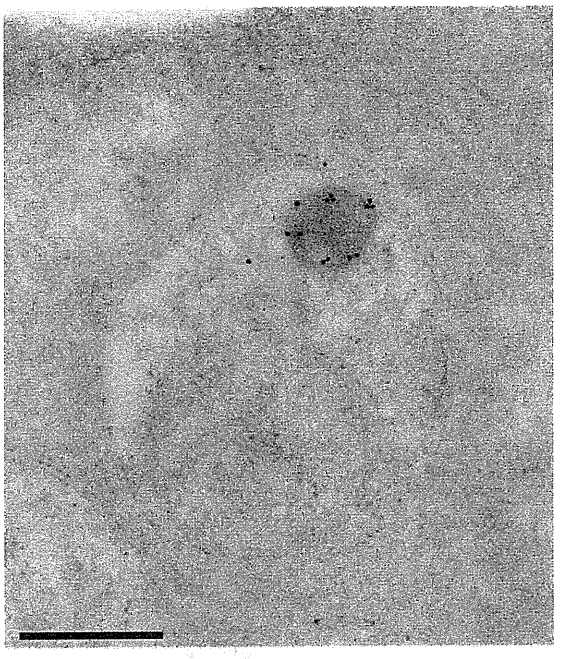

Fig. 5. Rhoptry body localization of *Pf*RON3 by immunoelectron microscopy. Longitudinally sectioned merozoites in mature schizonts were labeled with rabbit anti-*Pf*RON3_2 antibodies followed by secondary antibody conjugated with gold particles. The image shows that the gold particle signals were restricted to the merozoite rhoptry body. Bar represents 500 nm.

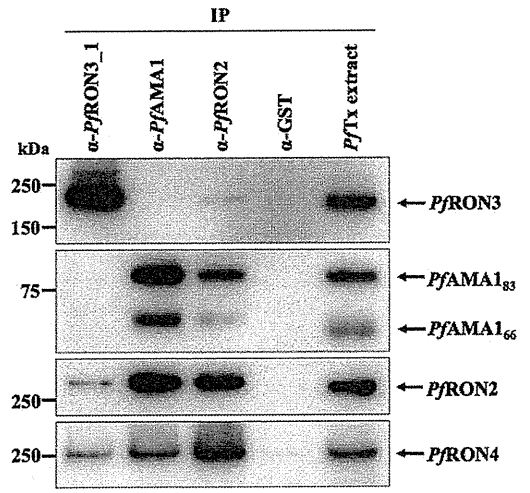

Fig. 7. *Pf*RON3 is not involved in the RON–AMA1 complex. Triton X-100 extracts of schizont-rich parasite (*Pf*Tx extract) were immunoprecipitated (IP) with rabbit sera against *Pf*RON3 (α-*Pf*RON3_1), *Pf*AMA1 (α-*Pf*AMA1), *Pf*RON2 (α-*Pf*RON2), or GST (α-GST), then stained with mouse antisera against *Pf*RON3, *Pf*AMA1, *Pf*RON2, or *Pf*RON4, respectively. Immunoprecipitation using anti-GST antibody was used as a negative control. No bands were detected in the anti-GST immunoprecipitate, indicating the exclusion of potential carryover of proteins due to insufficient or inadequate washing steps.

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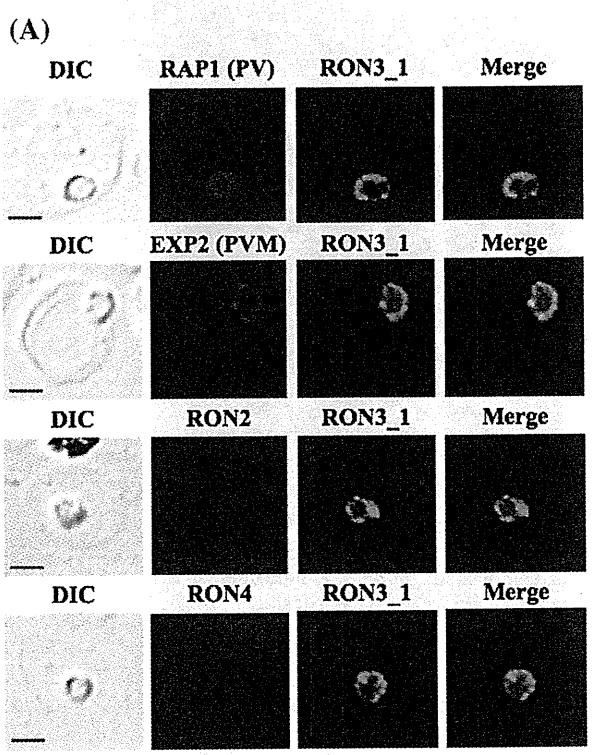

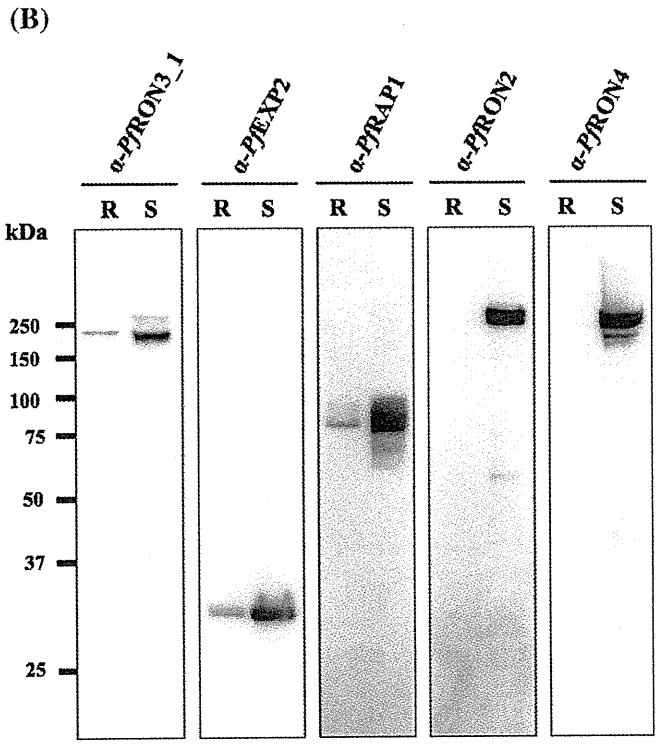

Fig. 6. *Pf*RON3 is found in the parasitophorous vacuole in ring stage parasites. (A) Ring stage parasites were dual-labeled with antisera against *Pf*RON3_1 and either *Pf*RAP1 (PV marker), *Pf*EXP2 (PVM marker), *Pf*RON2, or *Pf*RON4. Nuclei are visualized with DAPI in merged images shown in the right panels. Bars represent 2.5 μm. (B) Proteins from synchronized parasite cultures were harvested at the ring stage (R) and schizont stage (S), and separated by SDS-PAGE on a 12.5% gel under a reducing condition. After transfer of proteins onto a PVDF membrane, the membrane was stained using rabbit anti-*Pf*RON3_1, mouse anti-*Pf*RAP1, anti-*Pf*EXP2, anti-*Pf*RON2, anti-*Pf*RON4 antibodies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.parint.2011.01.001.

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Detection of *Plasmodium vivax* infection in the Republic of Korea by loop-mediated isothermal amplification (LAMP)

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ABSTRACT

Loop-mediated isothermal amplification (LAMP) is a novel technique that rapidly amplifies target DNA in isothermal conditions. In a previous study, the sensitivities and specificities of LAMP, microscopy, and nested PCR were compared in the context of rapid malaria detection. In the present study, LAMP detected vivax malaria parasites in 115 of 117 microscopically positive samples (sensitivity, 98.3%; 95% CI, 97.4–100%), which agreed well with the nested PCR results (sensitivity, 99.1%; 95% CI: 96.0–100%). No positive cases of malaria were detected by LAMP or nested PCR in 50 consecutive feverish patients other than malaria from malaria endemic areas. LAMP performed on DNA extracted from heat-treated blood had a sensitivity of 93.3% (28/30, 95% CI: 84.4–100%) and specificity of 100% (30/30, 95% CI: 100%). The present study shows that LAMP based assays have high sensitivity, specificity, and amplification efficiencies for *Plasmodium vivax* detection. The authors recommend that LAMP can be considered as a rapid nucleic acid amplification assay for the molecular diagnosis of *P. vivax* in both clinical laboratories and malaria clinics in areas where vivax malaria is endemic.

