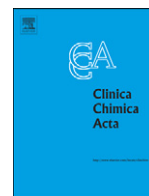




Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

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Biosensor as a molecular malaria differential diagnosis

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ARTICLE INFO

Article history:

Received 16 October 2012

Received in revised form 9 January 2013

Accepted 26 January 2013

Available online xxxx

Keywords:

Plasmodium

Malaria

Biosensor

Quartz crystal microbalance

Molecular diagnosis

ABSTRACT

Background: In malaria diagnosis, specific gene identification is required in cases with subclinical infection or cases with mixed infection. This study applied the biosensor technology based on quartz crystal microbalance (QCM) to differentially diagnose the most common and severe malaria, *Plasmodium falciparum* and *Plasmodium vivax*.

Method: The QCM surface was immobilized with malaria biotinylated probe. Specific DNA fragments of malaria-infected blood were amplified. Hybridization between the amplified products and the immobilized probe resulted in quartz frequency shifts which were measured by an in-house frequency counter. Diagnostic potency and clinical application of the malaria QCM were evaluated.

Result: The malaria QCM could differentially diagnose blood infected with *P. falciparum* from that infected with *P. vivax* (p -value < 0.05). No cross reaction with human DNA indicated the QCM specificity. Clinical application was evaluated using 30 suspected samples. Twenty-seven samples showed consistent diagnosis of the QCM with microscopy and rapid diagnosis tests (RDTs). Three samples reported "no malaria found" by microscopy showed *P. falciparum* infection by both QCM and the RDTs.

Conclusion: The malaria QCM was developed with high accuracy, specificity, sensitivity, stability and cost-effectiveness. It is field applicable in malaria endemic area and might be a promising point of care testing.

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1. Introduction

Malaria is a major infectious disease widely spread in tropical and subtropical regions. The World Health Organization (WHO) has put many efforts to control malaria leading to the significant reduction of malaria cases. However, transmission of about 30,000 malaria cases is still reported annually from non-endemic area, including industrialized countries [1]. Early and accurate diagnosis is needed to prevent severe cerebral malaria and death resulted from *Plasmodium falciparum* infection. Microscopic identification of the intraerythrocytic malaria is still the gold standard and commonly used in malaria diagnosis. Nonetheless, microscopy requires expertise and fails to detect mixed infections when one specie is present at low levels [2]. Therefore, molecular malaria diagnosis has been developed, mostly in reference centers. Several different polymerase chain reaction (PCR)-based methods have been reported [3–6]. Most of them have shown to be more sensitive than microscopy while some are able to detect mixed infections and infections with low parasite density [5,6]. Most of post-PCR identification used in previous reports was agarose gel electrophoresis [7,8]. However, this technique

needs staining with the carcinogenic ethidium bromide. This study aimed to use DNA biosensor based on quartz crystal microbalance (QCM) as the post-PCR analysis to differentially identify between *P. falciparum* and *Plasmodium vivax*. The QCM is a simple, sensitive, specific, and label-free technique and has been used in clinical applications [9] such as in diagnosis of α -thalassemia 1 (SEA deletion) [10,11], β -thalassemia [12], and *Vibrio cholerae* infection [13]. It was also applied in genotyping of *P. falciparum* [14]. Most QCMs used in previous works were gold-fabricated quartz surface. This study used silver fabricated QCM in order to reduce the production price. The cost effective molecular diagnosis is required in developing countries which are malaria endemic and epidemic areas [15]. The QCM was constructed using a specific avidin–biotin interaction to immobilize the malaria oligonucleotide probe on quartz silver electrode. Hybridization was assessed from shifts of the quartz oscillation frequencies due to surface mass changes. The malaria QCM potency in differential diagnosis of *P. falciparum* and *P. vivax* was evaluated and firstly reported in this study.

