca, N., Viani, I., Perandin, F., Piccolo, G., Calderaro, A., Galati, L., Ricci, L., Dettori, G., Turano, A., Chezzi, C., 2000. New Microbiol. 23, 339–346.
Mangold, K.A., Manson, R.U., Koay, E.S., Stephens, L., Regner, M., Thomson Jr., R.B., Peterson, L.R., Kaul, K.L., 2005. J. Clin. Microbiol. 43, 2435–2440.
Mayxay, M., Pukritrayakamee, S., Chotivanich, K., Imwong, M., Looareesuwan, S., White, N.J., 2001. Am. J. Trop. Med. Hyg. 65, 588–592.
Murray, C.K., Bennett, J.W., 2009. Interdiscip. Perspect. Infect. Dis 2009, 1–7.
Potipitak, T., Ngrenngarmlert, W., Promptmas, C., Chomean, S., Ittarat, W., 2011. Diagnosis and genotyping of Plasmodium falciparum by a DNA biosensor based on quartz crystal microbalance (QCM). Clin Chem Lab Med. 49 (8), 1367–1373.
Putaporntip, C., Hongsrimuang, T., Seethamchai, S., Kobasa, T., Limkittikul, K., Cui, L., Jongwutiwes, S., 2009. J. Infect. Dis. 199, 1143–1150.
Ram, M.K., Bertoncello, P., Ding, H., Paddeu, S., Nicolini, C., 2001. Biosens. Bioelectron. 16, 849–856.
Rantala, A.M., Taylor, S.M., Trottman, P.A., Luntamo, M., Mbewe, B., Maleta, K., Kulmala, T., Ashorn, P., Meshnick, S.R., 2010. Malar. J. 9, 269.
Rougemont, M., Van Saanen, M., Sahli, R., Hinrikson, H.P., Bille, J., Jaton, K., 2004. J. Clin. Microbiol. 42, 5636–5643.
Sauerbrey, G., 1959. Z. Phys 155, 206.
Shokoples, S.E., Ndao, M., Kowalewska-Grochowska, K., Yanow, S.K., 2009. J. Clin. Microbiol. 47, 975–980.
Swan, H., Sloan, L., Muyombwe, A., Chavalitshewinkoon-Petmitr, P., Krudsood, S., Leowattana, W., Wilairatana, P., Looareesuwan, S., Rosenblatt, J., 2005. Am. J. Trop. Med. Hyg. 73, 850–854.
Tombelli, S., Minunni, M., Mascini, M., 2005. Methods 37, 48–56.
Wangmaung, N., Promptmas, C., Chomean, S., Sanchomphu, C., Ittarat, W., 2013. Clin. Chem. Lab. Med. 51, 1199–1205.
Ward, M.D., Buttry, D.A., 1990. Science 249, 1000–1007.
Wongsrichanalai, C., Barcus, M.J., Muth, S., Sutamihardja, A., Wernsdorfer, W.H., 2007. Am. J. Trop. Med. Hyg. 77, 119–127.
World Helath Organization, 2012. World Malaria Report: Medical Surveillance Monthly Report (MSMR).