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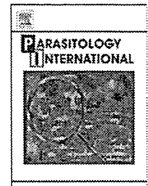

Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for clinical detection of *Plasmodium falciparum* gametocytes

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ABSTRACT

Plasmodium falciparum gametocytes are usually present in peripheral blood at a very low level, thus requiring a sensitive assay detection method. In this study, reverse transcription-loop-mediated isothermal amplification (RT-LAMP) was developed for clinical detection of *P. falciparum* gametocytes. Transcripts of Pfs16 of sexually committed ring and Pfs25 of mature gametocytes were detected by RT-LAMP in 82 clinical blood samples using nested RT-PCR as a gold standard. RT-LAMP demonstrated a detection limit of 1 parasitized red blood cell (RBC)/500 µl of blood for both Pfs16 and Pfs25 transcripts. For Pfs16 transcript, RT-LAMP detected all 30 samples positive by nested RT-PCR (100% sensitivity) and 1 in 52 samples negative by nested RT-PCR (98.1% specificity). For Pfs25 transcript, RT-LAMP detected all 15 samples positive by nested RT-PCR (100% sensitivity) and none of 67 samples negative by nested RT-PCR (100% specificity). Negative predictive value (NPV) and positive predictive value (PPV) of RT-LAMP for detection of Pfs16 transcript were 100% and 96.8%, respectively, and 100% for both when employing Pfs25 transcript. Detection rate of Pfs16 and Pfs25 transcripts by RT-LAMP in microscopically gametocyte-negative samples was 91.7% and 29.2%, respectively. Compared with nested RT-PCR, RT-LAMP had a higher sensitivity but similar specificity, with the advantage of a shorter assay time. As RT-LAMP requires very basic instruments and the results can be obtained by visual inspection, this technique provides a simple and reliable tool for epidemiological studies of malaria transmission and in gametocyte-targeted control programmes.

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1. Introduction

Asexual stages of the malaria parasite, *Plasmodium falciparum*, are responsible for the clinical symptoms, whereas the sexual stages (gametocytes) are transmitted from human hosts to mosquito vectors. The existence and infectivity of mature gametocytes in the blood circulation determine the accomplishment of transmission. Gametocytes arise from only a small fraction of asexual parasites and only a proportion of patients with a primary infection develop patent gametocytaemia [1]. Gametocytes are usually present in the blood circulation at a very low level. Several studies have shown that mosquitoes can be infected with gametocytes at densities lower than those detectable by conventional microscopy (<5 gametocytes/µl of blood) [2–9]. In the field, infected individuals can have *P. falciparum* gametocytaemia of less than 100 gametocytes/µl of blood and 1–10 gametocytes/µl of blood are enough to establish mosquito infection [10]. As *P. falciparum* gametocyte carriers account for a substantial proportion of the human infection reservoir due to an underestimation of such carriers by microscopy, a more sensitive and accurate method for gametocyte detection is needed in order to block transmission by appropriate treatment and in the epidemiological studies

In recent years, new technological methods have been developed as alternatives to microscopy, including nested reverse transcription-polymerase chain reaction (nested RT-PCR). Nested RT-PCR using *P. falciparum* gametocyte-specific genes, including Pfs25, is considered to be the most sensitive technique, capable of detecting 1 gametocyte/µl of blood, as well as being highly specific [11]. Although this technique is time-consuming and requires skilled technician, it can be used as a "gold standard" to evaluate other diagnostic tests.

Recently, a loop-mediated isothermal amplification (LAMP) technique that provides a rapid, simple, sensitive and inexpensive method for DNA amplification has been developed [12]. LAMP depends on autocycling

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strand-displacement DNA synthesis conducted by *Bst* DNA polymerase. The reaction is carried out without denaturation of DNA templates [13] and thus can be performed at an isothermal temperature. The amplified products are a series of stem-loop DNA of various lengths. The amplification result can be determined by visual inspection of a turbid solution due to precipitation of white magnesium pyrophosphate, a byproduct of DNA synthesis [14]. LAMP has been shown to have a similar sensitivity and a greater specificity than conventional microscopy, a gold standard, in clinical detection of four species of human malaria parasites: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, with sensitivity similar to that of nested PCR but with a shorter turnaround time [15].

In this study, we report the development of RT-LAMP for clinical detection of *P. falciparum* gametocytes using mRNA of *P. falciparum* gametocyte-specific genes, Pfs16 and Pfs25 as targets. We chose to compare the results of RT-LAMP to those of nested RT-PCR, a gold standard. Pfs16 is a 16.6 kDa protein located in the parasitophorous vacuole membrane. Pfs16 mRNA is firstly expressed in a ring stage and continues to be expressed throughout gametocytogenesis, in both male and female gametocytes [16,17]. Pfs16 mRNA expression indicates circulating sexual stage parasites composed of sexually committed rings and fully mature gametocytes (stage V) as other stages sequester in the vasculature. Pfs16 expression is considered as the earliest marker in *P. falciparum* sexual differentiation process [16–19]. Pfs25 is a 25 kDa major surface protein of zygotes and ookinetes. Pfs25 mRNA is expressed only in stage V gametocytes, making it suitable as a marker of mature gametocytes [11].

This study is the first study to use Pfs16 and Pfs25 for RT-LAMP. The RT-LAMP method developed here allowed detection of *P. falciparum* gametocytes in clinical blood samples with higher sensitivity than that achieved with nested RT-PCR or microscopy. Moreover, RT-LAMP detected gametocytes in over 29% of the blood samples missed by microscopy.

2. Materials and methods

2.1. Blood samples

EDTA-treated blood samples, 32 positive and 20 negative for *P. falciparum* infection by microscopy, were collected from participants at malaria clinics in Mae Sot and Mae Kasa, Tak, northwestern Thailand. Thirty additional blood samples negative for *P. falciparum* by microscopy were collected from residents in Bangkok, Thailand. Thick and thin blood films were made of all samples. Two milliliter of the remaining blood samples was centrifuged at 600 ×g for 5 min at room temperature and approximately 0.8 ml of packed red blood cells (RBCs) was suspended in 1 ml Trizol reagent and kept at −80 °C until used for RNA extraction. All participants signed informed consent forms before enrollment. Ethical approval for this study was obtained from Mahidol University ethics committee, Mahidol University, Bangkok, Thailand.

2.2. Conventional microscopy

Thick blood films were examined under 1000× magnification by an expert microscopist to identify malaria parasites. The results were confirmed from thin blood films by another microscopist. Percent of parasitized RBCs was determined by counting 10,000 RBCs and approximate number of parasites/µl of blood was calculated by assuming a RBC count of 5×10^6/µl of blood [20].

2.3. Total RNA isolation

Total *P. falciparum* RNA was isolated from infected blood samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction and treated with RNase-free DNase I (Fermentus Life Sciences, USA), followed by phenol-chloroform extraction. Any remaining parasite genomic DNA was eliminated by digestion with *Hinc*II and *Pci*I, and the samples were kept at −80 °C. Total RNA from gametocyte-producing *P. falciparum* laboratory strain AMB47 was used as a positive control. A negative control was total RNA from blood samples negative for *P. falciparum* by microscopy.

2.4. Detection of expression of gametocyte-specific genes, Pfs16 and Pfs25, by nested RT-PCR

Expression of A (asexual) type and S (sporozoite) type 18S rRNA was used as an internal positive control to confirm the presence of total RNA of the parasite, and for the presence of *P. falciparum* gametocytes, respectively [21]. Primers and amplification conditions were as described previously [11,22,23] with slight modifications. Details are given in the online Data Supplement (Table A1).

For the first PCR, 2 µg of RNA was added into a mixture containing 0.4 µl of random primers (500 µM) and 0.5 µl of RNase inhibitor (40 U/µl). Then, the mixture was incubated at 70 °C for 10 min and rapidly cooled on ice for 5 min to denature RNA secondary structures. For the RT step, 11 µl of the mixture was added to an RT-reaction mixture containing 4 µl of 5× Bart's buffer, 2 µl of 0.1 M DTT, 2 µl of 10 mM dNTPs, and 1 µl of AMV reverse transcriptase (RTase) enzyme (10 U/µl). To test for carry-over of genomic DNA, a negative control, without RTase, was included. RT-reaction was performed at room temperature for 10 min followed by incubating at 42 °C for 1 h, followed by 95 °C for 10 min to inactivate the enzyme. Two microliters of cDNA was used for the first RT-PCR, using GoTaq® Green Master Mix (Promega, USA) in Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany). The amplicon was diluted 100-fold in nuclease-free water and 2 µl was used in the second amplification as described previously [11,22,23]. Amplicons were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized under an ultraviolet light.

Table 1
Primer sets used for amplification of Pfs16 and Pfs25 genes in RT-LAMP.

Gene	Primer	Sequence (5′→3′)
Pfs16	F3	CCAGGAAGTTCTTCAGGT
	B3	TCATATTTGTGACAGCAGTAC
	FIP (F1c–F2)	GCAGAATCTTTTCCAGAAAGACCTTGCCTCTCTTCATGCTGTTG
	BIP (B1–B2c)	TGCCTTTAGAAACTCAGCTAGCTATAAGTTTCTGTTAACTGTCGTT
	LPF	GAGATAGTCCACCTAGATTAGG
	LPB	AGAAGAAATCAAGAGCTTATC
Pfs25	F3	TTTTAATTCAGATGAGTGGTCAT
	B3	CTTACATTCATTTGGTATACAAACA
	FIP (F1c–F2)	GTCTTTTCGTCACATTTCAGAACTTATGTAAATGTGAAAATGATTTGGTG
	BIP (B1–B2c)	ACCATGTGGAGATTTTTCCAAATGTCAAGATTACATTTACAAGCGTATG
	LPF	CTTCACATGTTTCTTCATTTACTAA
	LPB	ATTAAAATAGATGGAAATCC

2.5. Detection of expression of gametocyte-specific genes, Pfs16 and Pfs25, by RT-LAMP

The primer sets for RT-LAMP of Pfs16 (GenBank accession no. M64705) and Pfs25 (GenBank accession no. X07802) were designed using LAMP Primer Explorer V4 software (http://primerexplorer.jp/e/) (Table 1). RT-LAMP reaction was performed using a Loopamp RNA amplification kit (Eiken Chemical Co. Ltd, Tokyo, Japan). Parasite mRNA was converted into cDNA and amplified in one step in an enzyme mixture (AMV RTase and *Bst* DNA polymerase). RT-LAMP reaction mixture (25 µl) contained 1.3 µl of primer mix (FIP and BIP 40 pmol, Loop-F and Loop-B 20 pmol, F3 and B3 5 pmol), 12.5 µl of 2× reaction mix, 1 µl of an enzyme mixture, 2 µl (200 ng) of RNA template and 8.2 µl of distilled water. RT-LAMP amplification reaction was conducted at 65 °C for 90 min and the enzyme was inactivated at 80 °C for 5 min. Amplification was monitored by a Loopamp real-time turbidimeter (LA-200; Eiken Chemical Co. Ltd).

To detect genomic DNA contamination, total RNA sample was analyzed by conventional LAMP assay using a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd).

2.6. Determination of sensitivity and species-specificity of RT-LAMP

To establish the sensitivity of RT-LAMP, cultures of *P. falciparum* strain AMB47 containing rings or gametocytes (mostly stage V) were subjected to 10-fold serial dilutions from 1×10^7 to 1 parasitized RBC/500 µl of blood. Uninfected human RBCs at 40% haematocrit in heat-inactivated serum collected from non-infected blood were used to dilute the infected RBCs. Samples were subjected for total RNA isolation followed by RT-LAMP analysis. Species-specificity of RT-LAMP was conducted with total RNA of *P. falciparum* strain AMB47 and genomic DNA of *P. vivax*, *P. malariae* and *P. ovale* obtained from clinical blood samples collected from Mae Sot, Tak, Thailand. The specificity of amplification was confirmed by digestion of the RT-LAMP products with *XmnI* specific for both Pfs16 and Pfs25 amplicons. The digested products were subjected to 3% agarose gel-electrophoresis, followed by staining and visualizing as described above.

2.7. Statistical analysis

The specificity, sensitivity, negative predictive value [NPV; number of true negatives/(number of true negatives + number of false negatives)] and positive predictive value [PPV; number of true positives/(number of true positives + number of false positives)] of RT-LAMP were calculated based on 82 blood samples using nested RT-PCR as the gold standard method.