1. Introduction

Forty percent of the world's population is threatened by *Plasmodium vivax* and there are millions of clinical infections annually. Although *P. vivax* infections are often regarded as benign and self-limited, recent evidence indicates that the overall burden, economic impact, and disease severity of *P. vivax* infections have been underestimated (Price et al., 2007). Malaria control and treatment strategies depend largely on laboratory-confirmed diagnoses and the present lack of an affordable, reliable diagnostic method is a major obstacle because of misdiagnoses and treatment delays.

Microscopic examinations of blood smears are still considered to be the best means of detecting malaria. Such examinations are cheap and straightforward, but are also labor-intensive, time-consuming, and require well-trained personnel (Mens et al., 2007). However, a variety of rapid diagnostic tests (RDTs) for malaria have been recently developed. These tests have rapid turnaround times (15–20 min), are easy of use, and require no electricity supply or dedicated equipment, which means that inexperienced laboratory and clinical staff can make diagnoses (Moody, 2002). However, these tests are based on the recognition of *Plasmodium* antigens in blood and the current RDTs for *P. vivax* lack sufficient sensitivity for the detection of vivax malaria (van den Broek et al., 2006). On the other hand, PCR (polymerase chain reaction) based methods, such as nested PCR, use species-specific primers for DNA amplification and are able to detect parasitemias as low as 1 parasite/μl (Snounou et al., 1993), but protracted turnaround times, high costs, and the availability of a well-equipped laboratory render this technology unsuitable for routine diagnosis in hospital laboratories and field clinics in endemic areas (Hanscheid and Grobusch, 2002).

Recently, a new, simple, sensitive technique, loop-mediated isothermal amplification (LAMP), was developed to detect the highly conserved 18S ribosomal RNA gene of *P. vivax* (Notomi et al., 2000; Han et al., 2007). LAMP involves autocycling strand displacement DNA synthesis by *Bacillus stearothermophilus* (*Bst*) DNA polymerase using a set of six primers to produce stem-loop DNA

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structures (Nagamine et al., 2001, 2002). LAMP reactions can be conducted under isothermal conditions (ranging from 60 to 65 °C) and results can be determined by assessing turbidity visually (Mori et al., 2001; Nagamine et al., 2001). Furthermore, LAMP has been used successfully to detect *Plasmodium*, *Trypanosoma*, and *Babesia* infections in humans and animals (Kuboki et al., 2003; Ikadai et al., 2004; Poon et al., 2006; Han et al., 2007). In the present study, the sensitivity and specificity of LAMP for the analysis of blood samples of *P. vivax* patients, healthy subjects, and consecutive feverish patients in the Republic of Korea, were compared with those of microscopy and nested PCR.

2. Materials and methods

2.1. Patient samples

One hundred and seventeen blood samples from patients with vivax malaria were determined to be positive for vivax malaria by microscopy at Korea University Ansan Hospital and at local health centers and clinics in Gyeonggi and Gangwon Provinces in endemic areas of the Republic of Korea. Fifty blood samples from patients with consecutive fever of at least 37.5 °C and 30 blood samples from healthy patients were determined to be negative for vivax malaria by microscopy.

2.2. Microscopic examination

Thick blood smears were examined under 1000× magnification by microscopists with extensive experience in the identification of malaria parasites. The parasite density per 200 leukocytes was counted and then calculated as the number of parasites per microliter by assuming a leukocyte count of 7000/μl.

2.3. DNA extraction

Template DNA for nested PCR and LAMP assays was prepared from 200 μl of whole blood using QIAamp DNA Blood Mini Kits (QIAGEN GmbH, Hilden, Germany), which provided 200 μl aliquots of template genomic DNA. A straightforward, cheap DNA extraction was devised for *P. vivax* LAMP based on a previously described method (Poon et al., 2006). Briefly, 50 μl of whole blood were mixed with 50 μl of distilled water, heated at 99 °C for 5 min, and centrifuged for 2 min at 10,000 rpm. Two microliters of the supernatant were used as templates for 25 μl LAMP reactions. Thirty microscopically positive samples and 30 microscopically negative control samples were prepared using this heat-treatment.

2.4. Nested PCR assay

This assay targeted the *P. vivax* small subunit ribosomal RNA (SSU rRNA) gene and resulted in a primary product of 1626 bp and a secondary amplicon of 121 bp. The oligonucleotide primers (Table 1) used were previously described (Snounou and Singh, 2002). For the first PCR, 1 μl of template DNA was added to a 20 μl PCR mixture consisting of 0.4 μM of each primer (rPLU1 and rPLU5), 200 μM each deoxynucleotide triphosphates (dNTP) (Applied Biosystems, Foster City, USA), 2.5 mM $MgCl_2$, 1× PCR Gold Buffer (50 mM KCl, 15 mM Tris–HCl, pH 8.0), and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). DNA amplification was carried out under the following conditions: 95 °C for 10 min, 40 cycles of (95 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min), followed by a final extension at 72 °C for 5 min. One microliter of the first PCR product was used in the second amplification. The conditions and concentrations used for the second amplification were identical to those used for the first, except that rVIV1 and rVIV2 were used as for primers (Table 1) and amplification was performed over 30 cycles. Amplified products were visualized in 2% agarose gels stained with ethidium bromide. To prevent cross-contamination, different work areas and different pipettes were used for DNA template preparation, PCR mixture preparation, and DNA amplification. One uninfected blood sample was included as negative controls for every 10 samples processed.

Table 1
LAMP and nested PCR primer sequences used to detect *Plasmodium vivax*.

Method	Primer	Primer sequence (5′ → 3′)
LAMP[a]	FIP	CTATTGGAGCTGGAATTACCGC -TCCCAAAACTCAATTGGAGG
	BIP	AATTGTTGCAGTTAAAACGCTCG -TAAGCTAGAAGCGTTGCT
	F3	GGAATGATGGGAATTTAAAACCT
	B3c	ACGAAGTATCAGTTATGTGGAT
	LPF	GCTGCTGGCACCAGACTT
	LPB	AGTTGAATTTCAAAGAATCG
Nested-1st round[b]	rPLU1	TCAAAGATTAAGCCATGCAAGTGA
	rPLU5	CCTGTTGTTGCCTTAAACTTC
Nested-2nd round[b]	rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC
	rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA

[a] LAMP primers have been described by Han et al. (2007).
[b] Nested PCR primers have been described by Snounou and Singh. (2002).

2.5. LAMP assay

The LAMP primer sets (Table 1) used were designed based on the species-specific nucleotide sequences of the 18S rRNA genes of *P. vivax*, as determined by Han et al. (2007). LAMP was performed using Loopamp DNA amplification kits (Eiken Chemical Co., Ltd., Tokyo). Reaction mixtures (25 μl) contained 2.4 μM of both FIP and BIP primers, 0.2 μM of both F3 and B3c primers, 0.8 μM of both LPF and LPB primers, 12.5 μl of 2× reaction mixture [40 mM Tris–HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M betaine, and 2.8 mM of each dNTP], *Bst* DNA polymerase (1 μl), and 1–2 μl of DNA sample. LAMP was performed at 60 °C in a water bath for 60 min, and a sample formatting the visible turbidity was determined positive by the naked eye in a single blind manner. In addition, LAMP reaction mixes were stained with SYBR green I dye (Molecular Probes, Eugene, USA), and a positive sample was determined by the naked eye, under a UV trans-illuminator or using a hand-held black light.

2.6. Analytical sensitivity

To determine the lower detection limit (parasites per μl) of LAMP and nested PCR, we serially diluted 10 DNA templates extracted from vivax malaria specimens. Ten DNA samples obtained from health people were used as negative controls. Geometric means of analytical sensitivities were calculated.

Table 2
Microscopic examination, nested PCR, and LAMP results.

