An Improved Sample Preparation Method for the Detection of *Plasmodium falciparum* in Blood Samples

Thavamani Rajapandi*

Department of Natural Sciences, Coppin State University, Baltimore, Maryland

Research Article

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***For Correspondence**

Thavamani Rajapandi, Associate Professor of Biology, Department of Natural Sciences, Coppin State University, Science and Technology Center 204, 2500 West North Avenue, Baltimore, Maryland 21216, Tel: 410-951-4260.

E-mail: trajapandi@coppin.edu

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ABSTRACT

Microscopy remains the gold standard for the detection of malaria parasites in blood samples, while rapid diagnostic tests (RDTs) and polymerase chain reaction (PCR)-based methods are increasingly employed for field applications, each with limitations. Using *in vitro* cultured *Plasmodium falciparum* parasite isolates, we analyzed several sample preparation methods for PCR-based detection. Test samples subjected to hypotonic lysis, followed by a near complete depletion of hemozoin (Hz) and hemoglobin (Hb) and subsequent PCR amplification of the merozoite surface protein 2(*msp2*)gene resulted in a detection limit that was improved over samples prepared in isotonic buffer conditions. In addition, we amplified sequences of several target genes, including the *msp2*-, *msp1*-, *18S rRNA*- and *eba175* from parasite extracts prepared by hypotonic lysis. We identified a primer set which amplified a region within *eba175* that gave a greater detection limit of 1 parasite per 5 × 10^7 red blood cells (RBCs), as well as a primer set for *msp2* that gave a detection limit of 1 to 5 parasites per 5 × 10^7 RBCs by standard PCR analysis. The remaining targets exhibited maximum detection limits of 20 to 30 parasites per 5 × 10^7 RBCs. This newly developed method is 100 to 500 times more sensitive than currently available PCR-based methods which detect 30 to 100 parasites per 5 × 10^6 RBCs in 1 µl of blood). In addition, this method could be used in field deployable PCR-based diagnostics, as well as for parasite genotyping and quality control of failed RDTs.

INTRODUCTION

Detection of Plasmodium infections among pre-symptomatic and asymptomatic individuals plays a key role in the control of malaria transmission, treatment and the proper use of anti-malarial drugs. Microscopic observation, antigen detection by Rapid Diagnostic Tests (RDT) and Enzyme-linked immunosorbent assays (ELISA), and nucleic acid-based techniques are commonly used for diagnostic purposes. Microscopic observation of Giemsa-stained blood smears is the most commonly accepted practice for the diagnosis of malaria [1]. Another diagnostic method is RDT, based on the colorimetric detection of malaria parasite specific antigens. Currently, all field deployed RDTs detect *P. falciparum* specific histidine rich protein 2 (PfHRP-2), lactate dehydrogenase (PfLDH), or aldolase [2-4]. However, malaria parasite detection by RDTs requires large numbers of parasites in blood samples. The maximum detection limit by RDT is 100 parasites per µl of blood for all Plasmodium species [(0.001% infection in 5 × 10^6 RBC per µl of blood) [1]. Therefore, it is generally accepted that RDT is not optimal for the detection and diagnosis of malaria in blood samples with limited numbers of live parasites. Additionally, false positives may result due to the presence of parasite antigens in spite of parasite clearance following chemotherapy. Moreover, the detection limit of currently available PCR-based methods requires a minimum of 30 to 100 parasites in 1 µl of blood sample or 5 × 10^6 RBCs collected from *in vitro* cultured parasites [5]. These limitations highlight the need for a more sensitive and field-adaptable method for better diagnosis of clinical samples usually with low parasitemia. Yet, another consideration is the sample preparation method and selection of the best suitable target(s) for nucleic acid-based detection. These two parameters have not been extensively studied for field samples or samples prepared from *in vitro* cultured parasites. We were interested in improving the sample preparation method and PCR-based diagnosis by determining the optimal gene target for a successful detection of a very low number of plasmodium parasites in the clinical samples

as well as RDT-failed samples as a quality control measure.