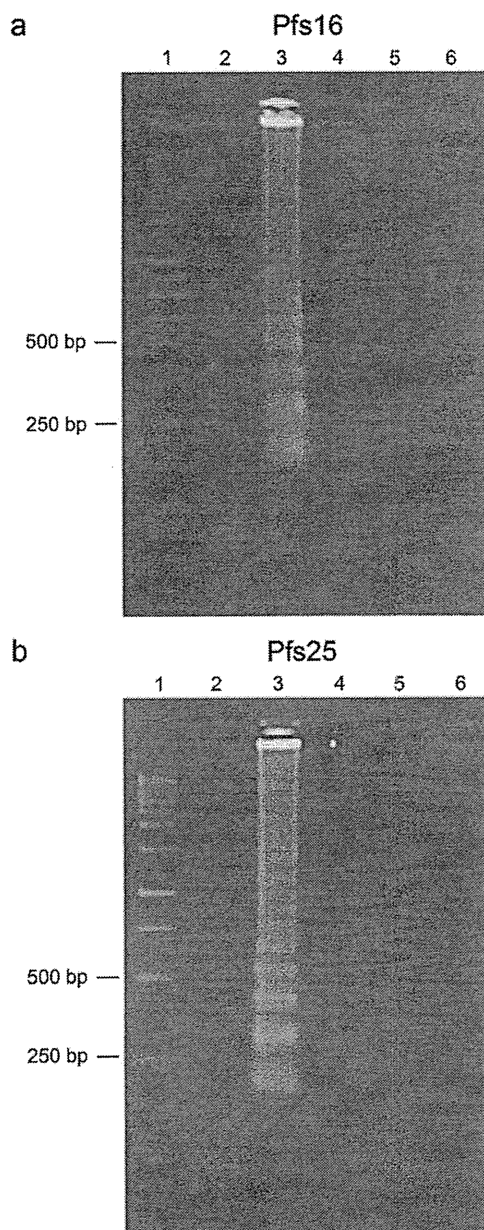

Fig. 1. Species-specificity of RT-LAMP assay. Each RT-LAMP reaction was carried out using six primers (FIP, BIP, F3, B3, LPF and LPB) for the detection of Pfs16 (a) or Pfs25 (b) with total RNA extracted from *P. falciparum* strain AMB47 and genomic DNA from *P. vivax*, *P. malariae* and *P. ovale*. Lane 1: 1 kb-DNA ladder size marker, Lane 2: negative control (no total RNA), Lane 3: *P. falciparum*, Lane 4: *P. vivax*, Lane 5. *P. malariae*, Lane 6: *P. ovale*.

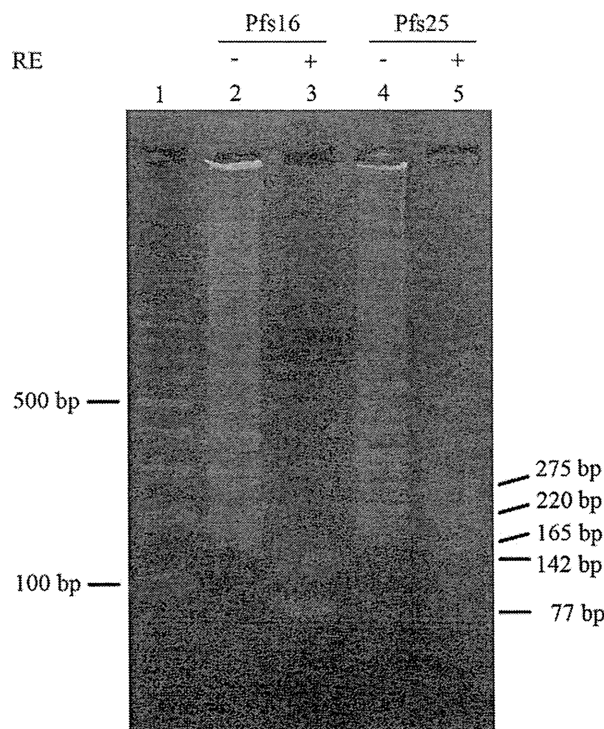

Fig. 2. Restriction enzyme-digestion of RT-LAMP-amplified products of Pfs16 or Pfs25. Lane 1: 100 bp-DNA ladder size marker, Lane 2: RT-LAMP product of Pfs16, Lane 3: *XmnI* digestion of RT-LAMP product of Pfs16, Lane 4: RT-LAMP product of Pfs25, Lane 5: *XmnI* digestion of RT-LAMP product of Pfs25. The digestion products were run on a 3% agarose gel.

3. Results

3.1. Plasmodium species-specificity of RT-LAMP

Plasmodium species-specificity of RT-LAMP for Pfs16 and Pfs25 was determined using total RNA from *P. falciparum* strain AMB47 and genomic DNA from *P. vivax*, *P. malariae* and *P. ovale*. RT-LAMP reaction with Pfs16 and Pfs25 primers specifically produced smeared amplified products and the typical ladder pattern at the bottom of the gel only when total RNA from *P. falciparum* was present in the reaction (Fig. 1a and b). No amplification was observed with DNA from *P. vivax*, *P. malariae*, and *P. ovale*. To verify that the amplified products contained Pfs16 and Pfs25 DNA sequences, the amplicons were digested with*XmnI*, generating the expected fragments of 77 and 142 bp for Pfs16 (Fig. 2, lane 3); for Pfs25, the predicted bands of 165, 220 and 275 bp were observed (Fig. 2, lane 5). These results demonstrated that the primer sets of RT-LAMP for Pfs16 and Pfs25 were highly specific for the detection of Pfs16 and Pfs25 mRNA.

3.2. Sensitivity of RT-LAMP

To evaluate the sensitivity of RT-LAMP, total RNA samples from 10-fold serial dilutions of ring and gametocyte cultures (1×10^7 to 1 parasitized RBC/500 µl of blood) of *P. falciparum* strain AMB47 were subjected to RT-LAMP. To check for carry-over of genomic DNA, RNA samples were amplified without RTase by conventional LAMP assay. Genomic DNA was detected in samples containing 1×10^3 to 1×10^7 parasitized RBCs/500 µl of blood for both Pfs16 and Pfs25 genes (Fig. 3, a and b). However, the turbidity of the amplified products from RTase-negative samples was less than that of RTase-positive samples and the detection time was 10–20 min longer. No carry-over of genomic DNA was detected in samples containing 1 to 1×10^2 parasitized RBCs/500 µl of blood for both Pfs16 and Pfs25 genes. Thus, amplifications observed in this range of parasite levels were from mRNA only. The detection limit of RT-LAMP was 1 parasitized RBC/500 µl of blood for both Pfs16 and Pfs25. This detection limit is equivalent to 0.002 parasitized RBCs/µl of blood or per 8,000 white blood cells, a typical number in a malaria endemic country [20].

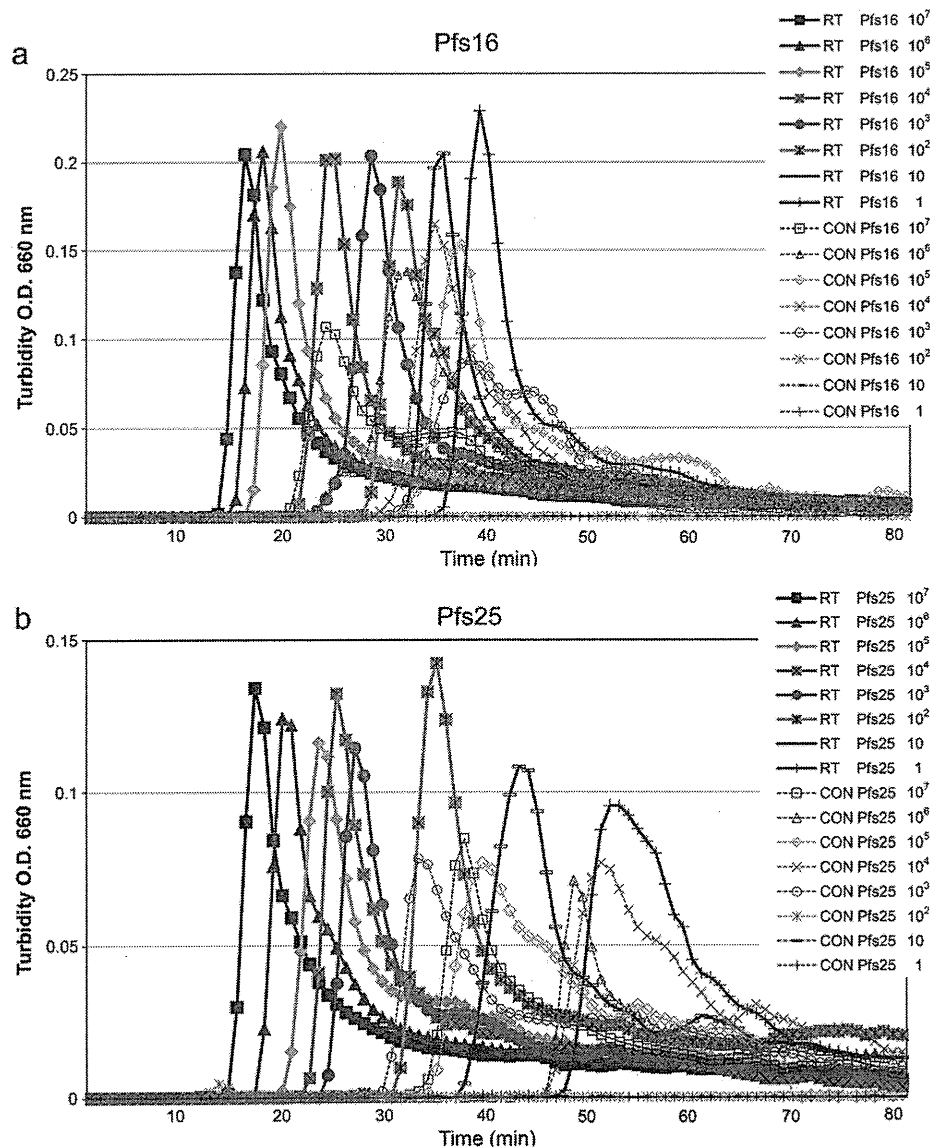

Fig. 3. Sensitivities of real-time RT-LAMP assay for Pfs16 and Pfs25 detection. RT-LAMP assay was carried out using total RNA purified from serially diluted samples of *P. falciparum* strain AMB47 (1×10^7 to 1 parasitized RBC/500 µl of blood). One representative result of three replicates is shown. RT-LAMP and conventional LAMP reactions of Pfs16 (a), RT-LAMP and conventional LAMP reactions of Pfs25 (b). RT and CON stand for RT-LAMP and conventional LAMP, respectively.

3.3. Detection of Pfs16 and Pfs25 gene expression from clinical blood samples by RT-LAMP and nested RT-PCR assays

Thirty-two and 50 clinical blood samples, positive and negative for *P. falciparum* infection by microscopy, respectively, were examined for the expression of Pfs16 and Pfs25 by RT-LAMP and nested RT-PCR. Total RNA purified from *P. falciparum* strain AMB47, a positive control, and from malaria-free blood samples, a negative control, were amplified in parallel. The A-type 18S rRNA, an internal positive control, and S-type 18S rRNA, an indicator of the presence of gametocytes, were amplified by nested RT-PCR. Parasitaemia of 32 microscopically *P. falciparum*-positive samples ranged from 0.001% to 2.43% and 8 samples were positive for gametocytes (Table 2). All 32 microscopically *P. falciparum*-positive samples were positive for A-type 18S rRNA and all 50 microscopically *P. falciparum*-negative samples were negative for A-type 18S rRNA, Pfs16 and Pfs25 by nested RT-PCR. Overall, both RT-LAMP and nested RT-PCR yielded similar results for Pfs16 except for 2 samples, PFSR98 and PFSR118, which were positive by RT-LAMP and negative by nested RT-PCR. PFSR98 was negative while PFSR118 was positive for S-type 18S rRNA indicating that PFSR118 was a true positive for Pfs16 expression as S-type 18S rRNA is expressed from early to mature gametocytes. As for Pfs25, 6 samples, PFSR99, -103, -104, -118, -120 and PFMS421, were positive by RT-LAMP but negative by nested RT-PCR. All these samples were positive for S-type 18S rRNA by nested RT-PCR, indicating that these samples were true positives for Pfs25 expression. Among the samples positive by RT-LAMP and negative by nested RT-PCR, 1 sample, PFSR118, was infective to mosquitoes (data not shown). These results clearly confirm the reliability of RT-LAMP for gametocyte detection. In total, among 32 microscopically *P. falciparum*-positive samples, nested RT-PCR showed that 30 and 15 samples were true positives for Pfs16 and Pfs25 mRNA, respectively, and 52 and 67 samples were true negative for Pfs16 and Pfs25 mRNA, respectively.