2. Material and method

2.1. Instrumentation and reagents

The immobilizing chemicals, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

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(EDC) were from Fluka (USA). Hydrogen peroxide, disodium ethylenediaminetetra-acetic acid, ethanol and all the reagents of buffers were from Merck (Italy). Three different buffers used were HEPES buffer (0.05 mol/l HEPES and 0.2 mol/l NaCl, pH 7.5); probe immobilization buffer (300 mmol/l NaCl, 20 mmol/l Na_2HPO_4 and 0.1 mmol/l EDTA, pH 7.4) and hybridization buffer (150 mmol/l NaCl, 20 mmol/l Na_2HPO_4 , and 0.1 mmol/l EDTA, pH 7.4). Other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO).

The specific malaria oligonucleotide probe and primers were synthesized by 1st BASE Pte Ltd (Singapore). The probe was designed using Primer 3 software [16] and tagged-biotin at the 5' end (5'-biotin-TTT TTT CAT AGG AAG GCA GCA GG-3').

The complementary *Plasmodium* target DNA was amplified by polymerase chain reaction (PCR) using 3 designed primers [17]. They are forward primer (FU: GGA GAG GGA GCC TGA GAA AT), reverse vivax primer (RV: AGC CGA AGC AAA GAA AGT CCT TA) and reverse falciparum primer (RF: AAA CCA AAA ATT GGC CTT GC).

The quartz crystals, 12 MHz AT-cut with 4 mm silver electrode (0.1257 cm^2 area), were from Kyocera-Kinseki Company (Thailand). Both sides of quartz silver surface were fabricated but each side was immobilized and analyzed in each measurement. The quartz resonance frequency (Hz) was recorded using an in-house oscillation counting device which was locally developed [18].

2.2. Specimen preparation

This study was reviewed and approved by the Mahidol University Institutional Review Board (MU-IRB 2011/012.0707). All malaria infected blood samples were collected from patients who live in the endemic area and have been diagnosed by Giemsa-stained thin and thick blood films by the certified microscopists. Both parasite species and density were determined. Uninfected blood was similarly collected and used as control.

Blood samples were collected by venipuncture and kept in the sterile tube containing EDTA as an anticoagulant. The EDTA blood (50 μl) was spotted onto three pieces of filter papers. The filter papers were air dried at room temperature and kept in a zip lock bag [14]. Patient code, sex, microscopic diagnosis and parasitemia were labeled. The filter papers were then transported to the QCM laboratory via a regular mail.

2.3. DNA extraction from blood spots

Malaria DNA from blood spots was extracted by Tris–EDTA buffer-based extraction as previously described [14,19]. Briefly, all areas of the blood spot were cut, chopped into small pieces and put in a microcentrifuge tube. The 65 μl of TE buffer was added and incubated at 50 °C for 15 min. To extract the malaria DNA, paper pieces were gently pressed for several times and heated immediately to 95 °C for 15 min. The eluted DNA could be used within a few hours or stored at –20 °C until used.

2.4. DNA amplification by the polymerase chain reaction (PCR)

The PCR reactions of both *P. falciparum* and *P. vivax* infected blood samples were optimized and performed in one reaction tube. The 3 primers, F, RF and RV were added in reaction tube containing malaria template genome and essential amplification reagents. One PCR reaction mixture (20 μl) consists of 5 μl of H_2O , 10 μl of PCR Master Mix Solution (Intron Biotechnology, Seongnam, Korea), 1 μl of each primer (5 $\mu\text{mol/l}$) and 2 μl of extracted DNA. The cycling reaction was performed in a DNA Engine Peltier Thermal Cycler (BIO-RAD, Hercules, CA). The DNA amplification was performed by gradient PCR with varying annealing temperature ranging from 51–60 °C. Then each PCR product was analyzed by agarose gel electrophoresis to obtain the optimal annealing temperature. Agarose gel electrophoresis was performed using 1% agarose gel in 0.5 $\times$ Tris/Borate/EDTA (TBE) buffer, at 100 V for 45 min. Then the amplified DNA was visualized under UV light [7,8]. The PCR products were kept at –20 °C until used. The uninfected blood collected on filter paper was used to test the specificity of the PCR reaction. DNA of uninfected blood was extracted and amplified under same condition as of malaria infected blood. The amplified product was analyzed by agarose gel electrophoresis and by the developed malaria QCM.