METHODS

Plasmodium falciparum in vitro culture and sample preparation for PCR by hypotonic lysis of RBCs and iRBCs (parasite infected RBC): *P. falciparum* cultures were maintained in RPMI containing 10% pooled heat-inactivated human serum as described previously [6]. 10 µl of packed RBCs (5×10^7 RBCs) harboring a non-detectable level of parasites by light microscopy (5-10 parasites/sample) was diluted in 0.5×10^8 uninfected RBCs (100 µl of 50% blood). The RBCs were collected by centrifugation and resuspended in 0.5 ml of isotonic (1X PBS) or hypotonic (0.1X PBS) buffers and mixed well by vortexing. The samples were incubated on ice for 5 min and the parasite pellet was collected by centrifugation. The parasite pellet was extensively washed with 5 ml of respective buffers. The supernatant was carefully removed without disturbing the pellet and the pellet was resuspended in 100 µl of water and heated at 95°C for 5 min. The various target genes from 10 µl of samples (equivalent to 5×10^6 RBCs) were amplified by PCR in 50 µl reaction mixes. Ten µl (1×10^6 RBC equivalents) of the samples were analyzed by SYBR® Green I detection following agarose gel electrophoresis.

PCR Amplification

Platinum Taq polymerase used in the PCR reaction was obtained from Thermo Fisher Scientific (cat # 10966026), and the PCR was performed with the primer sets listed in **Table 1**. In a typical PCR reaction, a master mix was prepared by adding MgSO4, dNTP mix, 1X PCR buffer and nuclease free water as described previously [5]. The required volume of the diluted buffer mix was aliquotted into multiple tubes containing the primer mixes and the template DNA. PCR reactions were performed by the PTC-200 (MJ Research Inc., USA) thermocycler under the conditions listed in **Table 1** for various target genes. Ten to 20 µl of the PCR amplified products were analyzed in a 0.85% SYBR® Green I-stained agarose gel and photographed by using a Gel Doc XR gel documentation system (Bio-Rad, cat # 1708195).

Table 1. Primers (sequence provided in the table) used for the amplification of the target genes, amplicon sizes and the conditions used for PCR amplification.

Gene target	Primer sequence 5'-3'	PCR condition	Amplicon size (bp)
msp2	F: ATGAAGGTAATTAAAACATTGTCT R: TTATATGAATATGGCAAAAGATAAAAC	1. 95°C for 5 min; 2.Annealing at 58°C for 2 min 3.Extension at 72°C for 2 min 4.Denaturation at 94°C for 1 min. repeat steps 2-4 30 times.	**819**
eba175	F: GAGGAAAACACTGAAATAGCACAC R: CAATTCCTCCAGACTGTTGAACAT	1. 95°C for 5 min; 2. Annealing at 58°C for 2 min 3. Extension at 72°C for 2 min 4. Denaturation at 94°C for 1 min. repeat steps 2-4 30 times.	**795**
ssu rna	F: TTAAACTGGTTTGGGAAAACCAAATATATT R: ACACAATGAACTCAATCATGACTACCCGTC	1. 95°C for 5 min; 2. Annealing at 58°C for 2 min 3.Extension at 72°C for 2 min 4.Denaturation at 94°C for 1 min. repeat steps 2-4 30 times.	**1200**