The sensitivity, specificity, NPV and PPV of RT-LAMP are summarized in Table 3. RT-LAMP detected Pfs16 and Pfs25 in all samples positive by nested RT-PCR, giving 100% sensitivity. Specificity of RT-LAMP for Pfs16 was 98.1% (1 sample detected among 52 samples negative by nested RT-PCR), and that for Pfs25 was 100% in 67 samples examined. NPV by RT-LAMP was 100% for both Pfs16 and Pfs25, and PPV was 96.8% for Pfs16 and 100% for Pfs25. Detection rates of Pfs16 and Pfs25 by RT-LAMP in microscopically gametocyte-negative samples ($n = 24$) were 91.7% and 29.2% respectively.

RT-LAMP detection time for Pfs16 and Pfs25 in clinical blood samples was 33.5 ± 8.8 min (mean ± SD; range, 22 to 63 min) and

Table 3
Sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of RT-LAMP for the detection of Pfs16 and Pfs25.

Variable	Pfs16	Pfs25
	n (%)	n (%)
Sensitivity	30/30 (100%)	15/15 (100%)
Specificity	51/52 (98.1%)	67/67 (100%)
NPV	51/51 (100%)	67/67 (100%)
PPV	30/31 (96.8%)	15/15 (100%)

Table 2
Detection of the expression of *P. falciparum* gametocyte-specific genes, Pfs16 and Pfs25, by nested RT-PCR and RT-LAMP.

Blood[a] sample	Microscopy		A-type 18S rRNA/ nested RT-PCR	S-type 18S rRNA/ nested RT-PCR	Pfs16/ nested RT-PCR	Pfs16/ RT-LAMP	Pfs25/ nested RT-PCR	Pfs25/ RT-LAMP
	Parasitaemia (%)	Gametocyte						
PFSR95	0.66	No	+	ND[b]	+	+	−	−
PFSR96	0.77	No	+	ND	+	+	+	+
PFSR98	0.02	No	+	−	−	+	−	−
PFSR99	0.09	No	+	+	+	+	−	+
PFSR100	0.08	Yes	+	ND	+	+	+	+
PFSR101	0.04	No	+	ND	+	+	−	−
PFSR102	0.13	No	+	ND	+	+	+	+
PFSR103	0.128	Yes	+	+	+	+	−	+
PFSR104	0.068	No	+	+	+	+	−	+
PFSR105	1.12	No	+	ND	+	+	−	−
PFSR106	2.43	No	+	ND	+	+	−	−
PFSR107	0.50	No	+	ND	+	+	−	−
PFSR109	0.41	Yes	+	ND	+	+	+	+
PFSR110	0.28	No	+	ND	+	+	−	−
PFSR111	0.038	No	+	ND	+	+	−	−
PFSR112	0.048	No	+	ND	+	+	−	−
PFSR113	1.87	No	+	ND	+	+	−	−
PFSR114	0.33	No	+	ND	+	+	−	−
PFSR115	0.001	No	+	ND	+	+	−	−
PFSR116	0.22	Yes	+	ND	+	+	+	+
PFSR117	0.035	No	+	ND	+	+	−	−
PFSR118	1.80	No	+	+	−	+	−	+
PFSR119	0.007	Yes	+	ND	+	+	+	+
PFSR120	0.009	No	+	+	+	+	−	+
PFSR121	0.47	No	+	ND	+	+	−	−
PFSR122	0.18	No	+	ND	+	+	−	−
PFSR123	0.22	No	+	ND	−	−	−	−
PFMS418	0.013	Yes	+	ND	+	+	+	+
PFMS421	0.02	Yes	+	+	+	+	−	+
PFMS423	0.35	Yes	+	ND	+	+	+	+
PFPP1	0.08	No	+	ND	+	+	+	+
PFPP2	0.02	No	+	ND	+	+	−	−
NSR1–NSR50	−	−	−	−	−	−	−	−

[a] Thirty-two and 50 samples positive and negative for *P. falciparum* by microscopy, respectively, were used in this study.
[b] Not done.

50.1 ± 11.4 min (mean ± SD; range, 35 to 76 min), respectively. Genomic DNA contamination was observed only in 7 of 31 samples positive for Pfs16. The detection time for carry-over of Pfs16 genomic DNA was 63.8 ± 21.8 min (mean ± SD; range, 32.5 to 76.3 min), but that for Pfs25 could not be estimated as only 2 in 15 samples contained carry-over of genomic DNA. Elimination of genomic DNA contamination with DNase I and a suitable restriction enzyme yielded a better result than treatment with DNase I alone.

4. Discussion

In areas where *P. falciparum* is endemic, detection of gametocytes is needed to prevent transmission to mosquitoes. As gametocytes are present in infected persons at low density, which often go undetected by standard microscopy, there is a need for a more sensitive, reliable and user-friendly method than microscopy to detect malaria parasite sexual stages. In this study, RT-LAMP, a simpler and more dependable DNA amplification method, was developed for detection of *P. falciparum* gametocytes. Pfs16 mRNA was used for identification of the earliest sexual stages and Pfs25 mRNA was used for mature gametocytes.

RT-LAMP technique developed here has a reliable specificity as there was no cross-reaction with genomic DNA from the other three species of human malaria parasites. Sensitivity of RT-LAMP for both Pfs16 and Pfs25 mRNA detection was 1 parasitized RBC/500 µl of blood, equivalent to 0.002 parasitized RBCs/µl of blood, a level undetectable by microscopy, which has a detection limit of 50 parasites/µl of blood [20]. Sensitivity of RT-LAMP in this study for the detection of Pfs25 mRNA is 500 times higher than that using quantitative nucleic acid sequence-based amplification assay (QT-NASBA) [24] and 12.5 times higher than QT-NASBA assay for *P. vivax* [25]. The QT-NASBA had been invented for the specific quantification of single-stranded RNA in the existence of DNA. Although the amplification of this assay can be done at a constant temperature, it still requires 2 different temperatures (65 °C, 2 min and 41 °C, 2 min) for the noncycling step, prior to the actual amplification step of 90 min [26].

For Pfs16 detection, RT-LAMP gave results comparable to those of nested RT-PCR. One (PFSR98) of 52 samples negative for Pfs16 by nested RT-PCR was positive by RT-LAMP and was negative for S-type 18S rRNA, the indicator for gametocytes, by nested RT-PCR. This discordant result may be due to a higher sensitivity of RT-LAMP compared to that of nested RT-PCR. It is likely that Pfs16 mRNA detected in PFSR98 by RT-LAMP is from sexually committed rings as no S-type 18S rRNA expression was observed. For Pfs25 detection, RT-LAMP gave results in good agreement with those of nested RT-PCR. However, RT-LAMP clearly demonstrated a superior sensitivity over nested RT-PCR, reflected in its ability to detect Pfs25 expression in samples negative by nested RT-PCR but infective to mosquitoes (PFSR118) or positive for gametocytes by microscopy (PFSR103 and PFMS421).

Seventy-three and 46.7% of samples positive for detection of Pfs16 and Pfs25, respectively, by RT-LAMP were microscopically gametocyte-negative. All samples positive for Pfs25 were also positive for Pfs16. As the detection rate of Pfs16 was higher than that of Pfs25 for both RT-LAMP and nested RT-PCR, this indicates that only a portion of sexually committed ring stages develops to become mature gametocytes. This finding is consistent with previous finding which demonstrate that sexual stage commitment can take place early in the blood-stage infection without successful maturation into infectious gametocytes [24]. This present study indicates that a large proportion of *P. falciparum*-infected patients in Mae Sot and Mae Kasa, Tak, northwestern Thailand harbors submicroscopic gametocytes, providing a larger reservoir of infected individuals with potential for malaria transmission than indicated by standard microscopy.

In summary, we have demonstrated that the RT-LAMP method developed for detection of *P. falciparum* gametocytes in clinical blood samples is superior to that of nested PCR and microscopy. RT-LAMP provides a rapid, simple, cheap, sensitive and specific method suitable in studies on *P. falciparum* epidemiology, transmission and drug control programmes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.parint.2010.05.008.

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Plasmodium vivax Ookinete Surface Protein Pvs25 Linked to Cholera Toxin B Subunit Induces Potent Transmission-Blocking Immunity by Intranasal as Well as Subcutaneous Immunization[▽]

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The nontoxic cholera toxin B subunit (CTB) was evaluated as a potential delivery molecule for the *Plasmodium vivax* ookinete surface protein, Pvs25. Recombinant Pvs25 was expressed as a secreted protein in the yeast *Pichia pastoris*, as a mixture of isoforms including multimers and the A and B monomers. The A isoform with the presumed native protein fold was the most abundant, accounting for more than 40% of all expressed protein. The molecularly uniform A isoform was chemically conjugated to CTB via its primary amines, and the fusion protein, retaining GM1-ganglioside affinity, was administered to BALB/c mice by the subcutaneous (s.c.) or intranasal (i.n.) route. Immunization of mice with conjugated Pvs25 without supplemental adjuvant induced antisera that specifically recognized *P. vivax* ookinetes *in vitro*. Furthermore, the antisera, when mixed with parasitized blood isolated from *P. vivax* patients from Thailand, was found to reduce parasite transmission to mosquitoes, conferring a 93 to 98% (s.c.) or a 73 to 88% (i.n.) decrease in oocyst number. Unconjugated Pvs25 alone conferred only a 23 to 60% (s.c.) or a 0 to 6% (i.n.) decrease in oocyst number. Coadministration of extraneous adjuvants, however, further enhanced the vaccine efficacy up to complete blockade. Taken together, we conclude that a weakly immunogenic Pvs25 by itself, when linked to CTB, transforms into a potent transmission-blocking antigen in both i.n. and s.c. routes. In addition, the present study is, to the best of our knowledge, the first demonstration of the immune potentiating function of CTB for a vaccine antigen delivered by the s.c. route.

Malaria is one of the most serious infectious diseases, with high mortality and morbidity, especially in tropical regions of the world. The disease causes 350 to 500 million clinical cases every year, and the estimated annual mortality exceeds 1.1 million (28). Implementation of many malaria control measures, including chemotherapy and insecticide-treated bed nets, have made a significant contribution to the reduction of malaria cases worldwide; however, these control measures are suboptimal, and hence new tools, particularly vaccines, should be used for local elimination and the ultimate eradication of malaria from the globe (9, 10, 24). The development of effective and affordable malaria vaccines is therefore likely to benefit global public health (Malaria Vaccine Technology Roadmap [MVTR], 2006 [http://www.malariavaccineroadmap.net/pdfs/Malaria_Vaccine_TRM_Final.pdf]).

Although *Plasmodium falciparum* causes the highest mortality rates among the four *Plasmodium* species known to infect humans (18), *P. vivax* malaria has the highest morbidity and is an important cause of recurrent malaria. This species is therefore an important target of malaria control efforts (4–6; MVTR). Furthermore, because global malaria eradiation is the ultimate goal, the value of developing vaccines against *P. vivax* cannot be underestimated (4–6; MVTR). Several promising vaccine candidates have been intensively investigated, such as those targeting the asexual stages, i.e., the sporozoite, hepatic and erythrocytic stages, which are designed to prevent infection and to reduce disease severity. On the other hand, transmission-blocking vaccines (TBVs) that target the sexual stage, in which the parasite undergoes sporogonic development in anopheline mosquitoes, prevent parasite transmission from mosquitoes to humans (7, 14, 25). TBVs induce antibodies that react with the ookinete surface proteins (OSPs) of malaria parasites within the mosquito midgut, and as such they do not directly protect vaccinated individuals from infection. They could, however, contribute to elimination of the disease by lowering the parasite transmission frequency below the threshold at which the parasite can maintain its life cycle (4, 6). In addition, TBVs, when combined with vaccines targeting other stages of the infection, could prevent transmission of parasites that have escaped the immune response. Furthermore, TBVs could also prevent transmission of drug-resistant

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parasites, which will likely to emerge when mass administration of primaquine is initiated. Therefore, TBVs might function as a "safety net" for pre-erythrocytic and erythrocytic vaccines, as well as other nonvaccine interventions.

We have recently tested whether a mucosal vaccination regime can be applied to TBVs, on the premise that noninvasive and easy-to-administer mucosal vaccines are advantageous in a mass vaccination campaign in a region where malaria is endemic (1–3). In these animal studies, we have demonstrated the potential of mucosal vaccines to block parasite transmission, but enhancement of the mucosal immunogenicity of recombinant antigens was found to be critically dependent upon the use of cholera toxin (CT) adjuvant. CT is well known for its high immune potentiating function for admixed antigens administered through the mucosal, particularly the intranasal (i.n.), route (13). However, its clinical application is hampered by its severe toxicity (26). Thus, alternative vaccine formulations not using the CT holotoxin are highly desirable.