2.5. Preparation of the malaria QCM

To reduce the strong acid oxidation, the silver electrode of quartz crystal was cleaned by the low acid strength Piranha solution (1:10 dilution of the conventional solution consisting of 3 parts of H_2SO_4 and 1 part of H_2O_2) as previously described [20]. It was then thoroughly washed with distilled water and dried. The baseline resonance frequency (f_0) was recorded. The clean silver surface was then soaked in 10 mM of mercaptopropionic acid (MPA) and rinsed with distilled water. The MPA coated surface was further treated with 20 mM EDC and 50 mM NHS, respectively.

The optimal concentration of avidin solution (0.1 mg/ml) as described previously [10,14,20] was spread on the silver surface and incubated for 30 min. After washing and drying, the resonance frequency (f_1) was recorded and used to calculate the avidin deposited mass and number by a Sauerbrey equation [21]. The avidin-coated silver surface was immobilized with the biotinylated probe at optimal concentration as described previously [10,14,20]. The resonance frequency (f_2) was recorded and used to calculate the mass and number of immobilized biotinylated probe. The QCM immobilized with biotinylated probe was used as the malaria QCM and was kept in a sealed cap at room temperature until used.

Each immobilization step was incubated at room temperature for 30 min in dark moist chamber to prevent solution evaporation. Drying process was done in a closed box connected to a MILLIPORE® air-pump (BENTON HARBOR, MI, USA) at 2.5 psi for 30 min [20]. The preparation of malaria silver QCM was summarized and illustrated in Fig. 1.

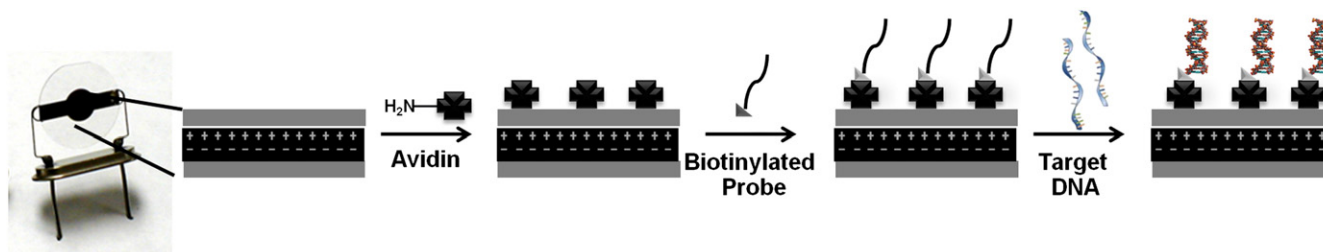


Fig. 1. The QCM preparation.

2.6. Evaluation of the malaria QCM

The genomic DNA from either *P. falciparum* ($n=3$) or *P. vivax* ($n=3$) infected blood was amplified, denatured at 95 °C for 10 min and cooled on ice. The optimal concentration of the amplified target DNA (10 µl of 50 µg/ml) was hybridized with the malaria QCM. After hybridization the QCM surface was gently washed and dried by an air-pump. The resonance frequency (f_3) was recorded. The frequency shifts (f_2-f_3) were compared and used in differential diagnosis. Uninfected blood samples were used as control to test for specificity of the malaria QCM.

The malaria QCM was evaluated for its detection limits by using amplified target DNA from samples infected with either *P. falciparum* or *P. vivax* at various concentrations (0, 10, 20, 40, 50 and 100 µg/ml). Each concentration was individually analyzed ($n=3$) by the malaria QCM and each frequency shift (f_2-f_3) was measured.

Storage stability of the malaria QCM was tested by keeping the probe immobilized QCM in a sealed cap at room temperature at various time intervals (0, 7, 14, 21, 28 and 60 days). Then the diagnostic ability was evaluated using target DNA amplified from blood infected with either *P. falciparum* ($n=3$) or *P. vivax* ($n=3$). The frequency shifts at each keeping time were recorded and compared to that of the freshly prepared malaria QCM.

