Tropical Biomedicine 32(4): 753–760 (2015)

A reproducible method for extraction of *Plasmodium falciparum* DNA by microwave irradiation and its potential for rapid molecular diagnosis

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Received 10 March 2015; received in revised form 21 August 2015; accepted 29 August 2015

Abstract. Malaria remains one of the most important communicable diseases. A rapid, simple and accurate method is a crucial part of malaria diagnosis. The aim of this study was to re-evaluate the microwave irradiation method to extract DNA from *Plasmodium falciparum* and compare with six other existing DNA extraction methods such as QIAamp DNA mini kit (Qiagen), FTA elute card, phenol-chloroform, Chelex, Chelex without proteinase-K and Rapid boiling. Two different *P. falciparum* isolates were used: (i) Laboratory strains with 0.3% parasitemia and (ii) clinical isolate with 0.6% parasitemia. Each DNA extraction method was validated for the presence of *P. falciparum* by a routine nested and real time PCR. In order to evaluate the sensitivity of the DNA extraction by microwave, double serial dilution of *P. falciparum* from *in vitro* culture at parasitemia that ranged from 0.0001 to 0.17% were used to extract the DNA by microwave and the *P. falciparum* DNA was then detected by nested and real-time PCR. The nested and real-time PCR were able to detect. *P. falciparum* DNA at the parasitemia level as low as 0.0003% and 0.0001%, respectively. Our results can reproduce the results from earlier studies and reveal microwave as a rapid and simple tool to extract *P. falciparum* DNA and subsequent molecular diagnosis of malaria.

INTRODUCTION

Malaria remains one of the most important communicable diseases in tropical countries (Khaminsou *et al.*, 2008). An estimated 135 to 287 million cases occur every year with approximately 672 000 deaths worldwide (WHO, 2014). *Plasmodium falciparum* may cause cerebral malaria and severe anemia particularly in infants, young children and pregnant women (WHO, 2014). *P. falciparum* is the most virulent species contributing to a larger extent to malarial deaths in Africa (Greenwood *et al.*, 2005). Recently, there have been reports on the occurrence of drug resistant strains of *P. falciparum* in Southeast Asia (Straimer *et al.*, 2015).

The gold standard for diagnosis of malaria is microscopy (Sultan *et al.*, 2009). However, there are some limitations, such as it requires considerable expertise (Guerin *et al.*, 2002). The detection of low parasitemia in malaria confirmed cases and tedious laboratory procedures remain among major challenges in differentiating *Plasmodium* species using microscopy and this is largely due to the technical expertise in blood smear examination. In addition, microscopy techniques fail to detect mixed infections (Mekonnen *et al.*, 2014). The application of

the polymerase chain reaction (PCR) to detect malaria has proven to be more sensitive compared to microscopy (Blossom *et al.*, 2005) and this method in particular has revealed a number of mixed infections in several endemic areas (Putaporntip *et al.*, 2009; Mekonnen *et al.*, 2014). Some PCR techniques can have a detection rate as low as <1 parasite/µL (Rubio *et al.*, 1999) while with the microscope 5-20 parasite/µL are normally required (Andrews *et al.*, 2005). An effective DNA extraction is an essential step for molecular diagnosis. In recent times many DNA extraction methods such as Phenol-chloroform and several commercial DNA extraction kits (i.e. Qiagen DNA extraction kit and FTA elute card) are available. Either conventional or commercial DNA extraction methods require a number of reagents, consumables and are often expensive. A simple, rapid, and cheap DNA extraction method that can yield high quality and quantity is crucial for molecular diagnostics. *Toxoplasma gondii* DNA extracted by microwave irradiation from serum and whole blood has been successfully used in PCR diagnosis (El-Awady *et al.*, 2000; Meganathan *et al.*, 2010). Recently, a rapid and reliable nucleic acid extraction procedure from human blood and malarial parasites using microwave irradiation as a promising platform is described (Port *et al.*, 2014).