RESULTS

P. falciparum detection by PCR using samples prepared by hypotonic lysis of iRBCs: Several methods have been widely used for the diagnosis of malaria such as microscopic evaluation of Giemsa-stained blood smears, RDTs and PCR-based amplification of parasite-specific DNA sequences. All these methods utilize heat-inactivated samples that include cytoplasmic contents prepared from whole blood or lysates prepared from parasite-infected RBCs devoid of WBCs. In this study, we devised an improved

sample preparation method from parasite-infected blood in the presence or absence of WBCs. Simply, the perturbance of the osmotic balance either shrinks the red blood cells or results in lysis. Under hypotonic condition (either water or 15 mM Phosphate buffer at pH 7.0), RBCs burst and release all the cytoplasmic contents including hemoglobin. In this procedure, the released cytoplasmic contents were removed by centrifugation and the genomic materials were pelleted down along with membranes. This crude pellet was heated to inactivate potential nucleases and the heated samples were used directly for the PCR-based amplification of parasite-specific target sequences. The test samples were prepared by various methods as indicated in **Figure 1** and representative samples are shown here. As shown in **Figure 1**, hypotonic lysis of samples in 0.1X PBS (15 mM phosphate buffer pH 7.0), followed by complete removal of released hemoglobin and most of hemozoin by repeated washing of the pellet in 0.1x phosphate buffer, resulted in greater sensitivity of detection of *P. falciparum* by PCR. Isotonic lysis of parasites in 1X PBS or hypotonic lysis without the removal of cytoplasmic contents did not increase the sensitivity compared to the hypotonic lysis combined with the removal of cytoplasmic contents (compare panels 1-12, 1'-12' and 1"-12" in **Figure 1**). The sensitivity of PCR amplification was determined based on the percentage of PCR positive samples obtained from the total number of samples tested. The results showed that 100% of samples were positive with the hypotonic lysis method while 20% of samples were positive with hypotonic lysis method without the removal of released cytoplasmic contents. Treatment of samples with the isotonic buffer method did not yield positive results by PCR.

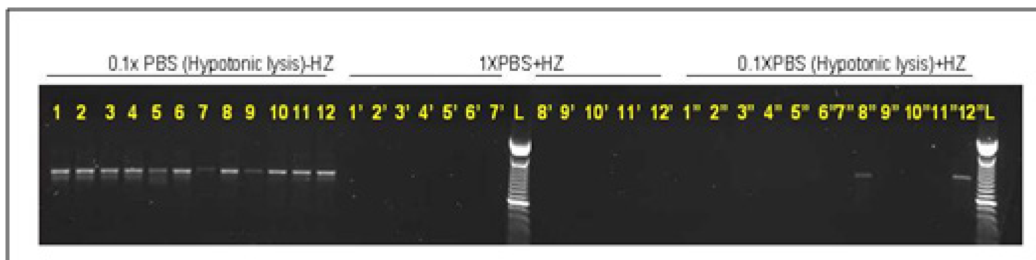

Figure 1. *P. falciparum* detection by PCR using samples prepared by hypotonic lysis of iRBC: The *msp2* gene was amplified by PCR- from multiple test samples as described in the Methods section. Samples were analyzed by agarose gel electrophoresis. Lanes 1-12, hypotonic-lysis of parasite-infected erythrocytes, and a near complete depletion of the trace amount of both hemozoin and hemoglobin by centrifugation (see Methods section); lanes 1'-12', addition of parasite-infected blood suspended in 1XPBS into the PCR reaction mix; and lanes 1"-12", addition of hypotonically-lyzed lysate with Hb and Hz.

Target selection for the sensitive detection of *P. falciparum* infection: Several targets have been analyzed and used for PCR-based detection and diagnostics of malaria, and the most commonly used targets are the small and large subunit 18S rRNA specific sequences. In order to select a sensitive target, we analyzed several oligonucleotide primer sets against multiple target genes, including *msp2-*, *msp1-*, *ssu rRNA-* and *eba175* for PCR amplification. As a first step, we used purified genomic DNA **(Figure 2)** as the target material. As shown in **Figure 2**, we identified a primer set specific to F or C type *eba175* sequence(s) that resulted in higher sensitivity of detection of *P. falciparum* DNA by PCR compared to primers specific for *msp2* and *ssrRNA*. The *eba175* primer set had the potential to detect genomic DNA equivalent to 0.5 parasites under standard PCR analysis. The next best target was *msp2*, with the potential to detect one parasite equivalent of genomic DNA. The remaining targets were limited to 20-30 parasite equivalents of genomic DNA (**Figure 2**, and data not shown).