Here, we extended our previous studies to test our hypothesis that the immunogenicity of a *Plasmodium vivax* malaria OSP, Pvs25, becomes substantially augmented when physically linked to the nontoxic B subunit of CT (CTB), even without supplementation with extraneous adjuvants. This should, in theory, effectively reduce parasite transmission to mosquitoes. Furthermore, we tested the TBV efficacy of the engineered fusion complex in a subcutaneous (s.c.) immunization regimen to test the immune potentiating function of CTB with this particular immunization route.

MATERIALS AND METHODS

Expression of Pvs25H protein from the methylotrophic yeast *Pichia pastoris*. The Pvs25 coding region (Ala_{23} to Leu_{195}) was amplified by PCR from plasmid Pvs25#26_SalI_pEU3, which harbors the coding region for the extracellular domain of the Pvs25 protein (12), with a sense primer (5′-GCCGTCACGGTA GACACC-3′) and an antisense primer containing an EcoRI site, a hexahistidine-coding sequence and a termination codon (5′-GGGAATTCTTAATGATGGT GATGGTGATGTGGTCCAAGGCATACATTTTTCTCTTTGTC-3′). The DNA fragment was amplified by using *Vent* DNA polymerase (New England Biolabs, Beverly, MA), was purified by using a PCR Purification Kit (Qiagen, Inc., Valencia, CA), and then digested with EcoRI. The fragment was subcloned into the SnaBI and EcoRI sites of the *P. pastoris* expression vector pPIC9K (Life Technologies, Carlsbad, CA) to construct the plasmid pPvs25H, which was designed to express an α-factor signal–Pvs25–hexahistidine fusion protein (see Fig. 1a).

P. pastoris recombination was performed according to the manufacturer's instructions (Life Technologies). Approximately 10 μg of linearized pPvs25H plasmid digested with SalI was electroporated into *P. pastoris* strain GS115 by using a Gene Pulser (1.5 kV, 200 Ω, 25 μF; Bio-Rad Laboratories, Inc., Redmond, WA). Immediately after electroporation, the cells were plated on minimal dextrose medium and incubated for 72 h at 29°C. Colonies were transferred to yeast extract-peptone-dextrose (YPD) medium containing increasing concentrations of Geneticin (G418; 1 to 5 mg/ml; Nacalai Tesque, Inc., Kyoto, Japan) for the selection of clones containing multiple copies of the desired gene. Clones that acquired a phenotype resistant to the highest level of G418 (5 mg/ml) were analyzed for production of the Pvs25H protein. Selected clones were cultured in BMGY medium with vigorous shaking in a baffled flask at 30°C until the optical density at 600 nm (OD_{600}) reached 2.0; the cultured cells were then transferred to BMMY medium containing 0.5% methanol to induce gene expression. Cells were cultured for an additional 72 h, with supplementation with 0.5% methanol for every 24 h of continued induction. The culture supernatant was collected by two rounds of centrifugation (9,600 × *g*) for 10 min, followed by filtration (FastCap filter with a 0.2-μm pore size; Nalgene Nunc International, Inc., Rochester, NY) to remove residual cells completely. Proteins secreted into the supernatant were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue or subjected to immunoblot analysis with anti-Pvs25 antiserum or anti-histidine tag monoclonal antibody (Roche, Basel, Switzerland). The supernatant was supplemented with 20 mM imidazole and applied to an immobilized metal ion affinity chromatography column (HisTrap FF Ni Sepharose 6 Fast Flow; GE Healthcare, Little Chalfont, United Kingdom). After a washing step with a buffer containing 20 mM imidazole, the Pvs25H protein was eluted with phosphate-buffered saline (PBS) containing 500 mM imidazole. The affinity-purified protein was used for size exclusion chromatography with a flow rate of 0.2 ml/min (HiLoad 16/60 Superdex 75-pg column; GE Healthcare) to separate the monomeric from the multimeric isoforms. The monomeric isoforms that were a mixture of A and B isoforms were then subjected to hydrophobic interaction chromatography (HIC; HiTrap Phenyl Sepharose HP; GE Healthcare), in which both the A and the B isoforms bound to the hydrophobic ligand by adjusting the concentration of ammonium sulfate in the solution to 2 M. Then, a two-step elution process was performed, using 1 M ammonium sulfate for elution of the A isoform, followed by PBS elution of the B isoform.

This highly purified A isoform (Pvs25H-A) was used for all immunization experiments. The endotoxin content of the Pvs25H-A protein was measured by the *Limulus* amebocyte lysate test (Pyrogent Single Test Vials; Cambrex, East Rutherford, NJ), and the endotoxin content was found to be <0.05 endotoxin unit (EU)/μg of protein.

Immobilized *tris*[2-carboxyethyl]phosphine hydrochloride on a beaded agarose support (TCEP; Pierce, Rockford, IL) was used to generate a reduced form of the sulfhydryl groups for recombinant protein samples. In addition, 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (Ellman's reagent; Pierce) and a cysteine hydrochloride monohydrate standard were used to estimate the amounts of free sulfhydryls.

The N-terminal protein sequences were analyzed by the Edman degradation method as described elsewhere, using a protein sequencer (Shimadzu, Kyoto, Japan).

All recombinant DNA experiments were conducted according to the Safety Guidelines for Gene Manipulation Experiments of the University of the Ryukyus.

Chemical conjugation between Pvs25H-A and CTB. Recombinant CTB was expressed and purified as described previously (11), and purified Pvs25H-A was chemically conjugated to CTB by using the heterobifunctional cross-linker *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Thermo Scientific, Inc., Rockford, IL). One milligram of Pvs25H-A (2 mg/ml in PBS-EDTA) was incubated with SPDP (0.6 mM, final concentration) for 1 h at room temperature to add pyridyl disulfide groups to the primary amines of the protein. The reaction mixture was then desalted and buffer exchanged to PBS by a size exclusion membrane filter (Amicon Ultra-15, MWCO 10,000; Millipore, Billerica, MA) to remove excess reagent and by-products (pyridine 2-thione). Similarly, 1 mg of CTB (2 mg/ml in PBS-EDTA) was incubated with SPDP (0.6 mM, final concentration) for 1 h at room temperature and then desalted and buffer exchanged to PBS. Pyridyldithiol-activated CTB was then incubated with dithiothreitol (DTT; 50 mM, final) for 30 min at room temperature to expose the sulfhydryl groups and then desalted and buffer exchanged to PBS as before. Finally, equal amounts of pyridyldithiol-activated Pvs25H-A and sulfhydryl-activated CTB were mixed, followed by incubation at room temperature overnight for conjugation. The conjugated sample was desalted as before, and the endotoxin content was measured to confirm that there had been no significant contamination during the conjugation process.

To evaluate the conjugation efficiency, untreated Pvs25H-A was separately incubated either with untreated CTB or pyridyldithiol-activated CTB (CTB^{SPDP}). Similarly, pyridyldithiol-activated Pvs25H-A (Pvs25H-A^{SPDP}) was separately incubated with either untreated or DTT-treated CTB (CTB^{DTT} or $CTB^{SPDP/DTT}$, respectively) (see Results and Fig. 2a for details). Each conjugation sample was analyzed by GM1–enzyme-linked immunosorbent assay (GM1-ELISA) as described previously (11). Briefly, 5 μg of monosialoganglioside GM1 (Sigma-Aldrich, St. Louis, MO)/ml, a receptor for CT, diluted with bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]; 50 μl/well) was coated onto a 96-well microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and the plate was incubated at 4°C overnight. After washing the plate twice with PBS containing 0.05% Tween 20 (PBS-T) and once with PBS, the plate was blocked with PBS containing 10% skim milk for 2 h at 37°C. Each conjugation sample (2 μg of total protein/well) was then applied to the wells, and the plate was incubated for 2 h at 37°C, followed by incubation with rabbit anti-CT antiserum (1/4,000; Sigma-Aldrich) or mouse anti-Pvs25 antiserum (1/500) for 2 h at 37°C. This was followed by the addition of anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase (1/4,000; Sigma-Aldrich). Finally, *p*-nitrophenylphosphate (Bio-Rad) was added, and the plate was incubated for 20 min at 37°C. The OD_{415} was measured by using a microplate reader (Bio-Rad).

To evaluate the conjugation state of the fusion complex, 1 mg of conjugation sample was subjected to size exclusion chromatography (HiLoad 16/60 Superdex

75-pg column; GE Healthcare). Molecular weight standards (Gel Filtration Calibration kits LMW; GE Healthcare) were used to estimate the molecular weights of CTB, Pvs25H-A, and their fusion complex by calculating the partition coefficient (K_{av}) values for each protein standard and sample protein.

Immunization with CTB-Pvs25H-A fusion protein and analysis of induced antibodies. Eight-week-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice (four or eight mice per group) were immunized via the s.c. or the i.n. route with 30 μg of Pvs25H-A, a mixture of CTB and Pvs25H-A (30 μg each) or 60 μg of CTB-Pvs25H-A fusion protein. Where indicated, incomplete Freund's adjuvant (IFA; Difco Laboratories, Detroit, MI) or 0.1 to 1.0 μg of CT (List Biological Laboratories, Campbell, CA) was used for s.c. or i.n. adjuvants, respectively. The mice were immunized three times, at weeks 0, 2, and 3.

Mice were anesthetized 1 week after the third immunization (week 4) by intraperitoneal injection of pentobarbital sodium salt (Nacalai Tesque, Inc.) and were sacrificed by exsanguination to collect serum. For specific serum antibody analysis, ELISA plates (Sumilon; Sumitomo Bakelite Co.) were coated with Pvs25H-A (5 μg/ml) in bicarbonate buffer by incubating the plate at 4°C overnight. The plate was washed twice with PBS-T and once with PBS. The plate was blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C. Twofold serial dilutions of the antisera starting with 50-fold dilution with 0.5% BSA in PBS were applied to the wells in duplicate, which were then incubated for 2 h at 37°C. The plate was then incubated with anti-mouse IgG conjugated to alkaline phosphatase (1/4,000; Sigma-Aldrich) for 2 h at 37°C. *p*-Nitrophenylphosphate (Bio-Rad) was added to the plate for color development, and the absorbance at OD_{415} was measured after 20 min of incubation at 37°C, using a microplate reader (Bio-Rad). The antibody titer was defined as the serum dilution that gave an OD_{415} value equal to 0.1 or as the serum dilution where a one magnitude higher dilution gave an OD_{415} value of <0.1.

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of the Ryukyus, and the experiments were conducted according to the Ethical Guidelines for Animal Experiments of the University of the Ryukyus.

Mosquito membrane feeding assay. Heparinized syringes were used for peripheral blood collection, with written informed consent, from *P. vivax* patients who came to a malaria clinic in the Mae Sod district in the Tak province of northwestern Thailand. Single species infection with *P. vivax* was confirmed by Giemsa stain of thick and thin blood smears. The levels of parasitemias and the gametocytemias were 0.22 and 0.02%, respectively, for the volunteer *P. vivax* patient 1, and 0.23 and 0.01%, respectively, for the volunteer *P. vivax* patient 2. Collected blood was aliquoted into tubes (300 μl/tube), and the plasma was removed after brief centrifugation. Pooled mouse antisera were mixed with an equal volume of heat-inactivated normal human AB serum prepared from malaria-naive donors. The test antiserum sample was mixed and incubated with *P. vivax*-infected blood cells (1:1 [vol/vol] ratio) for 15 min at room temperature. The mixture was then applied to a membrane feeding apparatus kept at 37°C to allow starved *Anopheles dirus* A mosquitoes (Bangkok colony, Armed Forces Research Institute of Medical Sciences) to feed on the blood meals for 30 min. Fully engorged mosquitoes were separated from unfed mosquitoes, maintained for a week in an insectary kept at 26°C, and provided with 10% sucrose water. For each blood sample-serum mixture, 20 mosquitoes were dissected, and the number of oocysts in the midgut was counted under a microscope after 0.5% mercurochrome staining.

All human subject research conducted in the present study was reviewed and approved by the Ethics Committee of the Thai Ministry of Public Health and the Institutional Review Board of the Walter Reed Army Institute of Research.