2.7. Clinical application of the malaria QCM

The clinical diagnostic potency of the malaria QCM was tested using 30 malaria suspected blood samples from endemic area. All samples were collected as blood spots on the filter papers. The DNA of each sample was extracted and specifically amplified using three primers as mentioned above. Optimal concentration of the amplified product was hybridized with the immobilized biotinylated probe on the QCM surface and the frequency shifts were measured. Results of the QCM were compared with agarose gel electrophoresis [10,14,20] and the commercial test kit (rapid diagnosis tests; RDTs) [22].

2.8. Statistic analysis

All data shown in this study were means of the triplicate experiments. All comparison data were analyzed by a 2-tailed Student's *t*-test.

3. Results and discussion

3.1. DNA amplification by the polymerase chain reaction (PCR)

DNA of the blood spots on filter papers was extracted and amplified using three designed primers under optimized PCR condition. The optimized DNA amplification used in this study was composed of DNA denaturation at 95 °C for 5 min, 45 cycles of DNA extension consisting of 95 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, followed by an additional extension at 72 °C for 5 min, and hold on 4 °C. The amplified product of either *P. falciparum* or *P. vivax* was analyzed by 1% agarose gel electrophoresis in TBE buffer at 100 V for 45 min and was visualized under the UV lamp. Uninfected human blood was amplified and analyzed under the same condition.

As shown in Fig. 2A, the amplified product of *P. vivax* was 392 bp while that of *P. falciparum* was 120 bp. These PCR products were consistent with the expected size when the primers were designed. The uninfected human blood and the control without DNA template showed no DNA band. The agarose gel analysis indicated an accurate primers design and the optimal PCR condition with no cross amplification of human DNA. The PCR conditions were used in all experiments.

It has been reported that in the development of QCM the tested DNA fragment should not be larger than 500 bp due to the possible steric hindrance effects on small reaction area of the QCM surface [23]. This study designed primers to obtain amplified products at 392 and 120 bp which are suitable for the development of malaria QCM.

3.2. Preparation of the malaria QCM

This study used 12 MHz AT-cut quartz crystal to create the malaria QCM since it provides a nearly zero frequency drift with room temperature and a high sensitivity to the surface deposited mass [24]. To reduce the production expense, the silver fabricated QCM was used instead of the standard gold fabricated QCM [14]. The silver is 10 times cheaper than the gold QCM. The cheap diagnosis is suitable for malaria endemic and epidemic areas where most of the population is poor and hard to access the expensive and sophisticated medical care [25]. However, silver is sensitive to strong acid oxidation leading to dark silver QCM surface [26] which interferes the QCM measurement. Using the silver QCM