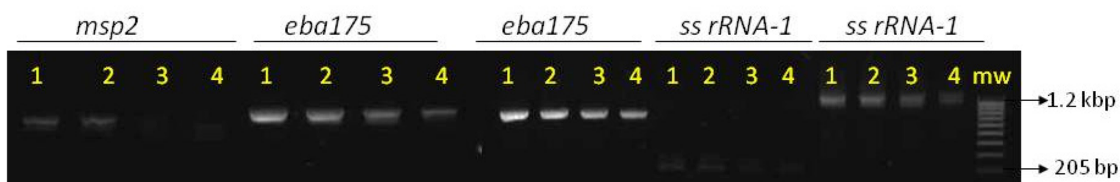

Figure 2. Target selection for the detection of *P. falciparum* infection: *msp2*, *eba175* and *ssu rRNA* genes were amplified from various concentrations of purified *P. falciparum* genomic DNA. PCR was performed in a 50 µl reaction and 10 µl from the reaction mix was analyzed by agarose gel electrophoresis. Lane 1: 2 ng; lane 2: 0.2ng; lane 3: 0.02 ng, and lane 4: 0.002 ng.

Table 2. Currently available tools to detect malaria parasites and a comparison of the sample.

Method	Target	Detection Limit Parasite/µl blood sample	Description of target and Limitation	Reference

Microscopy	Whole parasite	30	Detects asexual and sexual blood stage parasites of all species under microscope.	Kobayashi T [7]
			Reliable readings require skilled microscopists	
RDT	Antigen	>100	Detects malaria antigen by immunochromatographic assay with monoclonal antibodies to target parasite antigen. Antibody requirement	Kobayashi T [7]
			Antibody requirement	
PCR (conventional and real time)	DNA	10	Amplifies target parasite DNA. Depending on the target gene, genus- and species-level diagnoses are available. The result of conventional PCR is qualitative, whereas qPCR is quantitative. Purified DNA is required	Kobayashi T [7]
RT-PCR	RNA	10	Detects mRNA expressed at specific life cycle of the parasite. The test can be used to measure the transmissibility of the infection by quantifying the presence of mosquito-infective sexual stages.	Kobayashi T [7]
			Purified RNA is required	
NASBA	RNA	<1 parasite	Amplifies target RNA in a single step isothermal condition. Purified RNA is required.	Kobayashi T [7]
LAMP	DNA	5-10	Detecting infection by a turbidity meter after amplifying parasite DNA.	Kobayashi T [7]
			DNA extraction methods are key	
Serological tests	Malaria parasite specific antibody	>100	Detects and measures antibodies against malaria parasites as an indicator of recent and/or past exposure to parasites.	Kobayashi T [7]
			Antibody required	
Hypotonic lysis of whole blood	Whole blood	<1 parasite/>100 µl whole blood	*eba75* and *msp2*	This study

Preparation method used in this study. LAMP = loop-mediated isothermal amplification; NASBA = nucleic acid sequence–based amplification; RDT =rapid diagnostic test; qPCR = real-time quantitative PCR; RT-PCR = reverse transcriptase PCR

Determination of *P. falciparum* detection sensitivity limit by PCR using samples prepared by hypotonic lysis: In order to determine the PCR-based detection limit of *eba175* and *msp2* primers in crude samples obtained by hypotonic lysis of parasites, P. falciparum-infected RBCs were serially diluted and *eba175* and *msp2* were amplified from the samples prepared by hypotonic lysis. These results indicated that the primer set specific to a 1 kb sequence within *eba175* and *msp2* provided the most sensitive targets for diagnostics purposes and is suitable for both purified genomic DNA as well as the crude samples obtained from parasite cultures either in the presence or absence of human genomic DNA **(Figures 3A and 3B)** [8-10].