This study was carried out to re-evaluate the effectiveness of a microwave assisted extraction of DNA from *P. falciparum* in comparison with six existing DNA extraction methods such as QIAamp DNA mini (Qiagen) kit, FTA elute card, Phenol-chloroform, Chelex, Chelex without proteinase-K, and rapid boiling.

MATERIALS AND METHODS

Ethical considerations

Informed written consent was obtained from patients prior to the collection of blood samples. This study was approved by the Medical Ethics Committee of the review board of the University of the Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia, (Ethics reference number: 1010.23).

Sample collection and preparation

(A) *In vitro* culture of *P. falciparum*

The *in vitro* cultures of *P. falciparum* strain 3D7 was used in this study. *P. falciparum* (0.3% parasitemia) was used to extract DNA by all extraction methods. *P. falciparum* parasitemia samples that ranged from 0.0001-0.17% (approximate 2-6700 parasites/µL)were used to evaluate the sensitivity of the microwave extraction.

(B) Clinical sample

One clinical sample infected with *P. falciparum* (0.6% parasitemia) from the malaria clinic at the University of Malaya Medical Centre, Kuala Lumpur, Malaysia on December 2013 was use for DNA extraction by all methods.

DNA extraction methods

Twenty microliters of venous blood or an *in vitro* culture sample were used in all DNA extraction methods. Each method was done twice on different occasions. All DNA samples were kept at -20°C until further used.

(A) QIAamp DNA mini kit

DNA was extracted by the QIAamp DNA mini kit (QIAGEN, Germany) following the manufacturer's instructions.

(B) FTA elute card

DNA was extracted as previously described (Sultan *et al.*, 2009) with some modifications. Briefly, twenty microliters of venous blood or *in vitro* culture sample was spotted onto the FTA elute card (Whatman, Maidstone UK) and was allowed to dry for at least 24 hours. The FTA elute cards were subsequently punched and placed into the microcentrifuge tube, rinsed in with 500 µL of sterile distilled water by pulse vortexing three times. The water was discarded by a quick spinning process. Fifty µL of sterile distilled water was added and incubated at 95°C for 30 minutes.

(C) Phenol-chloroform

Twenty microliters of sample was suspended in 10 mL of NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris HCl pH 7.5). The next step was to add 10µL saponin (0.02% saponin, Sigma, Germany). The mixture was centrifuged at 5 000 rpm for 10 minutes. The pellet was transferred to a 1.5 mL microcentrifuge tube and suspended in 500 µL of NET buffer. The mixture was treated with 70µL of 1% N-lauroylsarcosine (Sigma, Germany) and 1µL RNAs A (100µg/mL). The mixture was incubated at 37°C for one hour. Then, the solution had 2 µL proteinase K (200 µg/mL, Qiagen, Germany) added and was incubated at 50°C for one hour. To this mixture was added 70µL phenol/chloroform/isoamyl alcohol (25:14:1) solution and was well mixed by inverting the tube. The solution was then centrifuged at 12 000 rpm for 10 minutes. The aqueous phase was transferred to a new tube. The phenol/chloroform/isoamyl alcohol precipitation was repeated three times. To the aqueous phase was added 30µL of 0.3M sodium acetate and 60µL of absolute ethanol. The mixture was centrifuged at 12 000 rpm for 30 min. The supernatant was discarded. The pellet was washed with 70% ethanol. The ethanol was removed and the DNA was dried and resolved in TE buffer. The solution were kept at -20°C until used. The protocol followed that of Mahittikorn *et al.* (2005) with some modifications.

(D) Chelex

This method was previously described by Mahittikorn *et al.* (2005). Briefly, twenty microliters of sample was mixed with 100 µL of Chelex-100 resin (Bio-Rad) and 2 µL of Proteinase K (20 mg/mL; Qiagen) in a 1.5 mL microcentrifuge tube. The mixture was incubated at 56°C for one hour, followed by incubation at 100°C for 15 minutes. The tube was briefly mixed then centrifuged at 10 000 rpm for 10 second. The supernatant was transferred to a new tube then centrifuged at 10 000 rpm for 10 minutes.