Microscopy (*n*)	Nested PCR		LAMP	
	Positive	Negative	Positive	Negative
Positive (117)	116	1[a]	115	2[b]
Negative (30)	0	30	0	30

[a] Negative determined by LAMP assay (60 parasites/μl).
[b] One sample was also determined to be negative by nested PCR (60 parasites/μl), whether the other was negative by LAMP alone (50 parasites/μl).

Table 3
Sensitivities, specificities, positive predictive values (PPVs), negative predictive values (NPVs) and *J* indices of nested PCR and LAMP.

Method	Sensitivity (%)	95% CI (%)	Specificity (%)	95% CI (%)	PPV (%)	NPV (%)	*J* index
Nested PCR	99.1	97.4–100	100	100	100	96.8	0.99
LAMP	98.3	96.0–100	100	100	100	93.8	0.98

Values are expressed with respect to microscopy findings.

2.7. Statistical analysis

Microsoft Office Excel 2003 was used to calculate test performances and acceptability evaluation indices using microscopic examination findings as the standard. The indices measured were the number of true positives (TP), number of true negatives (TN), number of false positives (FP), and number of false negatives (FN). Sensitivity was defined as TP/(TP+FN), specificity as TN/(TN+FP), positive predictive value (PPV) as TP/(TP+FP), and negative predictive value (NPV) as TN/(FN+TN). Reliability was quantified using *J* indices, which we calculated using: $(TP \times TN - FP \times FN)/(TP+FN)(TN+FP)$ (Mharakurwa et al., 1997). *J* indices have possible values between 0 and 1, and as values approach 1, the overall diagnostic ability approaches the ideal. Also, 95% confidence interval (95% CI) for sensitivity and specificity was calculated according to the following equation (Banoo et al., 2006):

$$p \pm 1.96 \times \sqrt{\frac{p(1-p)}{n}}$$

where p = sensitivity (or specificity) measured as a proportion (not a percentage) and n = number of samples from infected people (or, for specificity, from uninfected people).

3. Results

3.1. Parasite densities as determined by microscopic examinations

In total, 117 cases of microscopically positive blood samples (mean ± standard deviation (SD), 2648 ± 4406 parasites/μl; range, 50–22,599 parasites/μl) were subjected to LAMP assays and nested PCR. Of these, 10 cases (1373 ± 864 parasites/μl; range, 655–3622 parasites/μl) were used to determine the minimum detection limits of LAMP and nested PCR. In addition, 30 samples (4051 ± 5151 parasites/μl; range, 101–10,663 parasites/μl) were heat-treated and subjected to LAMP assays. No positive cases were found in the 50 consecutive feverish patients.

3.2. Minimum detection limits of LAMP and nested PCR

The minimum detection limit of LAMP was 30 ± 5 parasites/μl. LAMP did not produce any false-positive results in 10 DNA samples from health subjects. On the other hand, the minimum detection limit of nested PCR using diluted *P. vivax* malarial DNA was 3 ± 5 parasites/μl.

3.3. Sensitivities and specificities of LAMP and nested PCR

LAMP detected *P. vivax* in 115 of 117 microscopically positive samples (sensitivity, 98.3%; 95% CI, 97.4–100%) (Tables 2 and 3), whereas nested PCR detected *P. vivax* in 116 of 117 samples (sensitivity, 99.1%; 95% CI: 96.0–100%) (Tables 2 and 3). No false-positive results were obtained by LAMP or nested PCR among 30 blood samples from healthy subjects (a specificity of 100% for both). The PPV and NPV of LAMP were 100 and 93.8% (Table 3), respectively, whereas the PPV and NPV of nested PCR were 100 and 96.8%, respectively (Table 3). *J* indices (a measure of test reliability) were 0.98 for LAMP and 0.99 for nested PCR.

3.4. Detection of consecutive feverish patients by LAMP and nested PCR

Consecutive feverish patients other than malaria were collected from malaria endemic areas of the Republic of Korea. No *P. vivax* positive cases were shown in these 50 patients detected by both LAMP and nested PCR.

3.5. Detection in heat-treated samples by LAMP

For DNA extraction using the heating method, LAMP showed high sensitivity (93.3%; 95% CI: 84.4–100%) and specificity (100%). LAMP detected malaria parasites in 28 of 30 microscopically positive samples, and did not produce any false-positive results in the 30 blood samples of health subjects (Table 4). Using heat-treated samples as a source of template DNA, the *J* index by LAMP was 0.93 (Table 4).

3.6. Visual detection methods

Several visual detection methods were examined, namely, naked eye, naked eye under an UV trans-illuminator, and naked eye using hand-held black light (Fig. 1). The first of these involved the assessment of the presence of a white magnesium pyrophosphate (a byproduct of the amplification) precipitate (Fig. 1 (1)). The second involved the staining of *P. vivax* LAMP product DNAs with SYBR green I (Fig. 1 (2)), which is readily observed in a UV trans-illuminator (Fig. 1 (3)). The third also involved the addition of SYBR green I. After adding this dye, 'positive' reaction mixtures were colored bright green whereas 'negative' mixtures were colored weak green when examined with a hand-held black light (Fig. 1 (2) and (4)).

Table 4
Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and *J* index of LAMP assays for the detection of *Plasmodium vivax* in heat-treated samples.

Method	Sensitivity (%)	95% CI (%)	Specificity (%)	95% CI (%)	PPV (%)	NPV (%)	*J* index
LAMP	93.3 (28[a]/30[b])	84.4–100	100 (30[c]/30[d])	100	100 (28/28)	93.8 (30/32)	0.93

[a] Positive by LAMP.
[b] Positive by microscopic examination.
[c] Negative by LAMP.
[d] Negative by microscopic examination.

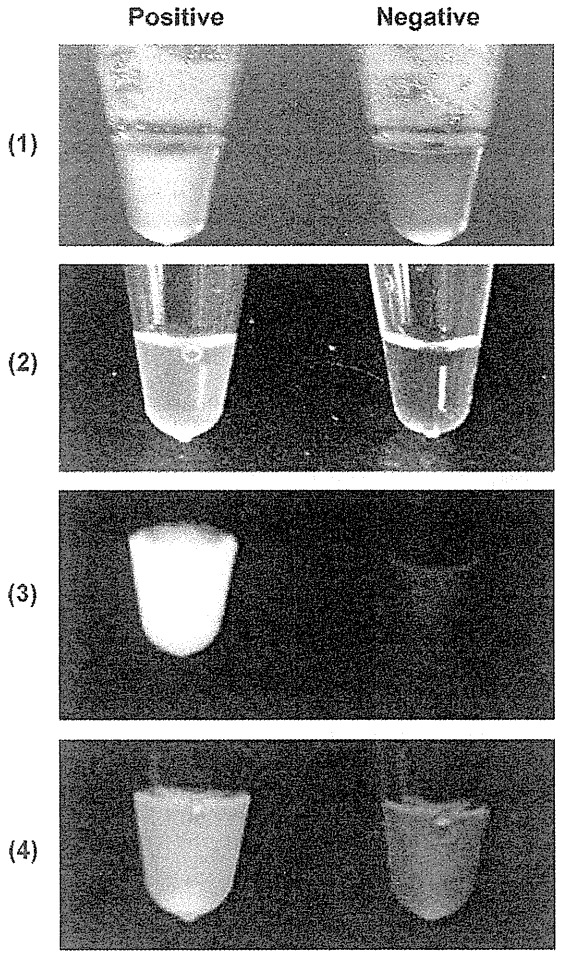

Fig. 1. Visual detection of *Plasmodium vivax* by LAMP from positive and negative samples. (1) Naked eye detection without SYBR green; (2) naked eye detection with SYBR green; (3) detection under an UV trans-illuminator using SYBR green; (4) detection under a hand-held black light using SYBR green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

P. vivax is the only malarial species known to infect humans in the Republic of Korea. An eradication campaign lead to its elimination in the late 1970s, but it re-emerged in 1993, and currently, 1000–2000 cases occur annually (Shin et al., 2008). Clinical suspicion of malaria should be confirmed by appropriate laboratory testing, which is mainly microscopic examination of Giemsa stained blood films. The sensitivity that can be achieved by an experienced microscopist using this method is about 50 parasites/μl of blood, which is equivalent to an RBC infection rate of 0.001% (assuming a total RBC count of 5×10^6/μl) (Moody, 2002). In the present study, nested PCR detected malaria parasites in patients with low level parasitemia of <0.0001% (approximately 3 parasites/μl), and LAMP was found to detect malaria parasites at <0.001% (approximately 30 parasites/μl), which is 1.6-fold more sensitive than that of microscopic examinations. Furthermore, although PCR is undoubtedly the most sensitive and specific method, it is not often available in areas with *P. vivax* transmission because of limited economic resources (Hanscheid and Grobusch, 2002).