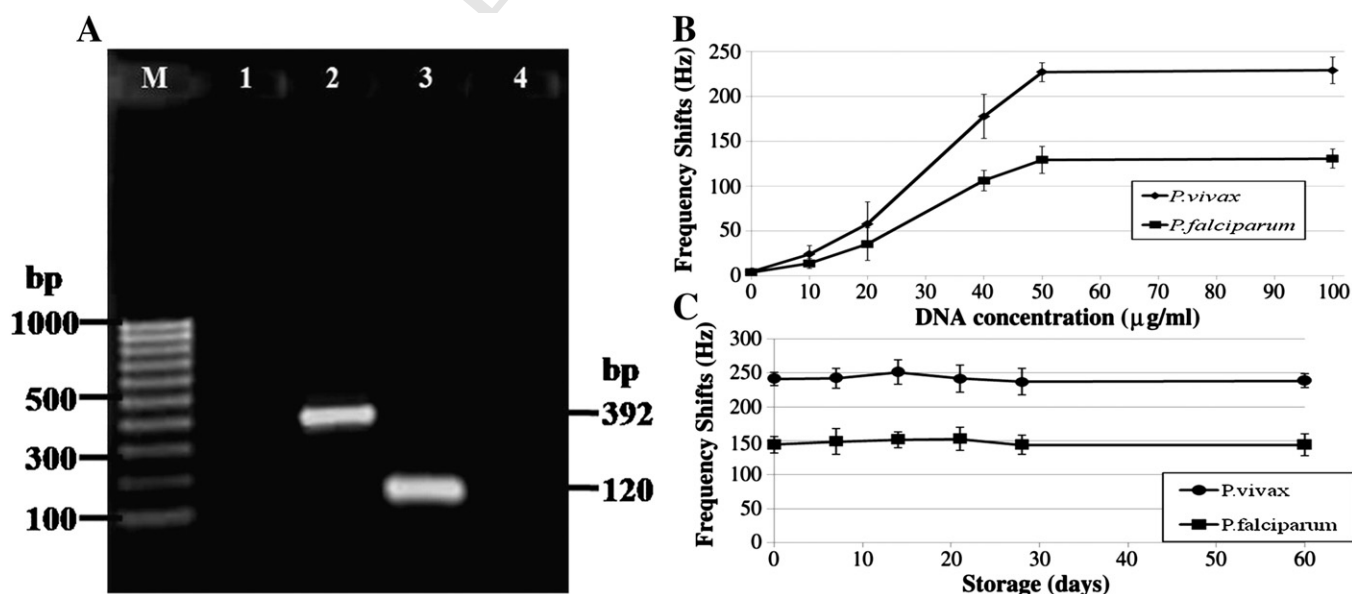


Fig. 2. A. Agarose gel electrophoresis of the malaria amplified target genes, *P. vivax* (lane 2) and *P. falciparum* (lane 3), using three designed primers. Uninfected human blood was also amplified and analyzed (lane 4). Control was reaction solution with no DNA template (lane 1). M is molecular weight marker. B. Evaluation of the malaria QCM using various concentrations of amplified target DNA from malaria infected blood samples. The frequency shifts of each concentration were measured and statistically analyzed. Each experiment was performed in triplicate. C. The storage stability of the malaria QCM after keeping in a sealed cap at room temperature up to 2 months. Potency to differentially identify between *P. falciparum* and *P. vivax* was also stable up to 2 months. Each experiment was done in triplicate.

needed 10× dilution of the conventional Piranha solution which could protect acid oxidation with no effect to the measurements.

The avidin and biotinylated probe could then be sequentially immobilized and the deposited numbers were estimated by a Sauerbrey equation [21]. The estimated immobilized avidin and biotinylated probe were 5.57×10^{11} and 14.32×10^{11} molecules, respectively. The binding ratio of avidin:biotin was 1:2.57. Generally, the free floating ratio is 1:4 [26]. But in this study the immobilized avidin might not be fully accessed by the biotin. Moreover, the biotin molecule was attached with 23 nucleotide probes which might cause steric hindrance during avidin–biotin binding. This QCM immobilized probe was evaluated using malaria infected and uninfected blood samples.

3.3. Evaluation of the malaria QCM

The potency of malaria QCM was evaluated using malaria infected blood samples diagnosed by the standard microscopic examination. DNA of the infected blood, with either *P. falciparum* or *P. vivax*, was extracted and amplified. Various concentrations (0, 10, 20, 40, 50 and 100 µg/ml) of the amplified target DNA were hybridized with the malaria QCM. Frequency shift of each concentration was measured. As shown in Fig. 2B, hybridization of the target DNA to the QCM was saturated when DNA concentration reached 50 µg/ml. This saturation binding implied surface monolayer immobilization which was required in the development of the QCM [27].

As also shown in Fig. 2B, the frequency shifts of both *P. falciparum* and *P. vivax* at 10 µg/ml concentration were significantly higher than control without target DNA (p value < 0.05). The results indicated sensitivity of the QCM at nanogram level (10 µl of the 10 µg/ml) to identify between malaria infected and non-infected blood. To differentially diagnose blood infected with either *P. falciparum* or *P. vivax* (p -value < 0.05) the target DNA not less than 200 ng (10 µl of the 20 µg/ml) is needed. However, this study used DNA concentration at 50 µg/ml since this concentration showed the highest frequency shifts.