(E) Chelex without proteinase-K

This procedure was similar to the above method **(Chelex method**) but proteinase K was not added.

(F) Rapid boiling

Twenty microliters of sample was mixed with 500 µL of ice-cold 5 mM sodium phosphate buffer (pH8.0) in a 1.5 mL microcentrifuge tube and was centrifuged at 14 000 rpm for 2 minutes. The supernatant was discarded and the pellet was washed twice. 50 µL of distilled water was then added to the pellet and boiled in a water bath for 15 min as previously described (Foley *et al.*, 1992).

(G) Microwave

Twenty microliters of sample was added into a 0.5 mL thin wall PCR tube and was exposed to the maximum temperature setting in a 800 watt microwave oven for 3 minutes, or until the blood was desiccated (Meganathan *et al.*, 2010). Approximately 50 µL of Tris-EDTA (TE) buffer pH 8.0 was added into the sample. Then, the tubes were briefly centrifuged at 10 000 rpm and the contents were kept for nested and real-time PCR.

Evaluation of the sensitivity of microwave method

In order to evaluate the performance of the microwave extraction technique, doubling dilutions (parasitemia that ranged from 0.0001-0.17%) of *P. falciparum* DNA obtained from *in vitro* culture were extracted by microwave as describe above. *P. falciparum* DNA was then detected with nested and real-time PCR.

Polymerase chain reaction (PCR) assay

(A) Nested Polymerase Chain Reaction (Nested-PCR)

Nested-PCR amplification was carried out according to the previously described method (Snounou *et al.*, 1993). Briefly, the PCR had a final reaction volume of 20 µL consisting of 10X master mix (Lucigen Corporation, USA), 250 nM of each of the primers, sterile distilled water and DNA extracted with *Plasmodium* genus-specific outer primers derived from the SSU rRNA genes (rPLU5 5'CTTGTTGTTGCCTTAA ACTTC3'and rPLU6 5'TTAAAATTGTT GCAGTTAAAACG3') for primary PCR. The thermal cycling profile was 94°C for 3 minutes; followed by 30 cycles of 94°C for

30 seconds, 58°C for 30 seconds, and 72°C for 90 seconds; followed by 72°C for 5 minutes. Two µL of this primary PCR product was used as the DNA template for the secondary PCR. The amplification reaction had a final volume of 20 µL consisting of 10X master mix including dNTPs, Taq DNA polymerase, magnesium, PCR buffer (Lucigen Corporation, USA), 250 nM of each of the primers, sterile distilled water and DNA template with *Plasmodium falciparum* species-specific inner primers (rFAL1 5'TTAAACTGGTTTGGGAAAACCAAATA TATT3' and rFAL2 5'ACACAATGAA CTCAA TCATGACTACCCGTC 3') and the thermal cycling profile was 94°C for 3 minutes; followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds; followed by 72°C for 5 minutes. The PCR products were checked after electrophoresis on a 1.5% agarose (BioRad Laboratories Inc., California USA) gel and stained with SYBR® safe DNA gel stain (Invitrogen, Germany).

(B) Real-Time Polymerase Chain Reaction (real-time PCR)

All genomic DNA were amplified in an assay targeting the *P. falciparum* lactate dehydrogenase (*ldh*) genes (forward primer LDH-F5'ACGATTTGGCTGGAGCAGA T3', reverse primer LDH-R 5'TCTCTATTCCA TTCTTTGTCACTCTT TC3' and Probe FAM-AGTAATAGTAACAGCTGGATTTACCAAGG CCCCA-TAMRA) were used as previously described (Pickard *et al.*, 2003). Total reaction mixtures were 20 µL, consisting of 10 µL of 1X SensiFAST Probe No-ROX mix, including dNTPs, stabilizers and enhancers (Meridian Life Science, USA), 6 µM forward primer; 6 µM reverse primer; 2 µM probe; 2 µL of DNA template and molecular grade water. All reactions were performed using the thermal cycler (BioRad, USA). The thermal cycling condition were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute as previously described (Pickard *et al.*, 2003) with some small modifications.