Detection of native Pvs25 in antisera from immunized mice. Peripheral blood from *P. vivax*-infected patients was collected as described above. The gametocytemic patient blood was used to grow zygotes and ookinetes *in vitro*, as described previously (23). They were spotted onto slides and fixed with acetone as described previously (1–3). The slides were blocked with 5% nonfat milk in PBS and incubated with mouse antisera derived from immunization with CTB-Pvs25H-A fusion protein emulsified with IFA or with the fusion protein supplemented with CT (1 μg), after dilution of the antisera 100-fold with 5% nonfat milk in PBS. The samples were washed with ice-cold PBS and incubated with Alexa 488-conjugated anti-mouse antibody (Invitrogen, Carlsbad, CA). After a wash with ice-cold PBS, the slides were viewed by confocal scanning laser microscopy (LSM5 Pascal; Carl Zeiss MicroImaging, Thornwood, NY).

Statistical analysis. The Wilcoxon-Mann-Whitney test was used to compare antibody titers or the number of oocysts per mosquito between the nonimmune and an indicated immunization group or between indicated two immunization groups. The Kruskal-Wallis test was used to compare antibody titers or the number of oocysts per mosquito among three groups (i.e., S, M, and L). The chi-square test was used to analyze the difference in the proportion of parasite-free mosquitoes out of the total number of mosquitoes examined between the nonimmune and an indicated immunization group or between indicated two immunization groups. All statistical analysis were conducted by using JMP software version 8.0 (SAS Institute, Inc., Cary, NC).

RESULTS

Expression and purification of recombinant Pvs25H-A isoform from *P. pastoris*. Ookinete surface proteins (OSPs) contain several intramolecular disulfide bonds, e.g., the 11 disulfide bonds in Pvs25, which are important for overall structural integrity and native antigenicity. For this reason, *E. coli* expression systems are unsuitable, and therefore yeast *Saccharomyces cerevisiae* expression systems are used (12, 15, 19, 22). We explored other, more efficient recombinant protein expression systems for Pvs25 and found a higher production efficiency in the yeast *P. pastoris*. We constructed a plasmid for secretory expression of Pvs25 as a C-terminal hexahistidine-tagged protein (Pvs25H) (Fig. 1a). The culture supernatant of recombinant *P. pastoris* was found to contain several protein species that were not present in the culture supernatant of vector-transformed clones (Fig. 1b), and these protein bands specifically reacted with anti-Pvs25 antiserum, as well as with an anti-hexahistidine tag monoclonal antibody (data not shown). Secreted Pvs25H was affinity purified on a nickel Sepharose column and further separated into large (fractions 16 to 19) and small (fractions 21 to 23) protein species by size exclusion chromatography (Fig. 1c and d). At least five protein bands were identified on SDS-PAGE (Fig. 1b) and their corresponding peaks by size exclusion chromatography (fractions 16, 17, 18-19, 21-22, and 22-23 in Fig. 1c and d). The apparent molecular masses of these protein species based on gel mobility on SDS-PAGE were estimated to be 63.2, 47.3, 33.2, 16.1, and 28.3 kDa, respectively. The calculated molecular mass of Pvs25H (based on its deduced amino acid sequence) is 20.2 kDa, to which the fraction 21 and 22 protein species corresponded most closely. We therefore concluded that this was monomeric Pvs25H. Furthermore, the apparent molecular masses of protein species found in fractions 18 and 19, 17, and 16 corresponded very well to multiples of the molecular mass of the apparent monomer, so we concluded that they represented the dimer, trimer and tetramer of Pvs25H, respectively. The protein species that appeared in fractions 22 and 23 exhibited markedly different gel mobility (28.3 kDa) from that of the monomeric protein (16.1 kDa) (Fig. 1d), although these two showed extensively overlapping chromatographic peaks on size exclusion chromatography (Fig. 1c). We concluded that the protein species in fractions 22 and 23 was a monomeric protein with a distinct hydrophobic character, and thus decided to further separate these two monomers based on hydrophobicity profile by HIC. HIC clearly separated the two monomeric isoforms (Fig. 1f), and we named the less hydrophobic isoform and the more hydrophobic isoform the A and B monomeric isoforms, respectively (19). The observed lower gel mobility for the A isoform than the B isoform on SDS-PAGE under nonreducing conditions (Fig. 1d) was consistent with the results of HIC: the more hydrophilic the molecule, the fewer SDS molecules bind, and the protein becomes less negatively charged, resulting in reduced gel mobility on SDS-PAGE.

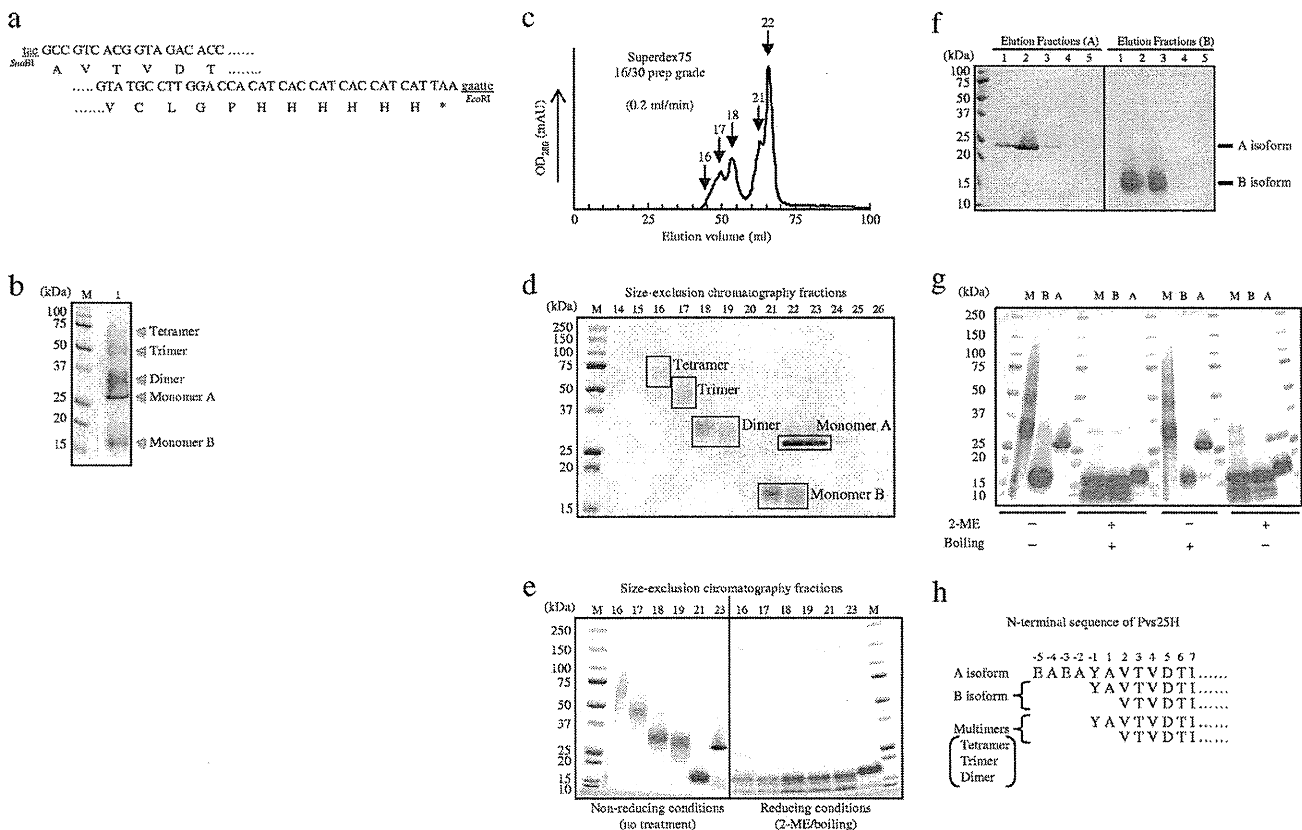

FIG. 1. Construction and expression analysis of Pvs25H in *Pichia pastoris*. (a) The 5′- and 3′-coding regions of the *Pvs25* gene sequence with its predicted amino acid sequence (Ala_{23} to Leu_{195}). The *Pvs25* gene fused to a hexahistidine tag at its C terminus was inserted into the SnaBI and EcoRI sites of pPIC9K, and the gene was integrated into the chromosomal DNA of *P. pastoris* strain GS115 by homologous recombination. (b) After selection of a high-producer clone that secreted the recombinant protein, nickel-affinity chromatography-purified Pvs25H was analyzed by SDS-PAGE (lane 1). M, molecular marker. (c) The affinity-purified Pvs25H was fractionated by size exclusion chromatography from which at least five chromatographic peaks were observed. (d) Size exclusion chromatography fractions 14–26 were subjected to SDS-PAGE (15% acrylamide). Based on the apparent molecular mass of each protein band, the fractions 16, 17, 18-19, 21-22, and 22-23 were defined as the tetramer, trimer, dimer, monomer B, and monomer A, respectively. (e) Selected chromatography peaks were subjected to 5 to 20% acrylamide gradient SDS-PAGE under nonreducing or reducing (10% 2-ME and boiling) conditions. (f) Hydrophobic interaction chromatography of the Pvs25H-A and Pvs25H-B monomeric isoforms. Elution fractions (A) are eluates of 2 M NH_4SO_4 (Pvs25H-A) and elution fractions (B) are eluates of PBS (Pvs25H-B). (g) Each isoform (M, a mixture of dimer, trimer and tetramer; B, B isoform; A, A isoform) was subjected to 15% acrylamide SDS-PAGE under various conditions, as indicated. (h) The N-terminal protein sequence of each isoform. Positively numbered amino acid residues (Ala_1 and the following) are residues of the Pvs25 protein; negatively numbered amino acids are derivatives of the pPIC9K α-factor secretion signal. The A monomer revealed a single, unique sequence, but the B monomer and the multimers showed a mixture of different N termini.

Molecular characterization of Pvs25H isoforms. The A isoform appeared as a single sharp band, but the other isoforms, including the multimers and the B monomer, appeared as diffuse bands on SDS-PAGE (Fig. 1b and d to g), indicating that the A isoform is constrained to a more uniform molecular configuration than the other isoforms. In addition, all of the isoforms, except the A isoform, generated at least three identical protein bands when samples were heat treated in the presence of SDS and β-mercaptoethanol (2-ME) prior to SDS-PAGE. The A isoform appeared as a single protein band with a slightly higher molecular mass than that for the largest protein band among the three bands observed for the other isoforms (Fig. 1e).

To characterize the molecular configuration of each isoform furthermore, mixtures of the multimers, including dimers, trimers, and tetramers and the A and B monomers, were subjected to SDS-PAGE after various treatments, as indicated in Fig. 1g. The gel mobility of all of the isoforms did not show any noticeable changes after boiling (100°C, 2 min), but 2-ME treatment (10% [vol/vol]) resulted in a banding pattern very similar to the pattern observed for complete denaturation (2-ME and boiling), except that the A isoform seemed to be slightly more resistant to the reducing agent than the other isoforms. This became particularly notable when lower concentrations of the reducing agent were used (data not shown). These results suggest that the multimers comprised B monomers self-cross-linked by intermolecular disulfide bonds and that the A isoform has a higher molecular rigidity than the other isoforms. Because all of the isoforms exhibited a strong resistance to the boiling and SDS, but not to the 2-ME and SDS treatment, it is plausible that the physical integrity of the protein is critically, if not completely, maintained by disulfide bonds.

Next, to evaluate the status of the covalent disulfide bonds in

the Pvs25H protein, Ellman's test was conducted for each isoform. No isoform reacted with the Ellman's reagent, suggesting either that no reduced sulfhydryls were present or that the molecules were inaccessible to the reagent. However, treatment with TCEP immobilized on agarose prior to the Ellman's test resulted in the detection of 4 to 6 molecules of reduced sulfhydryls per molecule of B monomer or multimeric isoforms, but fewer than 0.3 molecules of reduced sulfhydryls were detected per molecule of A monomer. These results indicated that the B and multimeric isoforms have disulfide bonds that are more accessible to surface-immobilized TCEP than those of the A isoform, indicating that the A isoform has more deeply buried disulfide bonds than the other isoforms. It is indicative of the higher molecular flexibility of the B isoform and the multimers than of the A isoform. Interestingly, however, denaturation of proteins with 2% SDS or 6 M guanidine hydrochloride, or TCEP agarose treatment in the presence of these denaturants prior to the Ellman's test, did not further increase the level of free sulfhydryls. These results strongly support the notion that all of the isoforms are tightly packed molecules and that their rigidity is maintained by intramolecular disulfide bonds and other noncovalent interactions.

Finally, the N-terminal protein sequences determined by the Edman degradation method for each isoform supported the results of SDS-PAGE, in that the multimers and the B isoform contain a mixture of polypeptide species with multiple N termini; however, the A isoform comprises a single polypeptide with a longer, unique N terminus (Fig. 1h). These results suggested that the multimers and the B isoform contain the same set of polypeptide species, with multiple primary structures and presumably various folding configurations. By using different combinations of structurally heterologous polypeptides, different isoforms might be generated.