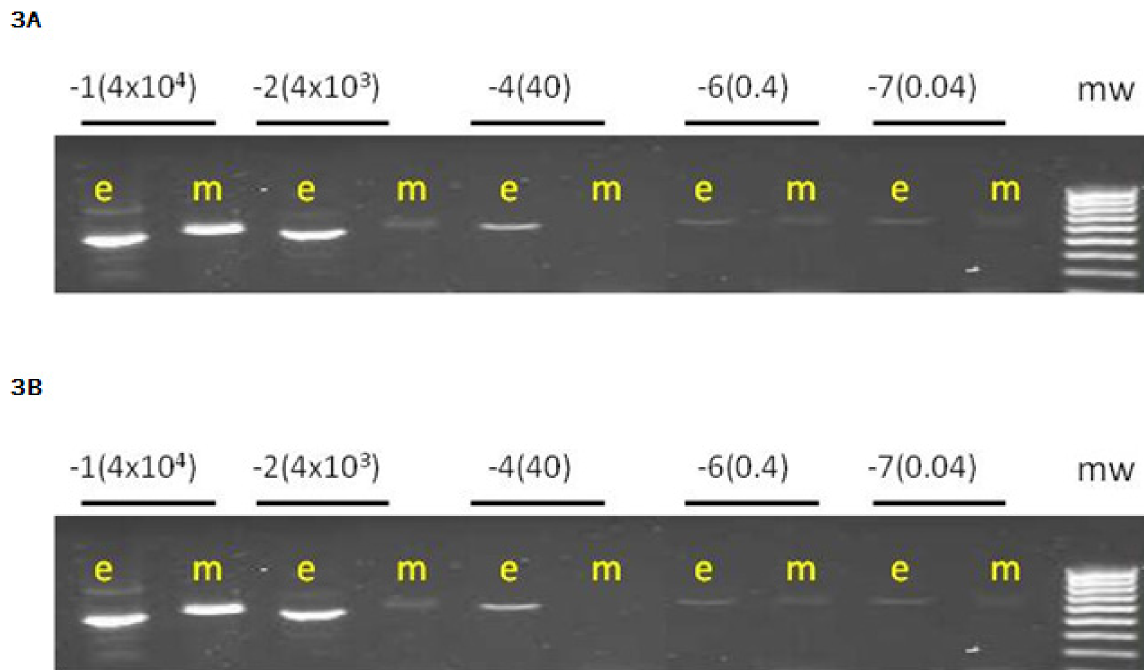

Figure 3. Determination of *P. falciparum* detection limit by PCR using samples prepared by hypotonic lysis. A, P. falciparum-infected erythrocytes were diluted from 4 × 10^4 to 0.04 parasites per reaction in culture medium or 1X PBS containing 1 × 10^9 uninfected erythrocytes. Samples were prepared by hypotonic lysis as described in the methods section. The *eba175* (e) and *msp2* (m) genes were amplified by PCR- in a 50 µl reaction mix and 20 µl of the samples were analyzed by agarose gel electrophoresis followed by SYBR® Green I staining. B, RBCs harboring 2.0% parasitemia in a 0.5 ml volume of blood (equivalent to 10 µl of packed cells) was diluted in 4.5 ml of whole blood containing human white blood cells (equivalent to 1 × 10^9 RBCs or 0.1 ml packed cells). This suspension was serially diluted up to 10^{-9} in 1 × 10^9 uninfected RBCs. One ml of serially diluted samples were processed as described in the Methods section and resuspended in 100 µl of water. The *eba175* gene was amplified by PCR in a 50 µl reaction mix and 20 µl of the samples were analyzed by agarose gel electrophoresis followed by SYBR® Green I staining.

