Evaluation of Recombinant *Plasmodium knowlesi* Merozoite Surface Protein-1₃₃ for Detection of Human Malaria

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Abstract. *Plasmodium knowlesi* is now known as the fifth *Plasmodium* species that can cause human malaria. The *Plasmodium* merozoite surface protein (MSP) has been reported to be potential target for vaccination and diagnosis of malaria. MSP-1₃₃ has been shown to be immunogenic and its T cell epitopes could mediate cellular immune protection. However, limited studies have focused on *P. knowlesi* MSP-1₃₃. In this study, an approximately 28-kDa recombinant *P. knowlesi* MSP-1₃₃ (pkMSP-1₃₃) was expressed by using an *Escherichia coli* system. The purified pkMSP-1₃₃ reacted with serum samples of patients infected with *P. knowlesi* (31 of 31, 100%) and non-*P. knowlesi* malaria (27 of 28, 96.43%) by Western blotting. The pkMSP-1₃₃ also reacted with *P. knowlesi* (25 of 31, 80.65%) and non-*P. knowlesi* malaria sera (20 of 28, 71.43%) in an enzyme-linked immunosorbent assay (ELISA). Most of the non-malarial infection (49 of 52 in by Western blotting and 46 of 52 in the ELISA) and healthy donor serum samples (65 of 65 by Western blotting and ELISA) did not react with recombinant pkMSP-1₃₃.

INTRODUCTION

Malaria is one of the most important infectious diseases and it causes high global mortality and morbidity. According to World Malaria Report year 2010, 106 countries or areas still have endemic malaria. Approximately 300 million malaria clinical cases are reported each year, resulting in approximately 1 million deaths. Approximately 2.5 billion persons or 40% of the world's populations are at risk.^{1,2} The four known species of human malaria parasites are *Plasmodium falciparum*, *P. malariae*, *P. vivax*, and *P. ovale*. *Plasmodium knowlesi* has recently been recognized as the fifth *Plasmodium* species that can cause malaria in human populations.^{3,4} *Plasmodium knowlesi* is naturally found in long-tailed (*Macaca fascicularis*) and pig-tailed (*M. nemestrina*) macaques. *Plasmodium knowlesi* malaria is widely distributed in counties in Southeast Asia, including Borneo,⁴ Myanmar,⁵ Thailand,⁶ the Philippines,⁷ Singapore,⁸ and Vietnam.^{9,10} High numbers of *knowlesi* malaria have been reported in Borneo Malaysia states of Sabah and Sarawak.^{4,11} Recently, the infection has been detected in several states in Peninsular Malaysia in rural or suburban areas.¹²

Plasmodium knowlesi has a rapid replication cycle, which may cause hyperparasitemia within a short period. Life-threatening complications such as respiratory distress, abnormal liver function, renal failure, and even death may occur.¹³ *Plasmodium knowlesi* could be misdiagnosed as *P. malariae* or *P. falciparum* by microscopy because early trophozoites of *P. knowlesi* morphologically resemble those of *P. falciparum*, and late and mature trophozoites, schizonts, and gametocytes of *P. knowlesi* are generally indistinguishable from those of *P. malariae*.¹⁴

Merozoite surface protein 1 (MSP-1) is a high molecular mass (185–225 kDa) glycoprotein expressed on the surface of blood stage merozoites of *Plasmodium* species.¹⁵ This molecule is involved in the maturation of merozoite and invasion into erythrocytes, thus making it a leading blood-stage malaria vaccine candidate.¹⁶ MSP-1 undergoes a two-step

processing by proteases, resulting in the production of several fragments. The first processing cleaves the MSP-1 precursor polypeptide into four major fragments, which are held together on the free merozoite surface by noncovalent contacts.^{17,18} The second processing further cleaves one of the fragments (MSP-1₄₂) into two fragments with molecular masses of 33 kDa (MSP-1₃₃) and 19 kDa (MSP-1₁₉). The soluble MSP-1₃₃, corresponding to the N-terminal region of MSP-1₄₂, sheds from the surface,^{19,20} whereas the membrane-bound MSP-1₁₉ remains anchored to the merozoite and is carried into the new erythrocyte.²¹

MSP-1₄₂ is one of the leading candidates for blood-stage malaria vaccines because it is able to induce cell-mediated and humoral immune responses,²² and its proteolytic product MSP-1₃₃ is responsible for the cellular immune protection.²³ For example, *P. yoelii* MSP-1₃₃ induces effector T cells that could assist in protective B cells response and mediate significant antiparasitic activity against *P. yoelii*.^{24,25} *Plasmodium falciparum* MSP-1₃₃ is naturally immunogenic because antibodies against this antigen could be detected in serum of *P. falciparum*-exposed blood donors.²⁶ It was further shown that T cell response and antibodies against MSP1 were induced in human clinical trials by using *P. falciparum* MSP-1, in which epitopes responsible for cytokine production and memory response development were localized to the 33-kDa region of MSP-1₄₂.^{27,28}

Hitherto, most of the studies on MSP-1₃₃ have focused on *P. falciparum*, *P. vivax*, or *P. yoelii*, but few on *P. knowlesi*. A large amount of *P. knowlesi* MSP-1₃₃ is required for its biochemical, structural, and immunologic studies. In this study, recombinant MSP-1₃₃ of *P. knowlesi* was expressed and purified by using an *Escherichia coli* system. The recombinant pkMSP-1₃₃ was evaluated by using sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assay (ELISA), and Western blot assays.

MATERIALS AND METHODS

Blood samples and serum collection. Malaria blood samples were collected from patients > 20 years of age who were admitted to the University of Malaya Medical Center in Kuala Lumpur, Malaysia during July 2008–December 2011.