Taken together, we concluded that the A isoform, which has a more uniform protein configuration, is less hydrophobic and has higher molecular rigidity than the B isoform, and perhaps it most closely resembles the native Pvs25 protein at the structural level. The proportions of each isoform expression were estimated to be 42% (A isoform), 27% (B isoform), 16% (dimer), 10% (trimer), and 5% (tetramer). Thus, the A isoform was produced most abundantly among all of the isoforms. The final protein yields of the total Pvs25H and the A isoform using our expression and purification method were 30 to 50 mg and 12 to 20 mg/liter of culture medium, respectively. We confirmed that the purified Pvs25H-A contained endotoxin at levels less than 0.05 EU/μg of protein. Based on these observations, we decided to use the A isoform (Pvs25H-A) as a TBV antigen to be linked to CTB.

Chemical conjugation of Pvs25H-A to CTB and its molecular evaluation. Recombinant CTB was expressed by *P. pastoris* strain GS115 and purified as previously reported (11). Pvs25H-A was chemically conjugated to CTB by using the heterobifunctional cross-linker SPDP (Fig. 2). Because CTB contains two cysteine residues per monomeric subunit, which, in the native form, are involved in an intramolecular disulfide bond, the existence of reduced sulfhydryls in our recombinant CTB was determined by using Ellman's reagent, and none was detected (data not shown).

Various conjugation schemes were evaluated for efficiency of linking Pvs25H-A to CTB via SPDP (Fig. 2a and b). Consistent with the results of Ellman's test for Pvs25H-A and CTB, SPDP modification of only one protein failed to generate the CTB-Pvs25H-A fusion complex (Fig. 2b). Thus, at least one partner protein had to be treated with the reducing agent to expose free sulfhydryls and make it reactive toward the pyridyldithiol groups added to the partner protein. Because intact disulfide bonds might be important for the overall structural integrity and native antigenicity of Pvs25 (12, 15, 19, 22), we avoided treating it with reducing agents. Therefore, the CTB or SPDP-modified CTB (CTB^{SPDP}) was treated instead with DTT (designated as CTB^{DTT} or $CTB^{SPDP/DTT}$ in Fig. 2a). Although both CTB^{DTT} and $CTB^{SPDP/DTT}$, when reacted with SPDP-modified Pvs25H-A (Pvs25H-A^{SPDP}), generated substantial levels of fusion complex with retained affinity for GM1-ganglioside, sequential treatment of CTB with SPDP and then with DTT resulted in an even higher specific reactivity toward Pvs25 antiserum (Fig. 2b).

Second, to evaluate the homogeneity of the fusion complex and the stoichiometry of each component within the complex, proteins before and after the conjugation process were analyzed by size exclusion chromatography (Fig. 2c). The two chromatographic peaks for Pvs25H-A and CTB in a mixed sample disappeared, and a new single peak emerged with an apparent molecular mass of 97.2 kDa. Because the molecular masses of Pvs25H-A and CTB are 29.8 and 53.4 kDa, respectively, based on the K_{av} values of chromatography standard proteins, the average stoichiometric ratio for CTB and Pvs25H-A was calculated to be 1:1.5, indicating that one CTB pentamer molecule carries one to two molecules of Pvs25H-A on its surface. Alternatively, if it is assumed that the fusion complex is highly homogeneous and its stoichiometric ratio is 1:1, the 14-kDa discrepancy between the observed and calculated fusion complex mass may be explained by irregularities in the molecular shape, resulting in a higher apparent molecular mass.

Taking all of the results together, we decided to use the Pvs25H-A^{SPDP} + $CTB^{SPDP/DTT}$ conjugation method (Fig. 2d) to generate the fusion complex for all immunization experiments.

Immunogenicity in mice of Pvs25H-A and its fusion protein with CTB when administered by the s.c. or the i.n. route. BALB/c mice were immunized with Pvs25H-A (designated as "S" in Fig. 3 and 4), a mixture of Pvs25H-A and CTB (designated as "M" in Fig. 3 and 4), or CTB-Pvs25H-A fusion protein (designated as "L" in Fig. 3 and 4), by the s.c. or the i.n. route, with or without the indicated adjuvants, at weeks 0, 2, and 3. Antisera were collected at week 4, and the Pvs25H-A-specific IgG titers were determined (Fig. 3a). We demonstrated that: (i) s.c. immunization tended to induce a higher response than i.n. immunization in both the absence and the presence of adjuvants; (ii) the fusion protein (L) consistently induced a higher response than antigen alone (S) or the mixture of proteins (M), regardless of adjuvant supplementation; (iii) supplementation with adjuvants was required for substantial augmentation of the IgG response for both immunization routes; (iv) IFA significantly augmented the response elicited by unfused or CTB-mixed antigen, but CT only marginally affected the response elicited by these antigens; and (v) CT did not exhibit a dose-dependent augmentation effect on the IgG response in the dose range used in the present study (0.1 to 1.0

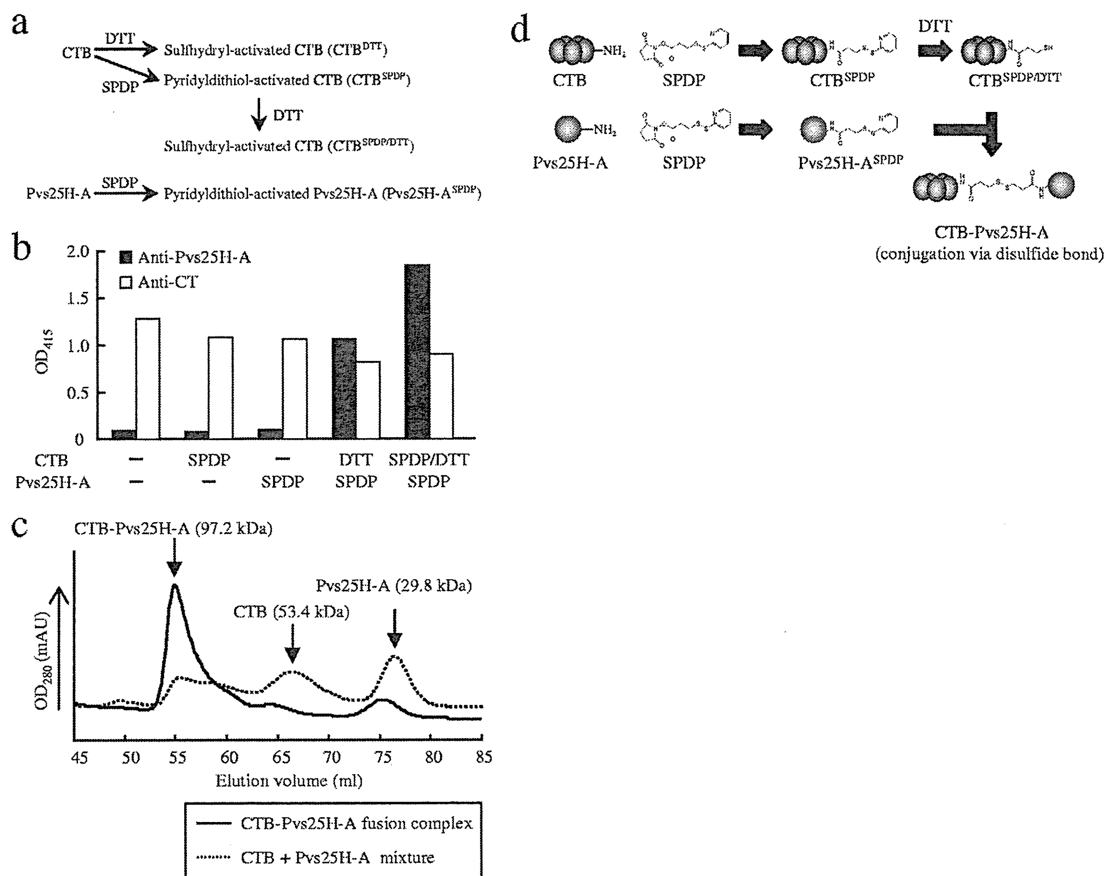

FIG. 2. Chemical conjugation of Pvs25H-A to cholera toxin B subunit (CTB). Various conjugation methods were evaluated for the generation of the CTB-Pvs25H-A fusion complex. The heterobifunctional cross-linker *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was used to link Pvs25H-A to CTB via the primary amines. (a) Either CTB or SPDP-modified CTB (CTB^{SPDP}) was first treated with DTT to expose free sulfhydryls (designated as CTB^{DTT} and $CTB^{SPDP/DTT}$, respectively), and then SPDP-modified Pvs25H-A (Pvs25H-A^{SPDP}) was separately mixed with each of them to generate the fusion complex. (b) The CTB-Pvs25H-A fusion complex was analyzed by GM1-ELISA using anti-cholera toxin (CT) (□) or anti-Pvs25H-A (■) antiserum. (c) The CTB-Pvs25H-A fusion protein (solid line) or a mixture of CTB and Pvs25H-A (dotted line) was subjected to size exclusion chromatography. For the fusion protein, the peaks for CTB and Pvs25H-A disappeared, and a new peak emerged, indicating generation of the fusion complex. (d) The conjugation scheme chosen for production of the CTB-Pvs25H-A fusion complex. See Materials and Methods for the detailed conjugation method.

μg). Finally, we confirmed that the antisera specifically recognized the *P. vivax* ookinete surface by immunofluorescence (Fig. 3b).

Transmission-blocking effect of the induced mouse antisera against field strains of *P. vivax* parasites. The TBV efficacy of the induced mouse antisera against *P. vivax* parasites in infected blood samples from patients was evaluated by the membrane feeding assay. The same experiments were performed twice, using blood samples from two volunteer donors (Fig. 4). The average number of oocysts observed per mosquito fed on patient blood mixed with antisera induced by s.c. immunization of mice with Pvs25H-A/IFA was reduced by >99.9% compared to the naive control serum (N). Omission of the adjuvant significantly abated the effect down to 20 to 60% reduction; however, conjugating the antigen to CTB resulted in a dramatic restoration of the vaccine efficacy back to >90%. A similar tendency, albeit with significantly lower efficacy, was observed for i.n. immunization, in that antisera induced by i.n. immunization with the fusion protein decreased the oocyst number by 70 to 90%, whereas unfused antigen conferred only a 0 to 6% blocking effect. As expected, CT supplementation augmented the effect for i.n.-administered antigens, in that both unfused and CTB-fused antigens conferred a blocking effect of >90%. Interestingly, however, addition of CTB to the mixture of antigen and CT significantly abated the vaccine efficacy down to 40 to 50%. The reason for this is unknown, and it could not have been predicted from the antibody titers (Fig. 3a). Taken together, we concluded that chemical coupling of Pvs25 to CTB is a potentially promising strategy to enhance the transmission-blocking efficacy in i.n. and s.c immunization regimes.