The diagnostic potency of malaria QCM was confirmed using clinical blood samples infected with either *P. falciparum* ($n=3$) or *P. vivax* ($n=3$). The uninfected blood samples were also tested for the QCM specificity. The DNA of all samples were extracted, amplified and investigated by the malaria QCM. As shown in Fig. 2B, the frequency shifts of *P. falciparum* (154.33 ± 11.01 Hz) were statistically different from those of *P. vivax* (248.67 ± 7.02 Hz) (p -value < 0.05). These frequency shifts were corresponded with the size of amplified target DNA (120 and 392 bp, respectively) (Fig. 2A). The uninfected blood samples showed no amplified DNA band by agarose gel electrophoresis (Fig. 2A) and no frequency shift by the malaria QCM (14.00 ± 4.58 Hz). All results

confirmed high potency of the malaria QCM in differential diagnosis between *P. falciparum* and *P. vivax*.

The storage stability of malaria QCM was tested by keeping the probe immobilized QCM in a sealed cap at room temperature for 0–60 days. At each time interval the diagnostic ability was evaluated using the known target DNA amplified from blood infected with either *P. falciparum* ($n=3$) or *P. vivax* ($n=3$). Fig. 2C showed stability of the malaria QCM at room temperature up to 60 days. The diagnosis potency of the QCM to differentially identify between *P. falciparum* and *P. vivax* was stable at all time intervals compared to freshly prepared QCM (day 0) (p -value < 0.05).

The QCM must be kept in a sealed cap at all times to keep the surface clean without non-specific deposit leading to non-specific mass change. Moreover, silver electrode could be oxidized in an open environment. The long storage stability at room temperature indicated the potent application of malaria QCM to be used in the remote area. It is possible to create the QCM in the reference laboratory and transported to the field setting as a package with an in-house frequency counter.

3.4. Clinical application of the malaria QCM

The clinical diagnostic potency of the malaria QCM was evaluated using 30 malaria suspected samples collected on filter papers. All samples had been diagnosed by the standard peripheral blood smear examination. Then DNA on the filter papers was extracted and amplified. The amplified products were examined by both agarose gel electrophoresis (Fig. 3) and by the malaria QCM (Table 1). *P. falciparum* infection was confirmed by the commercially available rapid diagnostic tests (RDTs).

Eleven cases reported falciparum infection by microscopic examination and RDTs showed DNA bands at 120 bp by agarose gel electrophoresis (Fig. 3) and frequency shifts at 133.69 ± 3.72 Hz by the malaria QCM (Table 1). Six cases reported vivax infection by microscopic examination showed consistent diagnosis by both agarose gel electrophoresis (392 bp) and the malaria QCM (249.55 ± 7.98 Hz). No malaria was reported by microscopic examination in 10 cases. These 10 cases were also consistent with both agarose gel electrophoresis and the malaria QCM (13 ± 4.64 Hz). Conclusively, 27 out of 30 cases showed consistent diagnosis by microscopic examination, agarose gel electrophoresis, RDTs and the QCM. However, there were 3 cases (sample numbers 6, 9 and 20) diagnosed to be *P. falciparum* infection by agarose gel electrophoresis, RDTs and the QCM but were falsely reported as “no malaria found” by microscopic examination.

In a comparative manner, the developed QCM is the best tool since it provides the most comprehensive molecular malaria diagnosis on a