Compared to the results of microscopic examination, LAMP had a sensitivity of 98.3% and a specificity of 100%, which were similar to the results of nested PCR (99.0 and 100%, respectively). These results also concur with those of a previous study that used of LAMP for vivax malaria diagnosis in northwestern Thailand and detected vivax malaria parasites in 31 of 34 microscopically positive samples (sensitivity 91.1%) (Han et al., 2007). In the present study, one sample was a false negative sample by both nested PCR and LAMP. In this sample, since the level of parasitemia (approximately 60 parasites/μl) was within the capabilities of nested PCR and LAMP and we believe that this negativity was due to a lower DNA extraction efficiency (Han et al., 2007). We also collected blood from 50 consecutive feverish patients from malaria endemic areas of the Republic of Korea. Because the areas belong to low malaria risk area, we did not find positive cases in these consecutive feverish patients detected by LAMP and nested PCR (Shin et al., 2008).

One of the attractive features of the LAMP is its ability to generate a large amount of white magnesium pyrophosphate precipitate for positive samples, which allows the presence of *P. vivax* to be easy identified by visual inspection (Mori et al., 2001). Four visual detection methods of LAMP assay were compared. It is important since one of the major advantages of the LAMP method is that there are no requirements for expensive materials. For prevention of contamination, it was also not recommended to open LAMP reaction tubes and add SYBR green after the reaction. The first method (naked eye detection without SYBR green) was the most useful when one positive control sample existed. In the present study, assessments of positivity based on precipitate formation in all 147 subjects (patients plus healthy controls) revealed a *J* index of 0.98. Another advantage of LAMP is that heat-treated DNA samples can be directly assayed. However, it should be noted that some inhibitors (for example, hemoglobin and lactoferrin) in blood can severely affect DNA amplification during PCR (Al-Soud and Radstrom, 2001), although it has been suggested that the *Bst* DNA polymerase used for amplification during LAMP is more resistant than other DNA polymerases (Poon et al., 2006).

Of the 30 heat-treated microscopically positive samples subjected to LAMP assays 28 tested positive, whereas all 30 heat-treated microscopically negative samples tested negative. The two false negative samples by LAMP are probably attributable to a lower purification efficiency of template DNA than that achieved with commercial DNA purification kits. It could be attributed to the heat-treatment of blood samples directly, as these may have contained cellular or other contaminants due to inadequate centrifugation, which could have mimicked turbidity, and thus, affected visual assessments (Paris et al., 2007). In the present study, 50 μl blood were mixed with 50 μl of distilled water and then heated, which would have reduced the contribution of the insoluble pellet fraction.

Performance and a rapid turnaround time are equally important aspects of any test, and clinicians and patients require a rapid on-site test that can produce a result in less than 1 h (Hanscheid and Grobusch, 2002). The described LAMP assay can achieve this, and thus, is much faster than routine nucleic acid amplification assays, like nested PCR.

The LAMP assay for *P. vivax* detection is more straightforward than nested PCR and microscopic examinations and has similar sensitivity and specificity in comparison with microscopic examinations. However, microscopic examination is highly technician dependent, requiring blinded confirmatory readings (Bell and Peeling, 2006). Results of microscopic examinations depend on the quality of the microscope and staining, on the technique with which the blood film is prepared and the parasites are counted, and on the concentration and motivation of the microscopist.

In contrast, the LAMP assay requires only simple preparation of the reaction mixtures, without a thermal cycler, time-consuming

DNA purification steps, and down-stream processing for amplicon detection. These features mean that the described LAMP method is suitable for the molecular diagnosis of *P. vivax* in both clinical laboratories and malaria clinics in areas where vivax malaria is endemic.

Acknowledgements

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Chapter 8

An Efficient Approach to the Production of Vaccines Against the Malaria Parasite

Takafumi Tsuboi, Satoru Takeo, Tatsuya Sawasaki, Motomi Torii, and Yaeta Endo

Abstract

In malaria vaccine research, one of the major obstacles has been the difficulty of expressing recombinant malarial proteins and it is mainly due to the lack of an efficient methodology for the synthesis of sufficient quantity of quality proteins. We demonstrate that the wheat germ cell-free protein synthesis system can be applied for the successful production of leading malaria vaccine candidate antigens and, thus, prove that it may be a key tool for malaria vaccine research.

Key words: Cell-free protein synthesis, Parasite, Malaria, Vaccine

1. Introduction

Malaria, a serious infectious disease that challenges the global health, causes millions of deaths annually, as well as illness in hundreds of millions of people. The most deadly form of the disease is caused by the inoculation of the malaria parasite, *Plasmodium falciparum*, by infected mosquito bites. The disease is re-emerging mainly due to the emergence of multidrug-resistant parasites and insecticide-resistant mosquitoes (1). Therefore development of malaria vaccine has been considered as one of the essential components for the malaria eradication (2). However, efforts to develop a successful vaccine have not yet accomplished (3). Since we need multiple vaccine candidate antigens to succeed in controlling malaria, post-genome malaria vaccine candidate discovery is necessary. One of the obstacles in this process is at the malaria protein production step and is mainly due to the lack of an efficient methodology to prepare quality proteins. *P. falciparum* genes have a very high A/T content (average 76% per coding

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sequence throughout the genome) and a number of them encode repeated stretches of amino acid sequences (4), and these features have been proposed as the major factors limiting *P. falciparum* protein expression in conventional cell-based systems (5–7). Moreover, the presence of glycosylation machinery in eukaryotic cell-based protein expression systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses (8–10). There are also constraints such as requirement of disulfide bond formation if the target protein requires it for its bioactivity and requirement of preparation of large quantities of antigen for immunization. The above limiting factors are impediments completely insurmountable in the case of eukaryotic cell-based expression systems such as yeast, baculovirus, or Chinese hamster ovary cell. But fortunately we found that the wheat germ cell-free system can surmount most of the above impediments in the way of finding and developing malaria vaccine candidates (11).

In this chapter, we describe how the wheat germ cell-free system is effective (1) in producing properly folded good quality protein (2) in sufficient quantities (3) that too without the need for codon optimization, using the leading malaria vaccine candidate Pfs25 as an example. And we also describe that (1) proteins produced by the wheat germ cell-free system can be easily purified using simple affinity chromatography, (2) they can be directly used for immunization, and (3) the antibody raised against the proteins are functional in our biochemical, immunocytochemical, and biological analyses. Therefore we hope the wheat germ cell-free system may have dramatic impact on malaria vaccine research.

2. Materials

2.1. Parasite cDNA Preparation, PCR Amplification, and Construction of the DNA Template for Transcription

1. Malaria parasite pellet, *P. falciparum* 3D7 strain (available from Malaria Research and Reference Reagent Resource Center managed by ATCC, Manassas, VA, see Note 1) stored at −80°C in the presence of Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland).
2. RNeasy Micro Kit (QIAGEN, Valencia, CA).
3. SuperScript III RT First-Strand Synthesis System (Invitrogen, Carlsbad, CA).
4. Oligonucleotide primers (see **Note 2**). Design the 5′ primers for the target to be amplified as follows: desired restriction site followed by a 30-mer of unique sequence covering the 5′ region of the open reading frame containing the start codon.

Design the 3′ primers, desired restriction site followed by a 30-mer of unique sequences covering the 3′ region of the open reading frame upstream of the termination codon. Either 5′ or 3′ primer may contain nucleotide sequence coding hexa-histidine tag for Nickel affinity purification.

5. Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA).
6. Thermal cycler (MJ Research, Waltham, MA) (see Note 3).
7. Plasmid of Ehime University (pEU)-E01 protein expression vector specialized for the wheat germ cell-free system.
8. Ligation high ligation reagent (Toyobo, Osaka, Japan).