DISCUSSION

Pvs25 is one of the top-priority *P. vivax* TBV candidates, and the production of stable and functional forms of the antigen in the most appropriate formulation is crucial (4; MVTR). In the present study, we investigated the methylotrophic yeast *P. pas-*

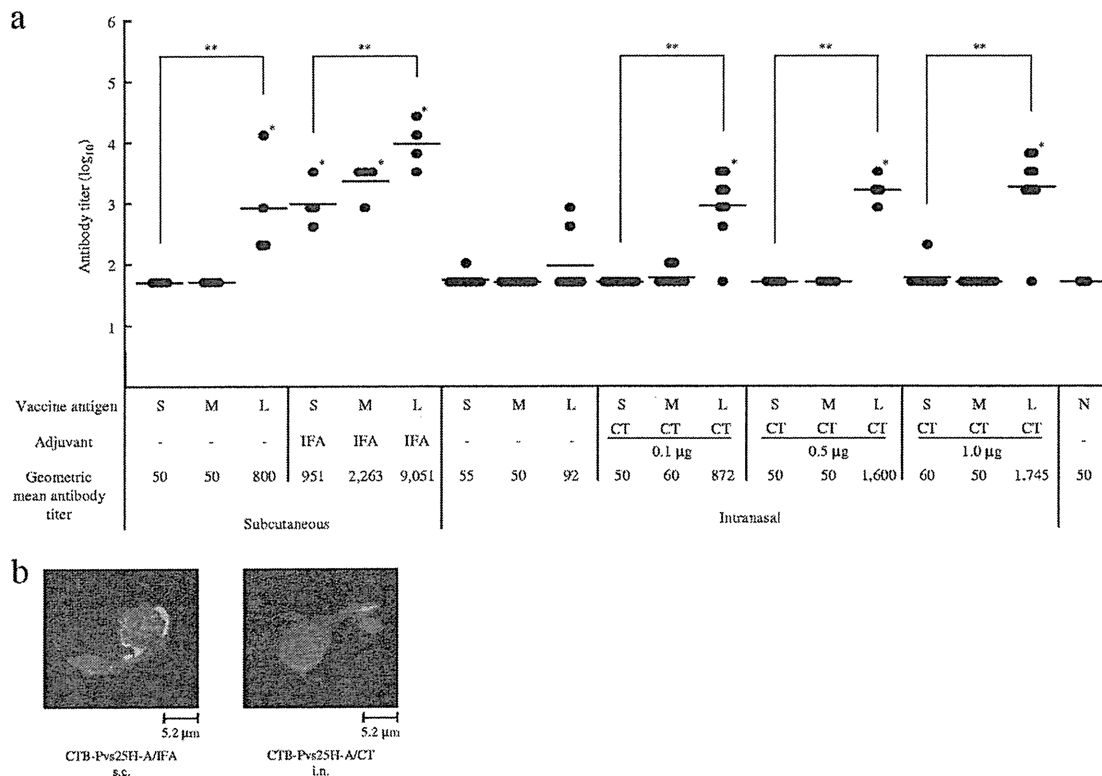

FIG. 3. Immunogenicity of the CTB-Pvs25H-A fusion protein for induction of a Pvs25-specific serum IgG response. (a) Female BALB/c mice (four or eight mice per group) were immunized with Pvs25H-A alone (30 μg) (S), a mixture of cholera toxin B subunit (CTB; 30 μg) and Pvs25H-A (30 μg) (M), or the CTB-Pvs25H-A fusion protein (60 μg), by the subcutaneous (s.c.) or the intranasal (i.n.) route (L), three times, at weeks 0, 2, and 3. Serum samples were collected a week after the third immunization and were evaluated for Pvs25-specific IgG titers. All mice received the same amount of Pvs25H-A antigen, i.e., 30 μg per injection. IFA and CT at various doses (0.1 to 1.0 μg) were used as s.c. and i.n. vaccine adjuvants, respectively. Nonimmune serum (N) was used as a negative control. Antibody titers were defined as the serum dilution that gave an OD_{415} of 0.1 or the serum dilution for which a one-point-higher dilution (2-fold) gave an OD_{415} of <0.1. *, Significantly different from nonimmune serum as determined by the Wilcoxon-Mann-Whitney test ($P < 0.05$); **, significantly different among the three groups (S, M, and L) as determined by the Kruskal-Wallis test ($P < 0.001$). (b) Ookinete-specific reactivity of induced antisera analyzed by immunofluorescence. The antisera derived from s.c. immunization with the CTB-Pvs25H-A fusion protein emulsified with IFA (CTB-Pvs25H-A/IFA), or the fusion protein administered i.n. with CT (1 μg) (CTB-Pvs25H-A/CT) specifically recognized native Pvs25 protein expressed on the surface of *Plasmodium vivax* ookinetes. Scale bar, 5.2 μm.

toris as a production host for Pvs25. The yield of Pvs25H was comparable to that reported previously for its expression in *S. cerevisiae* (19). When expressed in *S. cerevisiae*, this protein was also produced as a mixture of various isoforms (19). Although we observed a similar protein expression pattern, i.e., multimers and the A and B monomers, higher proportions of the molecularly homogeneous A isoform than the heterogeneous B and multimeric isoforms were produced when Pvs25H was expressed in *P. pastoris* and not in *S. cerevisiae*. This might present an advantage of using *P. pastoris* expression system for Pvs25 vaccine production rather than *S. cerevisiae* system. The *P. pastoris*-derived A isoform could be as conveniently and efficiently purified from the culture supernatant as reported for *S. cerevisiae*-derived A isoform, by a combination of affinity, size exclusion, and hydrophobic interaction chromatographies.

The next critical step in vaccine generation is the optimization of vaccine antigen formulations; a search for the optimal antigen formulation is often considered to be as important as choosing the best antigen among many vaccine candidate antigens. Pvs25H antigen adsorbed onto Alhydrogel (Brentag Biosector, Frederilssund, Denmark) has recently been shown to induce antibody effectively in human volunteers in a phase 1 clinical trial, and the antigen was found to be efficacious, as evidenced by significant transmission-blocking activity observed in the membrane feeding assay (17). That study confirmed that Pvs25 is a very promising TBV candidate; however, it is highly desirable to induce higher levels of transmission-blocking immunity for practical vaccine development (17). Another phase 1 clinical trial using Montanide ISA 51, a water-in-oil emulsion, has recently been completed; however, due to an unexpected frequent local reactogenicity, the vaccine efficacy has not been verified (29). Therefore, there seems to be an increasing demand for the development of a new immune-enhancing vaccine platform technology for malaria OSPs, because they are low-molecular-weight proteins that are by themselves not sufficiently immunogenic.

There have been several reports showing examples of chemical conjugation of *Plasmodium falciparum* OSPs with potential antigen carrier molecules such as the outer membrane protein of *Neisseria meningitidis* (30), exoprotein A of *Pseudomonas*

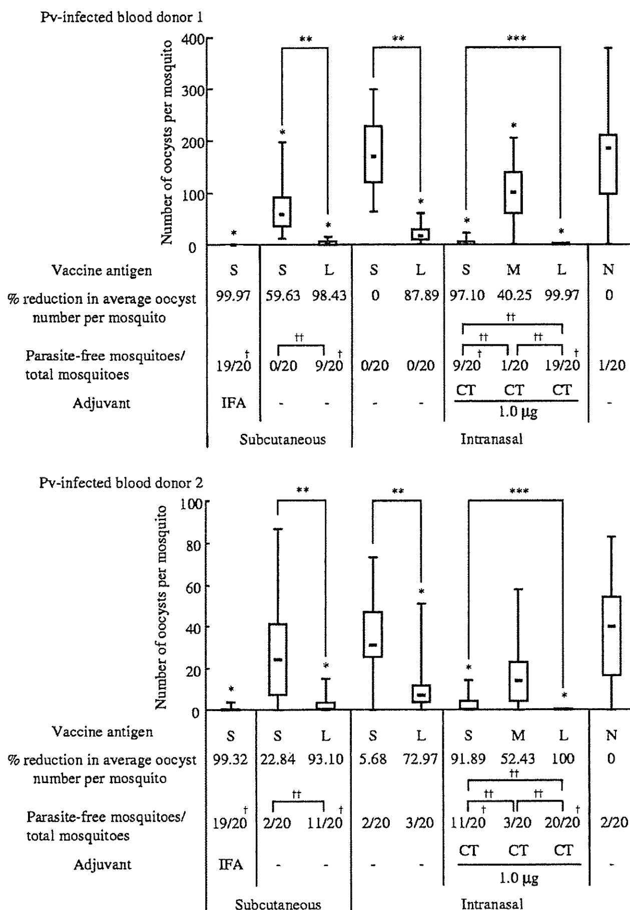

FIG. 4. Transmission-blocking vaccine (TBV) effects of the induced mouse antisera on *Plasmodium vivax* oocyst development in the *Anopheles dirus* A mosquito midgut. TBV effects on oocyst numbers induced by antisera (1/2 dilution) obtained from mice immunized with each antigen formulation (S, M, and L) as described in Fig. 3. N, nonimmune serum. Either IFA or CT was used as an adjuvant, as indicated. The data are expressed as the median values of oocyst number found per mosquito (bar within the box) with the 25 and 75% quartiles (the box) and ranges (whiskers above and below the box). The percent reduction was calculated as the reduction in the average oocyst number for each immunization group compared to the average oocyst number for the unimmunized control group (N). The number of parasite-free mosquitoes per total number of mosquitoes examined (20 mosquitoes) is provided. The analysis was performed twice, using different blood samples, as indicated in the upper panel (*P. vivax* [Pv]-infected blood donor 1) and the lower panel (Pv-infected blood donor 2). M groups without adjuvant supplementation, M and L groups with IFA supplementation, and all groups with 0.1- and 0.5-µg CT supplementation were excluded from membrane feeding analysis. *, $P < 0.001$ versus the nonimmune (N) group as determined by the Wilcoxon-Mann-Whitney test; **, $P < 0.001$ between the S and L groups as determined by the Wilcoxon-Mann-Whitney test; ***, $P < 0.001$ among the three groups (S, M, and L) as determined by the Kruskal-Wallis test; †, $P < 0.005$ versus the nonimmune (N) group as determined by the chi-square test; ††, $P < 0.005$ between the indicated two groups as determined by the chi-square test.

aeruginosa (16), ovalbumin (16), and a *P. falciparum* OSP itself by chemical crosslinking (16). All of these were demonstrated to increase TBV efficacy, but no attempts have yet been made to enhance the immunogenicity of *P. vivax* OSPs by coupling them to other proteins. In the present study we evaluated CTB as a potential carrier for Pvs25. First, to extend our previous study where CT was used as adjuvant (1–3), we tested our hypothesis that the mucosal immunogenicity of Pvs25 would increase when the protein was coupled to the nontoxic CTB subunit, even in the absence of CT supplementation. Second, to explore CTB's less-characterized immune potentiating properties for s.c.-delivered antigens, we immunized mice with the CTB-Pvs25H-A fusion protein by an s.c. route, in the presence or absence of IFA. Our principal finding was that the coupling of the antigen to CTB profoundly enhanced its immunogenicity in i.n. as well as in s.c. immunization regimes, without supplementation with extraneous adjuvants. However, the membrane feeding assay revealed that there was still much room for improvement (Fig. 4). For instance, although i.n. administration of the fusion protein alone conferred a relatively high transmission-blocking immunity (88 and 73% decreases in oocyst number for blood samples from donors 1 and 2, respectively) compared to unfused antigen alone or the unimmunized control group, only a few mosquitoes (0/20 to 3/20) were free of parasites. Supplementation of CT to the fusion protein increased the efficacy close to complete blockade (>99.9%), significantly increasing the number of mosquitoes free of parasites (19/20 to 20/20). Because supplementation of CT to unfused antigen resulted in an intermediate level of protection (97% [9/20] and 92% [11/20] decrease in oocyst number for blood samples from donors 1 and 2, respectively), we concluded that both the CT supplementation and the CTB-coupling strategies contributed to the increased vaccine efficacy, although the former was more efficient than the latter. A similar tendency was observed for the s.c. immunization regime: s.c. administration of the fusion protein alone conferred a more than 90% decrease in oocyst number, in which approximately half of the mosquitoes were free of parasites (9/20 to 11/20); however, the use of IFA with unfused antigen contributed more than the fusion method. We observed that the efficacy of the fusion protein administered alone by the s.c. route was almost equal to that attained by the unfused antigen administered i.n. with CT supplementation, in terms of the average numbers of oocyst per mosquito (>90%) as well as the number of mosquitoes free of parasites (9/20 to 11/20). Similarly, the vaccine efficacy for unfused antigen administered s.c. with IFA was almost equal to the level attained by the fusion protein administered i.n. with CT in terms of the average number of oocysts per mosquito (>99%) as well as the number of mosquitoes free of parasites (19/20 to 20/20). Although we did not assess the vaccine efficacy of the fusion protein emulsified in an oil adjuvant such as IFA, IFA was very effective in that almost complete blockade was observed for unfused antigen. It is likely that a combination of oil adjuvant and the CTB-coupling strategy would further enhance the efficacy. Taken together, we conclude that (i) s.c. immunization is more efficacious than i.n. immunization, inducing "one-level-higher" immunity than i.n. immunization, and (ii) a CTB-coupling strategy is substantially effective in enhancing transmission-blocking immunity, but supplementation with extraneous adjuvants is expected to induce an even higher immunity (Fig. 5).

The clinical use of CT, particularly as a nasal adjuvant, is hampered by its toxicity (26). Furthermore, the nontoxic CTB has yet to be proven a safe nasal vaccine delivery molecule.

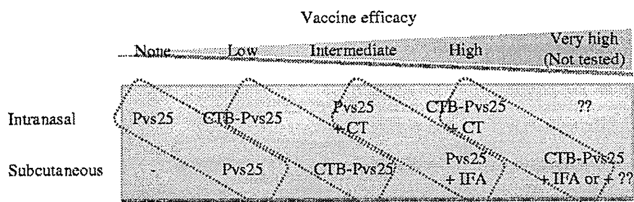

FIG. 5. Schematic summary of the observed or expected (but not tested) transmission-blocking vaccine (TBV) efficacy induced by each immunization regime. s.c. immunization tended to induce "one-level-higher" immunity than i.n. immunization in our experimental model. Identical or similar vaccine formulations are encircled by a dotted line. CTB, cholera toxin B subunit; CT, cholera toxin; IFA, incomplete Freund's adjuvant.