RESULTS

Nested PCR and real-time PCR for detecting *P. falciparum* DNA

P. falciparum DNA extracted by each method was successfully detected by both nested (Figure 1) and real-time PCR methods.

Evaluation of the sensitivity of microwave method

P. falciparum DNA from *in vitro* culture extracted by the microwave irradiation method were successfully detected by nested PCR and real-time PCR at densities as low as 0.0003% and 0.0001% parasitemia, respectively.

DISCUSSION

Malaria remains as one of the major public health problems in the world (Khaminsou *et al.*, 2008). A rapid, simple and accurate diagnosis is a crucial component for malaria

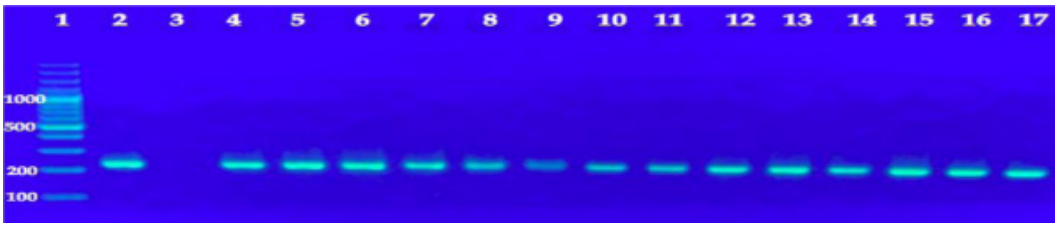

Figure 1. DNA extracted from malarial patient and amplified by nested PCR targeting ssRNA of *Plasmodium falciparum* (205bp). Lane 1: 100bp molecular weight marker; lane 2: positive *P. falciparum* control; lane 3: negative control (distilled water); lanes 4-5: DNA extracted by Qiagen; lanes 6-7: DNA extracted by rapid boiling; lanes 8-9: DNA extracted by chelex; lanes 10-11: DNA extracted by chelex without proteinase-K; lanes 12-13: DNA extracted by microwave treatment; lanes 14-15: DNA extracted by FTA elute card and lanes 16-17: DNA extracted by phenol-chloroform.

control strategies. Due to limitations of the microscopy method, the molecular techniques have now been regularly applied for diagnosis of malaria. Among the molecular techniques, PCR has many advantages as a malaria diagnostic tool as it can reveal a number of co-infections with other malarial species in several endemic areas (Putaporntip *et al.*, 2009; Mekonnen *et al.*, 2014). The PCR technique has a much higher sensitivity than microscopy (Blossom *et al.*, 2005). In molecular analysis, DNA extraction is the first key step to be used then successful DNA amplification is vital for the detection of a specific DNA target. Many DNA extraction methods are now available such as the conventional method (i.e. phenol-chloroform extraction), commercial DNA extraction kits (e.g. Qiagen DNA extraction kit, FTA elute card) and other methods (Chelex, Chelex without proteinase-K and a rapid boiling technique). However, the differences in the purity of the extracted DNA may be due to the presence of contaminants such lipids, polysaccharides or some extraction chemicals for example hexadecyltrimethylammonium bromide (CTAB) (Jasbeer *et al.*, 2009) so the ability of the extraction protocol to eliminate contaminant molecules (e.g. lipid, protein etc.) (Greenspoon *et al.*, 1998) can be critical.

Qiagen and FTA elute card are commercially available DNA extraction kits. They have been widely used since they are easy-to-perform, less time-consuming as well as safe (Mahittikorn *et al.*, 2005). However, these commercial DNA extraction kits are expensive. It has been reported that extracting DNA from blood samples using Qiagen kits provided lower PCR efficiency than the phenol-chloroform extracted DNA (Viltrop *et al.*, 2010). Alternatively the FTA elute card has been developed. This method has improved the sensitivity of PCR due to the relative purity of the extracted DNA by this method being free from PCR inhibitors which becomes attached to the FTA matrix before release of the DNA (Sultan *et al.*, 2009). In addition the FTA elute cards require a short processing time, allow long-term sample storage and inhibit microbial growth (Greenspoon *et al.*, 1998).