2.2. Antigen Scale Cell-Free Protein Synthesis and Affinity Purification

1. pEU-E01 plasmid that contains target cDNA.
2. 5× transcription buffer (TB): 400 mM HEPES-KOH (pH 7.8), 80 mM magnesium acetate, 10 mM spermidine, and 50 mM DTT.
3. Nucleotide tri-phosphates (NTPs) mix: a solution containing 25 mM each of ATP, GTP, CTP, and UTP.
4. SP6 RNA polymerase and RNasin (80 U/mL, Promega, Madison, WI).
5. 40 mg/mL creatine kinase.
6. 240 OD/mL WEPRO®1240H (CellFree Sciences, Matsuyama, Japan).
7. 1× Translational substrate buffer (SUB-AMIX): 30 mM HEPES-KOH (pH 7.8), 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 4.0 mM DTT, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate.
8. 6-well microplate (Greiner, Frickenhausen, Germany).
9. Nickel-nitrilotriacetic acid agarose beads (Qiagen).
10. Poly-Prep chromatography column (Bio-Rad, Hercules, CA, USA).
11. Phosphate buffered saline (PBS): Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4 (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by diluting one part with nine parts of water.
12. 1× Wash buffer: 1× PBS supplemented with 30 mM of imidazole and 300 mM NaCl.
13. 1× Elution buffer: 1× PBS supplemented with 500 mM of imidazole and 300 mM NaCl.

2.3. Characterization of the Target Molecules by Confocal Immunofluorescence Microscopy

1. Female BALB/c mice 6–8 weeks of age (Kitayama Labes, Ina, Japan).
2. Freund complete and incomplete adjuvant (Wako Pure Chemical, Osaka, Japan)
3. CF11 cellulose powder (Whatman, Maidstone, UK)
4. 50% Percoll: Percoll (GE Healthcare Bio-Sciences, Piscataway, NJ) diluted 1:1 (v/v) with 2× PBS
5. Ookinete culture medium: RPMI1640 medium (Invitrogen) supplemented with 50μg of hypoxanthine per mL, 25 mM HEPES, 20% heat-inactivated fetal calf serum (Invitrogen), 24 mM NaHCO3, %U of penicillin per mL, and 5μg of streptomycin per mL (pH 8.4).
6. Eight-well Multitest slides (Flow Laboratories, McLean, VA).
7. Blocking and dilution buffer: 5% (w/v) nonfat dry milk in PBS prepared freshly.
8. Secondary antibody: Antimouse IgG conjugated to Alexa488 (Invitrogen) (see Note 4).
9. Nuclear stain: 2 mg/mL 1,000× DAPI (4,6-diamidino-2-phenylindole) stock solution in 100% methanol.
10. Mounting medium: ProLong Gold Antifade Reagent (Invitrogen) (see Note 5).

2.4. Malaria Transmission-Blocking Vaccine Efficacy Assay

1. Malaria parasite-infected blood specimen collected from malaria patient, under informed consent, at the clinics in Thailand.
2. *Anopheles dirus* mosquitoes reared in the insectary, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand (see Note 6).
3. Anti-Pfs25 sera produced in BALB/c mice.
4. Malaria naïve human AB-type serum obtained from volunteers lived in Bangkok.
5. Custom made water-jacketed membrane-feeding apparatus.
6. Parafilm M (Alcan Packaging, Neenah, WI)

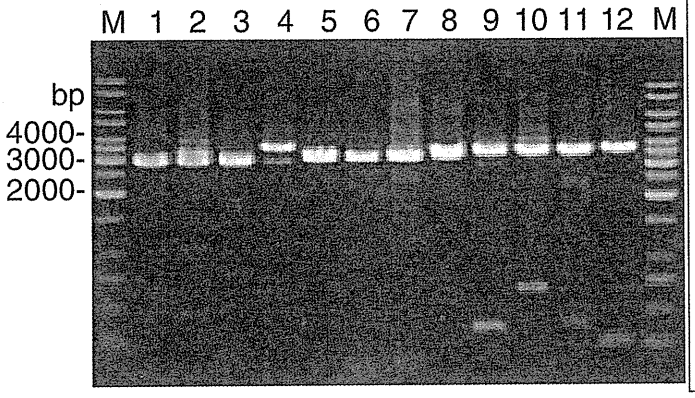
3. Methods

One of the well-known difficulties in malaria research is the PCR amplification of the target sequence because nucleotide sequences of *P. falciparum* genes are highly A/T rich. This feature requires longer primers than those for the G/C rich organism to amplify

the target sequences by PCR. Moreover, there are a number of malaria proteins that contain stretches of amino acid repeat motifs and also a number of malaria genes that contain multiple adenine-rich nucleotide sequences (A-islands) in the coding region. This makes the cDNA cloning dramatically difficult, because these A-islands sometimes cause different number of A-nucleotides in each plasmid clone that causes frameshift. Therefore, the initial hurdle for the malaria protein production is the cDNA-cloning; these factors also require consideration on the plasmid construct design. The following methods explain (1) PCR amplification and cloning of the parasite ORF into the pEU expression vector, (2) synthesis of mRNA using expression vector, (3) translation of prepared mRNA in bilayer system, and (4) biological qualification of the synthesized proteins, which includes the recognition of the parasite molecule with the antibody raised against the recombinant malaria protein. This can be accomplished through indirect immunofluorescence microscopy and vaccine efficacy assay.

3.1. Parasite cDNA Preparation and PCR amplification of DNA-Template Construction for Transcription

1. Extract total RNA from cultured *P. falciparum* 3D7 parasites, then reverse transcribe the mRNA into cDNA by using SuperScript III RT First-Strand Synthesis System.
2. PCR amplify the desired target using Phusion™ High-Fidelity DNA Polymerase.
3. Clone the PCR products (see Fig. 8.1) into the pEU-E01 plasmid using Ligation high ligation reagent.
4. Confirm the sequence of the cloned insert (see Note 7).
5. Purify the plasmid.



	Size (bp) Marker	A/T(%)
M:		
1:	3018	76.3
2:	3048	78.8
3:	3102	81.1
4:	3222	79.4
5:	3342	78.9
6:	3345	75.3
7:	3393	73.0
8:	3540	75.7
9:	3663	78.6
10:	3678	78.9
11:	3696	79.7
12:	3771	75.0

Fig. 8.1. Efficient PCR amplification of the *Plasmodium falciparum* cDNAs. Twelve cDNA targets were selected from *P. falciparum* 3D7, randomly. The cDNA targets were PCR amplified using Phusion™ High-Fidelity DNA Polymerase, under the following conditions: 98°C for 2 min and then 40 cycles at 98°C for 15 s, 55°C for 20 s, and 68°C for 2 min, followed by a final extension at 68°C for 10 min. The amplified products were visualized in a 0.8% agarose gel stained with ethidium bromide. All of the targets ranging from 3,018 bp (*lane 1*) to 3,771 bp (*lane 12*) were successfully amplified even in the presence of very high A/T contents (i.e., 73.0–81.1%).

3.2. Antigen Scale Cell-Free Protein Syntheses of Malaria Protein and Affinity Purification

An example of the methods and the results for Pfs25 is described.

1. Prepare a plasmid clone which has an insert as shown in Fig. 8.2A.
2. Incubate 200 µL of transcription mixture containing 20 µg of the plasmid DNA, 1× TB, 2.5 mM each of NTPs, 200 U of SP6 RNA polymerase, and 200 U of RNasin for 6 h at 37°C.