Therefore, an alternative approach for using CTB as a vaccine antigen carrier is highly desirable. Although there have been numerous reports demonstrating enhanced mucosal immunogenicity of various antigens by coupling to CTB (13), very few systematic studies have been conducted to assess CTB's antigen carrier capacity for s.c.-delivered antigens. Our present study clearly demonstrated the potential of CTB in s.c. vaccine platform design. Furthermore, it is notable that, unlike antigens emulsified with oil adjuvant such as IFA or antigens administered with an aluminum hydroxide adjuvant, the protein-only CTB-coupled antigens are likely to be much less reactogenic. It is believed that recent innovations in effective but much less locally reactogenic and safer oil adjuvants such as MF59 (Chiron Corp., Emeryville, CA) (20, 21), the Montanide ISA series (Seppic, Inc., Fairfield, NJ) (20, 29), and the GlaxoSmithKline adjuvant systems (GlaxoSmithKline, Brentford, United Kingdom) (8, 27), will expedite malaria vaccine development. It is also possible that protein delivery molecules will ultimately be combined with effective oil or other adjuvants, including aluminum hydroxide. This is supported by our recent unpublished study in which a recombinant malaria antigen administered with an alum adjuvant only marginally enhanced its immunogenicity, whereas the same antigen loaded onto carrier molecules became highly immunogenic when applied together with the alum.

In the present study, we did not assess the molecular mechanisms of the immune potentiating function of CTB. However, our observation that simple mixing of Pvs25H-A with CTB did not produce a profound immune enhancement implies that its immunogenicity results from the antigen delivery, rather than a physiological cell activation, as occurs for CT. Further experiments are ongoing to characterize the immune potentiating function of CTB using the C-terminal 19-kDa fragment of merozoite surface protein 1 from *Plasmodium yoelii*. The results of these studies will help us judge whether CTB could contribute to a new platform technology for the design of s.c.-delivered subunit vaccines against infectious diseases such as malaria.

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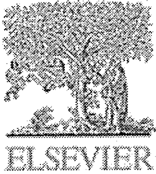

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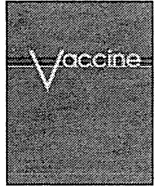

Intranasal and intramuscular immunization with Baculovirus Dual Expression System-based Pvs25 vaccine substantially blocks *Plasmodium vivax* transmission

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ABSTRACT

We have recently developed a new experimental vaccine vector system based on *Autographa californica* nucleopolyhedrosis virus (AcNPV) termed the "Baculovirus Dual Expression System", which drives expression of vaccine candidate antigens by a dual promoter that consists of tandemly arranged baculovirus-derived polyhedrin and mammalian-derived CMV promoters. The present study used this system to generate a *Plasmodium vivax* transmission-blocking immunogen (AcNPV-Dual-Pvs25). AcNPV-Dual-Pvs25 not only displayed Pvs25 on the AcNPV envelope, exhibiting aspects of its native three-dimensional structure, but also expressed appropriately immunogenic protein upon transduction of mammalian cells. Both intranasal and intramuscular immunization of mice with AcNPV-Dual-Pvs25 induced high Pvs25-specific antibody titres, notably of IgG1, IgG2a and IgG2b isotypes, indicating a mixed Th1/Th2 response. Importantly, sera obtained from subcutaneously immunized rabbits exhibited a significant transmission-blocking effect (96% reduction in infection intensity, 24% reduction in prevalence) when challenged with human blood infected with *P. vivax* gametocytes using the standard membrane feeding assay. Additionally, active immunization (both intranasal and intramuscular routes) of mice followed by challenge using a transgenic *P. berghei* line expressing Pvs25 in place of native Pbs25 and Pbs28 (clone Pvs25DR3) demonstrates a strong transmission-blocking response, with a 92.1% (intranasal) and 83.8% (intramuscular) reduction in oocyst intensity. Corresponding reductions in prevalence of infection were observed (88.4% and 75.5% respectively). This study offers a novel tool for the development of malarial transmission-blocking vaccines against the sexual stages of the parasite, using the Baculovirus Dual Expression System that functions as both a subunit, and DNA based vaccine.

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1. Introduction

Malaria is a serious, acute and sometimes relapsing disease, caused by protozoan parasites of the genus *Plasmodium*. It is responsible for high morbidity and mortality in tropical and subtropical regions, causing approximately 1 million deaths per year, the majority of whom are African children under the age of five [25]. Given the complex life cycles of the different plasmodial species and the distinct host immune responses to each developmental stage, *Plasmodium* provides many potential targets for the development of prophylactic vaccines against the parasite.

An anti-malarial transmission-blocking vaccine (TBV) that prevents fertilization and/or ookinete/oocyst development within the mosquito is an attractive strategy to limit the transmission of malaria. The ookinete proteins Pvs25 and Pvs28 which are expressed on the surface of the sexual and early sporogonic forms of *Plasmodium vivax* are presently lead targets for the development of a *P. vivax* TBV [15–18]. A variety of expression vectors (e.g., *Escherichia coli*, *Pichia pastoris* and DNA) have been used to express Pvs25 protein which has been administered alone or in combination with adjuvants (e.g., Freund's adjuvant, aluminum hydroxide and cholera toxin) [19,20,26,33,34]. To date these studies suggest that the recombinant protein currently requires both not only linear, but conformation dependent epitopes, and a strong adjuvant to induce transmission-blocking antibodies. Phase I human trials with a clinical-grade recombinant Pvs25 produced by *P. pastoris* administered with an alum adjuvant produced antibodies that inhibit transmission of the parasite by ~80% (intensity) and 20–30% (prevalence) [19].

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To improve the safety and efficacy of current Pvs25-based TBV candidates, new vaccine vehicles and/or delivery systems (e.g., needle- and adjuvant-free, long-lasting and cost-effective) need to be considered. In addition, a suitable small-animal model for the *in vivo* assessment of *P. vivax* TBV-induced functional immune responses might provide a useful tool for evaluating its TBV efficacy before proceeding to human clinical trials. Recently, we have developed a new vaccine vector system based on the baculovirus *Autographa californica* nucleopolyhedrosis virus (AcNPV) termed the "Baculovirus Dual Expression System", which drives expression of vaccine candidate antigens by a dual promoter that consists of tandemly arranged baculovirus-derived polyhedrin and mammalian-derived CMV promoters. It has been shown that AcNPV, an enveloped double-stranded DNA virus that naturally infects insects, possesses strong adjuvant properties that can activate dendritic cell-mediated innate immunity through MyD88/TLR9-dependent and -independent pathways [30]. When applied to *P. berghei* circumsporozoite protein (PbCSP) as a model for malaria pre-erythrocytic stage vaccine, this immunogen elicited high PbCSP-specific antibody titres and PbCSP-specific $CD8^+$ T-cell responses without extraneous immunological adjuvants in mice, and conferred complete protection against sporozoite challenge [8]. The Baculovirus Dual Expression System therefore constitutes an innate immunostimulating complex, and functions as a subunit and DNA vaccine that generates strong humoral and cellular immune responses. In addition, the AcNPV-based vaccine has another great potential for adjuvant-free intranasal (i.n.) administration. For blood-stage malaria vaccine development, we have also shown that i.n. immunization with AcNPV-based vaccine expressing *P. yoelii* merozoite surface protein 1 19 kDa fragment ($PyMSP1_{19}$) induced not only strong systemic humoral immune responses with high titre of $PyMSP1_{19}$-specific antibody but also natural boosting of $PyMSP1_{19}$-specific antibody responses at a short time following challenge, and conferred complete protection [28].

Here we evaluate a second-generation transmission-blocking Pvs25 immunization protocol based on the Baculovirus Dual Expression System (AcNPV-Dual-Pvs25). We show that the AcNPV-Dual-Pvs25 elicits highly effective Pvs25-specific humoral immune responses, and confers significant transmission-blocking activity, assessed by the standard membrane feeding assay (SMFA) on peripheral blood from *P. vivax* infected patients, and by active immunization of mice challenged with *P. berghei* expressing Pv25 (Pvs25DR3), which has been specifically generated as a murine model for the *in vivo* assessment of Pvs25-based TBV-induced functional immune responses [12].

The data reported here demonstrates the successful implementation of the Baculovirus Dual Expression System using both i.n. and intramuscular (i.m.) methods of delivery, giving equivalent responses for each method of immunization. Our results show that the Baculovirus Dual Expression System provides a simple, nontoxic, safe and adjuvant-free vaccine delivery platform, and as such could be considered as a powerful tool for the development of future TBVs against *Plasmodium* spp.

2. Materials and methods

2.1. Cell lines, mice and parasites

Sf9 cells were maintained at 27 °C in SF900-II medium (Invitrogen, San Diego, CA) supplemented with antibiotics. HepG2 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, and antibiotics. Female BALB/c or Theiler's Original (TO) mice, 7–8 weeks of age at the start of the experiment, were purchased from Harlan (UK). *P. berghei* clones ANKA 2.34 and Pvs25DR3 [12] were used for challenge infections. General parasite maintenance was carried out as described previously [24].

2.2. Recombinant baculovirus

The DNA sequence corresponding to amino acids Ala_{23}-Leu_{195} of Pvs25 (*P. vivax* Salvador I strain) was amplified from pEU3-Pvs25 [27] using the primers pPvs25-F1 (5′-GAATTCATGGCTAGCGCCGTCACGGTAGACACC-3′) and pPvs25-R1 (5′-CCCGGGGCCCAAGGCATACATTTTTCTCTTT-3′). The PCR product was ligated into the *Eco*RI/*Sma*I sites of pTriEx–PbCSP–gp64 [8] to construct a baculovirus transfer vector, pTriEx–Pvs25–gp64 (Fig. 1). The recombinant baculovirus AcNPV-Dual-Pvs25 was generated in Sf9 cells by co-transfection of the recombinant transfer plasmids pTriEx–Pvs25–gp64, with BacVector-2000 DNA (Novagen), according to the manufacturer's protocol. Purification of viral particles was performed as described previously [8]. The purified baculovirus particles were free of endotoxin (<0.01 endotoxin units/10^9 PFU), as determined by the Endospecy® endotoxin measurement kit (Seikagaku Co., Tokyo, Japan).

2.3. Recombinant proteins

A 0.5-kb fragment of the *Pvs25* gene (encoding amino acids 23–159) was excised from pTriEx–Pvs25–gp64 by digestion with *Eco*RI and *Sma*I, and inserted into the *Eco*RI/*Sma*I sites of pGEX-4T-1 (GE Healthcare) to construct the recombinant expression plasmid, pGEX-Pvs25. Recombinant Pvs25, created as a fusion protein with glutathione S-transferase (GST-Pvs25), was expressed in *E. coli* and purified using a GST affinity column (GE Healthcare) as described previously [1]. Resultant protein was used as an immunogen for vaccination of mice and as antigen for isotype analysis.

2.4. Western blotting and indirect immunofluorescence assay (IFA)

Western blotting was carried out as described previously [2]. HepG2 cells were seeded at a density of 5×10^4 cells/well in collagen-type-I-coated eight-well chamber slides (BD Biosciences) and transduced with purified baculovirus particles at an m.o.i. of 10. After 48 h incubation, cells were fixed for 15 min in acetone/methanol [6,4] at −20 °C, and incubated with anti-Pvs25 mAb N1-1H10 (MR4, Manassas, VA) and then with FITC-conjugated goat anti-mouse IgG (Biosource International, Camarillo, CA).

For preparation of *P. vivax* ookinetes, peripheral blood was collected in heparinized syringes under written informed consent from patients who attended malaria clinics within the Mae Sod district in the Tak province of northwestern Thailand. The use of all human materials in this study was reviewed and approved by the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the Walter Reed Army Institute of Research, USA. For IFA, cultured *P. vivax* parasite preparations rich in zygotes and small numbers of ookinetes were spotted on slides and fixed with acetone. Sera obtained from immunized mice were tested by IFA on the fixed parasite material. To confirm the position of all parasites in the IFA, the slides were stained with DAPI (4′,6-diamidino-2-phenylindole) (Wako Pure Chemical, Osaka, Japan). Bound antibodies and labeled nuclei were recorded by confocal scanning laser microscopy (LSM5 PASCAL; Carl Zeiss MicroImaging, Thornwood, NY).

2.5. Immunization

Mice were immunized four times at 3-week intervals with 5×10^7 PFU of AcNPV-Dual-Pvs25 either by the i.m. or i.n. routes.