DISCUSSION AND CONCLUSION

In this study, a simple and improved sample preparation method using blood samples harboring a microscopically non-detectable level of Plasmodium parasites was developed. The test sample was prepared by hypotonic lysis of iRBCs and a near complete removal of hemozoin (Hz) and hemoglobin (Hb) by centrifugation. These two key steps enhanced the PCR-based detection limit of malaria parasites to several hundred-fold from blood samples with low parasitemia. This phenomenon may be explained by high contents of Hb or Hz which inhibited the rate of amplification of both *eba175* and *msp2* gene sequences (data not shown). This is supported by our results using samples prepared by hypotonic lysis and removal of Hb and Hz which improve the level of detection. Also, samples prepared in 1x phosphate buffer which contains ~ 155 mM sodium phosphate which provides an isotonic condition, which is equivalent to physiological osmotic condition and RBCs and iRBCs will not be lysed. The high concentration of hemoglobin and or hemozoin will potentially inhibit the polymerase activity in the PCR reaction. The hyponic lysis method released all the hemoglobin and most of the hemozoin are washed out, and in particular the potential inhibitors are removed in this sample preparation method. This crucial and simple step improved the sensitivity of detection of parasite DNA by PCR. Interestingly, PCR-based amplification of *eba175* was affected in one of the test samples containing high number of parasites that was subjected to hypotonic lysis. The selection of targets such as *eba175* and/or *msp2* resulted in higher levels of detection by PCR compared to other commonly used targets in previously published studies **(Table 2)** [10-12]. It should be noted that interpreting PCR-based diagnostic results in clinical samples can be problematic, similar to antigen-based detection approaches. This may due to a small fraction of parasite DNA that remains in the bloodstream after the infection is cleared by anti-malarial drug therapy and the large excess amount of human genomic materials. Therefore, the differentiation of an active infection from a recently cleared infection is arguable. However, the quantity of parasite DNA from recently cleared parasites should be almost negligible when parasitemia is at a non-detection level. The sensitivity level obtained from the samples prepared by hypotonic lysis is several folds greater than previous published studies. Therefore, this newly developed method will help to solve many challenges in the diagnosis of malaria by microscopy, RDTs and PCR-based methods. In conclusion, adapting this simple method to a field setting by using a portable PCR machine is also amenable with greater accuracy.

REFERENCES

1. Murray CK, et al. Update on rapid diagnostic testing for malaria. Clin Microbiol Rev. 2008;21:97-110.

2. Ishengoma DS, et al. Using rapid diagnostic tests as source of malaria parasite DNA for molecular analyses in the era of declining malaria prevalence. Malar J. 2011;10:6.

3. Makler MT and Hinrichs DJ. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. Am J Trop Med Hyg. 1993;48:205-210.

4. Makler MT, et al. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. Am J Trop Med Hyg. 1993;48:739-741.

5. Maher SP, et al. A highly sensitive, PCR-based method for the detection of *Plasmodium falciparum* clones in microtiter plates. Malar J. 2008;7:222.

6. Trager W and Jensen JB. Continuous culture of Plasmodium falciparum: its impact on malaria research. Int J Parasitol. 1997;27:989-1006.

7. Kobayashi T, et al. Malaria diagnosis across the International Centers of Excellence for Malaria Research: Platforms, Performance, and Standardization. Am J Trop Med Hyg. 2015;93:99-109.

8. Bell D, et al. Ensuring quality and access for malaria diagnosis: how can it be achieved?Nat Rev Microbiol. 2006;4:682-695.

9. Moody A. Rapid diagnostic tests for malaria parasites. Clin Microbiol Rev. 2002;15:66-78.

10. Goodyer ID and Taraschi TF. *Plasmodium falciparum*: A simple, rapid method for detecting parasite clones in microtiter plates. Exp Parasitol. 1997;86:158-160.

11. Touré FS, et al. Erythrocyte binding antigen (EBA-175) of Plasmodium falciparum: improved genotype determination by nested polymerase chain reaction. Trop Med Int Health. 2001;6:767-769.

12. Snounou G, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 1993;61:315-320.