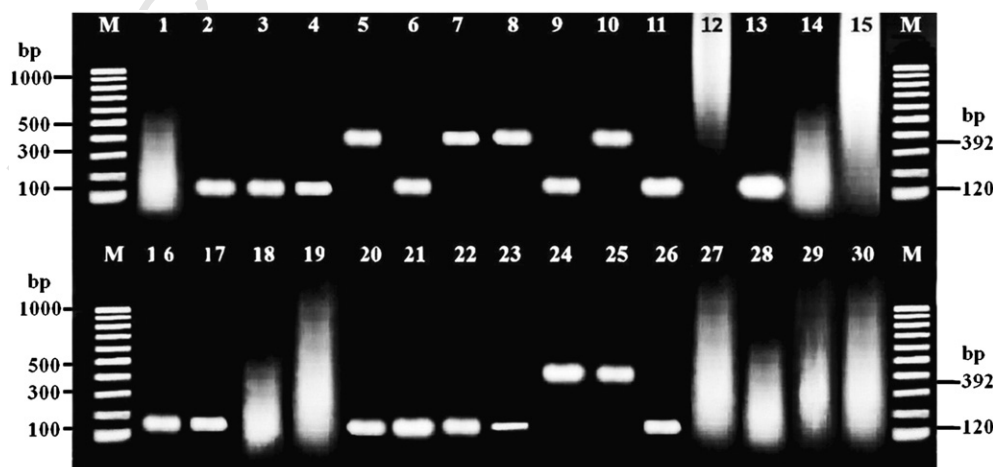


Fig. 3. Diagnosis of malaria suspected blood samples ($n=30$) by the agarose gel electrophoresis. M was a molecular ruler. Three cases (numbers 6, 9 and 20) were reported “no malaria found” by microscopic examination showed DNA bands at 120 bp by agarose gel electrophoresis.

Table 1

Clinical application of the malaria QCM in diagnosis of 30 suspected blood samples. Results were compared to the standard microscopic examination, rapid diagnostic tests (RDTs) and agarose gel electrophoresis.

	Microscopic examination	Malaria QCM (Hz)	Rapid diagnostic tests (RDTs)	Agarose gel
<i>P. falciparum</i> infection (n = 11)		133.69 ± 3.72	<i>P. falciparum</i>	120 bp
<i>P. vivax</i> infection (n = 6)		249.55 ± 7.98	–	392 bp
"No malaria found" (n = 10)		13 ± 4.64	–	No DNA band
"No malaria found" (n = 3)		140.00 ± 2.83	<i>P. falciparum</i>	120 bp

single test format. It is sensitive at the nanogram level, specific genetic test, rapid, cheap and high throughput. Microscopic examination needs expertise of the microscopist especially in cases with low parasitemia or with mixed infection. False negative is often reported as shown in 3 cases (Table 1). Agarose gel electrophoresis needs staining with the carcinogenic ethidium bromide and analyzing under the UV light. The RDTs can identify only *P. falciparum* infection since it detect antigen specific to *P. falciparum* such as HRP-II, pLDH and aldolase [22].

The malaria silver QCM developed in this study has opened a new and exciting era in malaria diagnosis. Success of using silver QCM and of improved QCM drying steps [20] increases its potency in clinical used. On the day of blood collection, diagnosis can be done in 4 h and can thus help accurate and rapid treatment decision. The QCM can be manually used to analyze approximately 30–50 samples/4 h of analysis time [20].

Using silver fabricated QCM is 10 times cheaper than the conventional gold fabricated QCM [10,14,20]. One sensor has two functioning reaction sites, each site on each side. The silver fabricated QCM could not be reused or re-probed [20]. However, it costs only \$1 per one tested sample. Cost effectiveness made it suitably applied in the world region where most of the people cannot afford an expensive health care.

Long stability at room temperature made the malaria QCM to be transported and used in the remote area away from the production site. The frequency counter can be simply produced and transported as a package of test kit. It is possible that the malaria QCM will achieve maximum potential as an affordable point of care test in the near future. The role of QCM in the management and control of malaria appears to be promising.

4. Conclusions

This study created a new DNA biosensor based on quartz crystal microbalance (QCM) to differentially diagnose malaria infection by either *P. falciparum* or *P. vivax*. The malaria QCM was developed by sequentially immobilized avidin and biotinylated probe on the silver electrode of quartz surface. The target fragments of either *P. falciparum* or *P. vivax* were amplified using three designed primers under optimized PCR condition. Then the amplified product was hybridized with the immobilized biotinylated probe and the quartz frequency shifts were measured. It was found that the new malaria QCM was sensitive, specific and stable after keeping at room temperature up to 2 months. Clinical application was also evaluated and found that the malaria QCM could clearly differentially identify *P. falciparum* from *P. vivax*. It is possible to develop the malaria QCM to be a promising point of care testing in malaria diagnosis.

Acknowledgments

This work was co-supported by the Mahidol University under the National Research Universities Initiative, the Graduate Studies and the Faculty of Medical Technology, Mahidol University, Bangkok, Thailand.

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