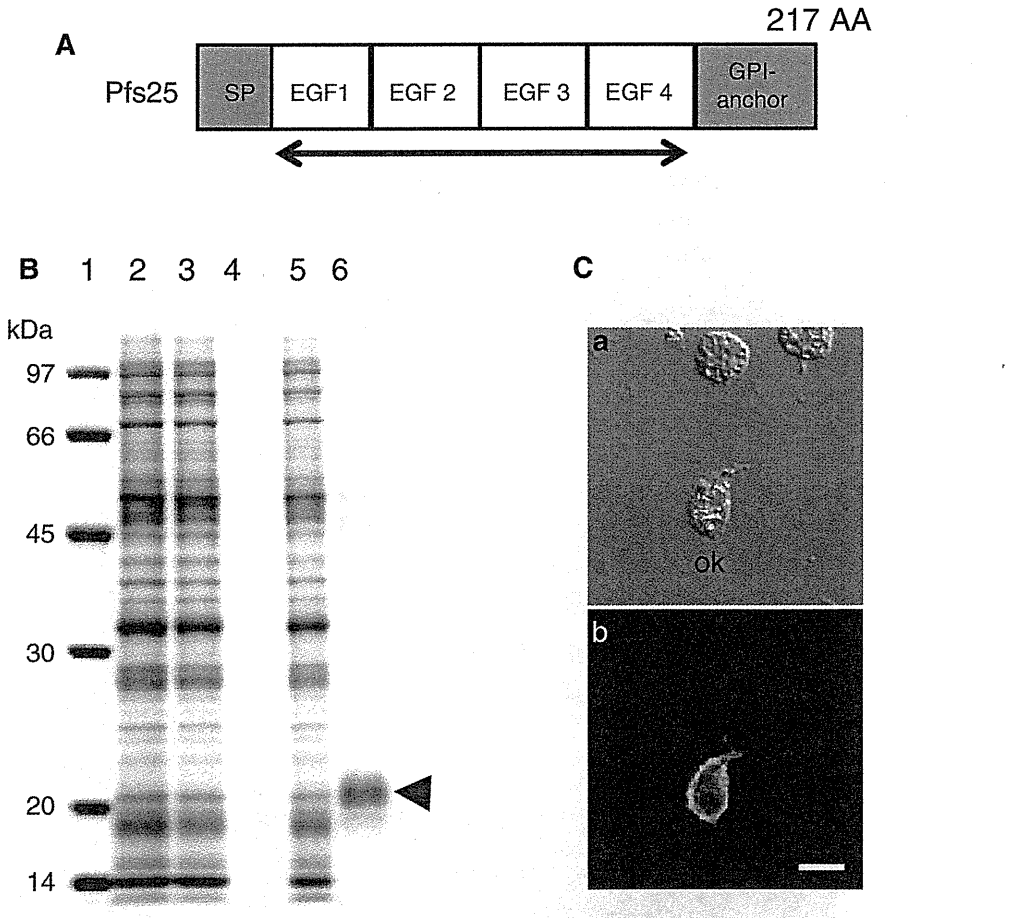


Fig. 8.2. Cloning, expression, and immunolocalization of Pfs25, a leading malaria transmission-blocking vaccine candidate. (**A**) Schematic representation of Pfs25. Pfs25 was expressed without the nucleotide sequences coding for the signal peptide (SP) and the GPI anchor (*arrow*). This Pfs25 gene was amplified by PCR from the *P. falciparum* 3D7 strain using antisense primer that contains nucleotide sequence encoding for hexa-histidine tag at the C terminus and subcloned into pEU-E01 plasmid at the EcoRV site. The A/T content of the *pfs25* insert was 70%. (**B**) The expression, affinity purification, and SDS-PAGE of recombinant Pfs25. Recombinant Pfs25 was expressed using the wheat germ cell-free method, affinity purified by Nickel affinity chromatography, and size-fractionated by 12.5% SDS-PAGE under reducing condition and stained with CBB. *Lane 1*, molecular mass markers in kDa; *lane 2*, total translation mixture; *lane 3*, supernatant fraction of the translation mixture; *lane 4*, pellet fraction of the translation mixture; *lane 5*, flow-through fraction after the affinity purification, *lane 6*; affinity-purified recombinant protein (*arrow head*). (**C**) The immune serum raised against recombinant Pfs25 specifically recognized native Pfs25 proteins expressed on the surface of *P. falciparum* immature ookinetes (ok). (**a**) Differential interference contrast and (**b**) fluorescence confocal images were obtained on a LSM5 PASCAL microscopy (Carl Zeiss MicroImaging, Thornwood, NY) using a 63× oil-immersion lens. *Bar*=5 µm.

3. Mix the transcription solution containing transcribed mRNA (see Subheading 3.2, step 1) with 200 µL of WEPRO®1240H (240 OD/mL) supplemented with 0.4 µL of creatine kinase (40 mg/mL). Add 4.4-mL of 1× SUB-AMIX into a single well of a 6-well plate and then underlay the above transcription mixture and incubate at 17°C for 16 h.
4. Add imidazole (pH 8.0) in the translation reaction mixture (final concentration, 20 mM) and then add 80 µL of 50% slurry of Ni-NTA beads.
5. Incubate the tube for 16 h on a continuous rotator, at 4°C, for the binding of proteins on to the beads.
6. Transfer the solution with the beads into a Poly-Prep column.
7. Wash the beads by 0.4 mL of wash buffer three times and then elute the recombinant protein with 80 µL of elution buffer five times.
8. Analyze the purified protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition, and the bands were visualized with Coomassie brilliant blue (see Fig. 8.2B).

3.3. Preparation of Antiserum and Parasite Antigen for Indirect Immunofluorescence Assay

3.3.1. Antiserum Preparation

1. Immunize female BALB/c mice (6–8 weeks of age) by subcutaneous injection with affinity purified recombinant proteins emulsified with the same volume of Freund's complete adjuvant as a priming dose and then administer two additional booster doses with Freund's incomplete adjuvant at 3-weeks interval.
2. Collect blood by cardiac puncture a week after the final boost under anesthesia and then separate sera after the coagulation of the blood (see Note 8).

3.3.2. Preparation of Parasite Antigens from the Cultured Ookinetes of Plasmodium falciparum

1. Collect peripheral blood with heparinized syringes from malaria patients under written informed consent. Purify gametocytes by passing 5–10 mL of blood through CF-11 column to remove leukocytes followed by 50% Percoll density gradient centrifugation (350 × *g* for 25 min at room temperature).
2. Collect the interface rich in gametocytes on the Percoll cushion into a new tube. Wash them twice with PBS and then culture this parasite pellet to ookinete in 1 mL of ookinete medium for 24 h at 24°C in air.
3. Wash the cultured parasite preparations rich in ookinetes twice with PBS and then spot them on 8-well Multitest slides and fix them with ice-cold acetone for 5 min.
4. Store the slides at −80°C until use.

3.3.3. Staining Procedure for Immunofluorescence Assay

1. Take the desired number of antigen slides out from the freezer and then place them quickly in a desiccator until they are brought to room temperature.
2. Block the slides with blocking buffer for 30 min at 37°C in the humidified chamber.
3. Incubate with anti-Pfs25 immune sera (1:100 dilution with blocking buffer) for 1 h at 37°C (see Note 9).
4. Wash the slides with ice-cold PBS for 5 min and incubate with secondary antibody (1:500) and DAPI (1:1,000) diluted with blocking buffer for 30 min at 37°C, followed by washing with ice-cold PBS for 5 min. Mount the slides with Prolong Gold Antifade Reagent and incubate the slides for overnight at room temperature to allow complete solidification of the mounting medium.
5. View the slides under confocal microscopy. Excitation at 488 nm induces the Alexa Fluor 488 fluorescence (green emission) for the Pfs25 (Fig. 8.2Cb), while the differential interference contrast image (Fig. 8.2Ca) is also captured. For example, the fluorescent signal for Pfs25 on the surface of ookinete is shown in Fig. 8.2Cb.

3.4. Malaria Transmission-Blocking Vaccine Efficacy Assay

1. Collect peripheral blood into heparinized syringe from a volunteer patient.
2. Aliquot the collected blood into tubes (300 µL/tube) and remove plasma by a brief centrifugation.
3. Dilute mouse immune sera into 1:2, 1:8, and 1:32 (v/v) with heat-inactivated normal human AB serum prepared from Thai malaria naïve donors.
4. Mix each diluted test serum with *P. falciparum*-infected blood cells (1:1, v/v) and incubate for 15 min at room temperature.
5. Place the mixture into a water-jacketed membrane feeding apparatus (see Fig. 8.3a) whose bottom is sealed by Parafilm M kept at 37°C with circulating water outside of the feeder to allow starved *A. dirus* mosquitoes (Armed Forces Research Institute of Medical Sciences) to feed on the blood meals for 30 min (see Fig. 8.3b).
6. Remove unfed mosquitoes by manual aspiration and maintain only fully engorged mosquitoes for a week by giving 10% sucrose water in the insectary.
7. Dissect 20 mosquitoes for each mouse test immune serum and count the number of oocysts developed within the mosquito midgut under the microscope by staining with 0.5% mercurochrome. The transmission-blocking vaccine efficacy is accessed by the number of oocysts per mosquito (see Fig. 8.3c).