Phenol-chloroform is a well-established DNA extraction method (Mahittikorn *et al.*, 2005; Viltrop *et al.*, 2010). It is able to extract the DNA in high concentrations and with a high purity. However, this method requires toxic chemicals such as phenol or chloroform, laborious work and is time-consuming. This method is moreover not considered suitable for large-scale laboratory diagnosis (Griffiths *et al.*, 2006; Mahittikorn *et al.*, 2005; Yamamura *et al.*, 2009).

Chelex and Chelex without proteinase-K methods have been used to extract DNA from protozoan parasites such as *T. gondii* (Mahittikorn *et al.*, 2005) and *P. falciparum* (Bereczky *et al.*, 2005). The Chelex methods were found to be simple, and does not require toxic chemicals (Bereczky *et al.*, 2005; Mahittikorn *et al.*, 2005). It has been suggested that this method is suitable for low parasitemia in low endemic settings (Morris *et al.*, 2013). However, the disadvantages of Chelex is that there is a chelating agent which could lead to the denaturation of double-stranded DNA and is not suitable for downstream work that requires double-stranded DNA such as Restriction Fragment Length Polymorphism (RFLP) analysis (Mahittikorn *et al.*, 2005). DNA extracted using Chelex methods is susceptible to degradation during sample freeze-thawing so it is not suitable for long-term storage (Greenspoon *et al.*, 1998). The boiling method has been used to extract *Plasmodium* DNA (Sultan *et al.*, 2009). The boiling method is inexpensive but time consuming and it does not produce DNA suitable for a long-term storage (Sultan *et al.*, 2009).

Microwave has been applied to extract DNA from many different types of samples including those from the environmental (Orsini & Romano-Spica, 2001) and clinical samples such as Hepatitis B virus DNA (Cheyrou *et al.*, 1991; Costa *et al.*, 1995) *Toxoplasma gondii* DNA (El-Awady *et al.*, 2000; Meganathan *et al.*, 2010) and also recently for human and malarial parasites (Port *et al.*, 2014).The present study confirmed the effectiveness of microwave treatment to use for DNA extraction since both nested and real-time PCR were able to

detect the parasite as low as 0.0003 and 0.0001% parasitemia, respectively. This finding is in agreement with the other studies that reported that *P. falciparum* DNA extracted by microwave treatment could be used to detect by loop mediated isothermal amplification as low as one parasite/µL (Port *et al.*, 2014). The microwave is considered to be an effective method, as it is easy-to-perform, less time-consuming and does not require any chemicals to be added (Cheyrou *et al.*, 1991; Costa *et al.*, 1995; Meganathan *et al.*, 2010; Orsini & Romano-Spica, 2001). Additionally, all reagents used for this method are widely available and microwave ovens are commonly seen in many laboratories (Orsini & Romano-Spica, 2001).

CONCLUSIONS

In conclusion, we report a rapid and simple method for extracting *P. falciparum* DNA from blood using a microwave-based method and has been revalidated as described by earlier studies. This is an alternative method that can be used in the routine laboratory. Future studies should involve the application of such a method in other sample sources (e.g. blood spot on filter paper, long term storage of sample etc.).

Acknowledgement. We thank Khaw Loke Tim, Yap Nan Jiun for provided the *in vitro* culture of *P. falciparum* 3D7. This study was supported by the university of Malaya High Impact Research Grant (UM/MoHE High Impact Research; H-20001-00-E000062) from the Ministry of Higher Education, Malaysia and the University of Malaya Research Grants (UMRG 544/14HTM, 362-15AFR and PG055-2014A). The authors thank Dr. Brian Hodgson for his assistance with English and comments.

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