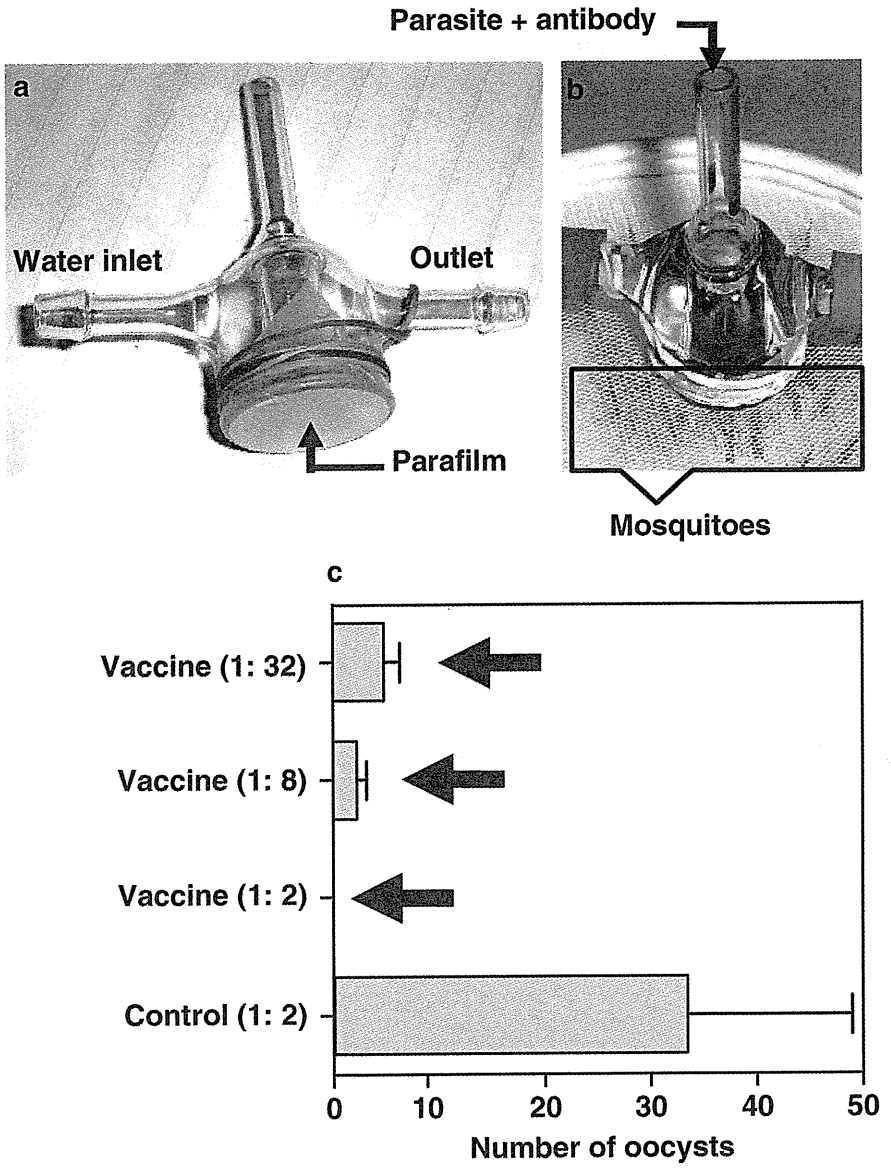


Fig. 8.3. Transmission-blocking efficacy of antibodies against *Plasmodium falciparum* parasites. (**a**) A membrane feeding apparatus with its bottom sealed by Parafilm M. (**b**) The apparatus is kept at 37°C with circulating water outside of the feeder to allow mosquitoes to feed on the infected blood mixed with test and control sera. (**c**) The median numbers of oocysts per mosquito ($n=20$) (*bars*) with interquartile ranges (*lines on top of the bars*) were compared among groups of mosquitoes fed on either anti-Pfs25 serum serially diluted or control mouse serum. Dilution range of test immune serum used is shown as 1:2 to 1:32. *Arrows* indicate statistically significant differences compared to the control group analyzed using Kruskal–Wallis test ($P<0.05$).

4. Notes

1. The Malaria Research and Reference Reagent Resource Center (MR4) is a central source of quality reagents to the malaria research community. Materials available to registered users include parasites, mosquito vectors, antibodies, antigens, gene

libraries, etc. MR4 Web site (http://www.mr4.org/Home/tabid/93/Default.aspx) also provides a lot of useful information such as online protocol book named *Methods In Malaria Research* (version 5.2). This book includes protocols that are useful to malaria research, for example, parasite culture methods, immunological assays, or molecular biological techniques.

2. For the efficient PCR amplification, the melting temperature of oligonucleotide primer should be higher than 70°C and it can be roughly calculated by the following formula; $2°C \times (A+T) + 4°C \times (G+C)$. This is one of the essential factors for the efficient PCR amplification of *P. falciparum* because the *P. falciparum* genome is A/T rich.
3. Thermal cycler, which has gradient temperature function, is useful when optimizing the PCR condition, such as testing a lot of different annealing temperatures.
4. For the multiple labeling experiment, check the quality of the secondary antibodies in advance. For example, some of the commercially available secondary antibodies recognizing mouse IgG are highly cross-adsorbed using bovine, goat, human, rabbit, and rat IgG and also human serum. This secondary antibody can be useful for the double labeling with both mouse and rat primary antibodies.
5. This antifade reagent will solidify, and the sample can be saved for months after mounting, and it also offers enhanced resistance to photobleaching. This criterion is very important for the high-quality imaging because this reagent allows the repeated scanning with the confocal laser microscope until the satisfactory images are obtained.
6. *A. dirus* is a major malaria vector mosquito species in Thailand. This mosquito line is established and adapted as one of the laboratory lines.
7. Pay special attention to confirm the number of adenine nucleotides in the A-islands present in the target region. Otherwise wrong number of adenine nucleotides cause frameshift. If a plasmid clone contains wrong number of adenine nucleotides, verify another plasmid clone which sometimes might have a correct number of adenine nucleotides in the A-island.
8. The antisera for immunofluorescence assay can be saved by addition of sodium azide (final concentration, 0.02%) (Caution highly toxic) and stored at 4°C to prevent repeated freeze thaw cycles which damage the antibodies. However, the antisera for the biological assay such as membrane feeding should not contain any preservatives. In this case, all the antisera are kept frozen at –80°C.

9. One well in the 8-well Multitest slide can hold up to 20 μL of the blocking or antibody solution. In the case of 24-well Multitest slide, one well can hold up to 10 μL of the solution.

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The wheat germ cell-free protein synthesis system: A key tool for novel malaria vaccine candidate discovery

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ABSTRACT

Malaria kills more than a million people a year, causes malady in about three hundred million people and poses risk to approximately 40% of the world's population living in malarious countries. This disease is re-emerging mainly due to the development of drug-resistant parasites and insecticide-resistant mosquitoes. Therefore, we are now forced to resort to remedy through vaccination. Until now, not even a single licensed malaria vaccine has been developed despite intensive efforts. Even the efficacy of RTS,S, the most advanced and promising vaccine candidate in the pipeline of malaria vaccine development, was only around 50% based on a number of clinical trials. These facts urge malaria researchers to urgently enrich this pipeline, as much as possible, with potential vaccine candidates. With the availability of malaria genome database, the enrichment of this pipeline is possible if we could now employ an efficient protein expression technology to decode the malaria genomic data, without any codon optimization, into quality recombinant proteins. Then, these synthesized recombinant proteins can be characterized and screened for discovering novel potential vaccine targets. The wheat germ cell-free protein synthesis system will be a promising tool to this end. This review highlights the recent successes in synthesizing quality malaria proteins using this tool.

1. Introduction

Malaria kills more than a million people a year, causes malady in about three hundred million people and poses risk to around 3.3 billion people (WHO, 2008). Despite the parasite's complex life cycle, high level of antigenic diversity and mechanism of immune evasion, naturally acquired immunity to malaria does develop after repeated exposure over a period of several years and this immunity confers protection, against symptomatic disease, high-density parasitemia and death (Doolan et al., 2009; Genton, 2008; Gupta et al., 1999; Marsh and Kinyanjui, 2006), through protective antibodies (Cohen et al., 1961; McGregor, 1964). In addition to the naturally acquired immunities, sterile, long-lasting protective immunity has been convincingly proved in many studies, such as vaccination with radiation-attenuated sporozoites (Clyde, 1975; Hoffman et al., 2002; Nussenzweig et al., 1967), and inoculation of infective sporozoites to human volunteers under a prophylactic regimen of chloroquine (Roestenberg et al., 2009). These facts strongly support the reasoning of incorporating malaria vaccines as one of the components of malaria control measures.

2. Malaria vaccine development

The efficacy of malaria control through current interventions that employs drugs and insecticides may not be sustained too long since they rely on too few compounds (Genton, 2008). In fact, the disease is re-emerging mainly due to the emergence of drug-resistant parasites (Greenwood and Mutabingwa, 2002). Very recently, parasites have developed resistance even to the hitherto-promising artemisinin (Dondorp et al., 2009). Therefore, we have to develop and employ malaria vaccines as one of the essential components towards the malaria eradication (Greenwood, 2009). The fact that, in spite of intensive efforts, not even a single licensed malaria vaccine has been developed, urges malaria research community to employ efficient post-genomic approaches (Richie and Saul, 2002). Malaria vaccines are generally divided into three groups based on stages of the parasite life cycle targeted by the vaccine. They are pre-erythrocytic vaccines, asexual blood-stage vaccines, and transmission-blocking vaccines. Fig. 1 gives a quick description about vaccine categories, the stages they target, along with malaria vaccine candidates in clinical trials. Detailed descriptions about

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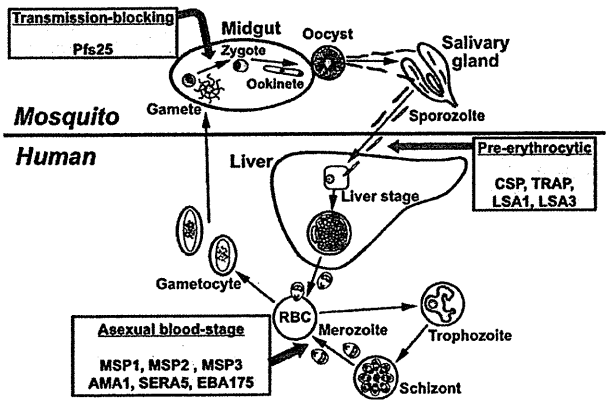

Fig. 1. Malaria vaccines in clinical trials mapped on the parasite life cycle. Malaria vaccines are categorized into three groups based on the target stages of the parasite life cycle. They are pre-erythrocytic, asexual blood-stage, and transmission-blocking vaccines. RBC: red blood cell, CSP: circumsporozoite protein, TRAP: thrombospondin-related adhesion protein, LSA1: liver stage antigen 1, LSA3: liver stage antigen 3, MSP1: merozoite surface protein 1, MSP2: merozoite surface protein 2, MSP3: merozoite surface protein 3, AMA1: Apical membrane antigen 1, SERA5: Serine repeat antigen 5, and EBA175: erythrocyte binding antigen 175. Detailed descriptions about these vaccines were given in other published reviews (Genton, 2008; Richards and Beeson, 2009).

these vaccines are given in other published reviews (Genton, 2008; Richards and Beeson, 2009).

2.1. Pre-erythrocytic vaccines

Pre-erythrocytic vaccines have been designed to prevent entry of sporozoites into hepatocytes and the development of liver stage parasites (Greenwood et al., 2008; Waters, 2006).

2.2. Asexual blood-stage vaccines

Asexual blood-stage vaccines are designed to reduce merozoite invasion, multiplication and growth in order to protect against clinical symptoms and particularly severe disease. It is widely understood that this vaccine induces antibodies that may have roles in prevention of merozoite invasion, clearance of infected erythrocytes, prevention of adhesion and sequestration of parasitized erythrocytes in the vasculature. It is also possible that, in addition to preventing clinical illness, an effective blood-stage vaccine may also contribute to malaria eradication by reducing the efficiency of the transmission of parasites from human host to mosquito by interrupting the blood-stage life cycle in the human body (Duffy, 2007; Genton, 2008; Moll et al., 2007; Richards and Beeson, 2009).

2.3. Transmission-blocking vaccines

Transmission-blocking vaccines are aimed at interrupting the parasite life cycle in the mosquito blood meal. These vaccines elicit antibodies against antigens that are expressed by the sexual stages of the parasite and, thus, stop their subsequent development in the mosquito midgut (Carter, 2001; Tsuboi et al., 2003). These transmission-blocking vaccines, if used in combination with pre-erythrocytic or asexual blood-stage vaccines, might play a key role in finally breaking the transmission of parasites, leading to eradication of the diseases (Targett and Greenwood, 2008).

It is an accepted view that an effective malaria vaccine need to target several stages of parasite and several components of the different stages of parasite (Lasonder et al., 2002) and it must induce protective immune responses equivalent to, or better than, those provided by naturally acquired immunity or immunization with attenuated whole parasite (Gardner et al., 2002). In order to accelerate the discovery of such vaccine candidates, it is indispensable now to establish and exploit two things. One is the optimal recombinant protein synthesis system for synthesizing malaria proteins on a whole-proteome scale and other is the efficient post-genomic high-throughput approaches for sifting potential vaccine candidates out from this whole malaria proteome.

3. Post-genome approaches for novel malaria vaccine candidate discovery research

As described in Fig. 1, decades of research in the pre-genomic era have identified only a handful of vaccine candidates. With the recent completion of the genome projects of human malaria parasites, *Plasmodium falciparum* (Gardner et al., 2002), *P. vivax* (Carlton et al., 2008), primate malaria parasite, *P. knowlesi* (Pain et al., 2008), and rodent malaria parasite, *P. yoelii* (Carlton et al., 2002), we are now in the post-genome era. However, to tangibly reap the benefits from these genomic data, it is indispensable to thoroughly analyze these data using at least two post-genomic high-throughput approaches. One is the functional approach (otherwise called as reverse vaccinology (Flower, 2008)), and the other is the immunoscreening approach.

3.1. Functional approach

In the functional approach, initially putative vaccine candidates are selected from the genome database based on either patent or latent functional criteria. Then these putative candidates are expressed *in vitro* using effective and efficient protein synthesis system to obtain quality proteins for further downstream vaccine candidate assessment studies (Hall et al., 2005). For example, the genes that are essential for the parasite's survival revealed by gene knockout studies (Cowman and Crabb, 2006), or the genes with signatures of strong immune selective pressure revealed by polymorphisms and diversity studies (Mu et al., 2007), or the genes involved in host cell invasion, or the genes whose products are localized on the cell surface or in the apical organelles of the sporozoite, merozoite, and ookinete could be putative vaccine candidates.

3.2. Immunoscreening approach

In the immunoscreening approach, initially putative vaccine candidates are selected based on their immuno-reactivity with the protective antibodies that are elicited in humans, after natural or experimental infection or after vaccination with attenuated organisms (Hoffman et al., 2002; VanBuskirk et al., 2009). Here extensive set of quality recombinant malaria proteins are synthesized, in small scale, using protein synthesis system and are screened intensively, using immunoassays such as enzyme-linked immunosorbent assay, protein microarray, with a large number of human serum samples obtained from non-immune and immune individuals (Doolan et al., 2008). Then, these immuno-reactive putative vaccine candidates that correlate with protection will be synthesized, in large scale, for further downstream vaccine research.

4. What is the optimal recombinant protein synthesis system for malaria proteins?

Whatever may be the approach employed for novel vaccine candidate discovery research, we do need to have an effective and