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Patient blood samples infected with malaria were confirmed by microscopic examination, polymerase chain reaction (PCR) based on *Plasmodium* small subunit ribosomal RNA genes,⁴ and the BinaxNOW[®] malaria rapid diagnostic test (Inverness Medical International, Stockport, United Kingdom). Non-malarial parasitic infections were confirmed by commercial ELISAs. Patient serum samples were categorized as follows: *P. knowlesi* human malaria; non-*P. knowlesi* human malaria (*P. falciparum*, *P. vivax*, *P. ovale*); non-malarial parasitic infection (filariasis, amebiasis, cysticercosis, toxoplasmosis, toxocarosis); and healthy donor.

DNA extraction and construction of recombinant expression plasmid. *Plasmodium knowlesi* DNA was extracted from blood sample by using a blood extraction kit (QIAGEN, Hilden, Germany). A primer pair, MSP1₃₃_F: 5'-GAGCTCG AGAATCACGTGGCTGCATTCA-3' and MSP1₃₃_R: 5'-GAGCTCCTACATCTGAGTTTGTACATTTAAC-3' was designed based on the *P. knowlesi* H strain MSP-1₃₃ nucleotide sequence (GenBank accession no. XM_002258546). Polymerase chain reaction was performed with an initial denaturing step at 95°C for 4 minutes, followed by 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds. A final elongation step at 72°C for 10 minutes was added to the last cycle. The PCR product was purified by using the QIAquick Gel Extraction Kit (QIAGEN), and the nucleotide sequence of the amplified fragment was confirmed by sequence analysis. The amplified fragment was then cloned into pCR 2.1-TOPO plasmid vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The plasmid was digested with restriction enzyme *SacI* at 37°C for 3 hours, and the digested target fragment was ligated with expression vector pRSET A (Invitrogen) at 4°C overnight. Transformation into maintenance host *E. coli* TOP10F' strain was performed with the resulting ligation mixture for propagation and maintenance of the plasmid. Before expression, the plasmid was transformed into expression host *E. coli* BL21 (DE3) pLysS strain.

Expression of recombinant pkMSP-1₃₃. A single recombinant BL21 (DE3) pLysS colony was inoculated and propagated overnight in Luria-Bertani broth containing ampicillin (100 µg/mL) and chloramphenicol (35 µg/mL) at 37°C with shaking. The overnight culture was diluted with Luria-Bertani broth to an optical density (OD) of 0.1 at 600 nm, and the culture was allowed to grow to an OD₆₀₀ of 0.4–0.6. The culture was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown at 37°C with shaking (250 rpm) for two hours. Cells were harvested by centrifugation at 5,000 × *g* for 10 minutes. The recombinant proteins were analyzed and identified by SDS-PAGE and Western blotting.

Optimization of recombinant pkMSP-1₃₃ expression conditions. Concentration of IPTG used for induction and collection time after inductions were monitored to determine the optimum expression conditions of recombinant pkMSP-1₃₃. The IPTG concentrations of 0.1 mM, 0.5 mM, and 1.0 mM were used for induction. Cell culture (1 mL) was collected before induction and every hour after induction up to 5 hours. Cells were harvested and analyzed by SDS-PAGE and Western blotting.

Purification of recombinant pkMSP-1₃₃. Small-scale protein purification was performed by using 1 mL of cell culture with the MagneHis purification system (Promega, Madison, WI). Bacteria cells were lysed using FastBreak[™] Cell Lysis

Reagent. Paramagnetic precharged MagneHis[™] Ni-Particles were then added to the lysate to bind on the polyhistidine tag at the N-terminal of the recombinant pkMSP-1₃₃. Washing buffer was added to remove unbound proteins. The purified protein was recovered by elution with 100 mM imidazole. For larger-scale protein purification, recombinant pkMSP-1₃₃ was purified with nickel-NTA agarose resin by using the ProBond[™] purification system (Invitrogen). An OD₆₀₀ for culture after two hours of induction with IPTG was measured. Two conditions were used for purification, which include native condition and hybrid condition.

For purification with native condition, cell pellet from a 50 mL culture (0.5 grams) was resuspended in native binding buffer (pH 8.0) containing 10 mM imidazole, and 16 mg of lysozyme was added per gram of cell paste. The cells were sonicated on ice with three 5-second pulses at high intensity to disrupt and break the bacterial cell wall. Protein lysate was added to a purification column that contained agarose resin and washed four times with native wash buffer (pH 8.0) containing 20 mM imidazole. The purified recombinant pkMSP-1₃₃ was eluted with native elution buffer (pH 8.0), which contained 250 mM imidazole.

For purification with the hybrid condition, cell pellet from a 50 mL culture (0.5 grams) was resuspended in 6 M guanidinium lysis buffer (pH 7.8) by using 16 mL of guanidinium lysis buffer per gram of cell paste. Sonication was used for cell wall disruption. Protein lysate was added to a purification column, which contained agarose resin, washed twice with denaturing binding buffer (pH 7.8) containing 8 M urea, twice with denaturing wash buffer (pH 6.0) containing 8 M urea, followed by four washes with native wash buffer (pH 8.0) containing 20 mM imidazole. The purified recombinant pkMSP-1₃₃ was eluted with native elution buffer (pH 8.0), which contained 250 mM imidazole and stored at –80°C.

SDS-PAGE and Western blotting. Non-purified and purified recombinant pkMSP-1₃₃ was analyzed by using 12% SDS-PAGE and Western blotting. The proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue (Bio-Rad, Hercules, CA). The separated proteins were also transferred by electroblotting onto polyvinylidene difluoride membranes (Bio-Rad). Blocking of the membranes was performed by incubation in Tris buffered saline (TBS) containing 5% skimmed milk overnight at 4°C. The membranes were washed three times with TBS containing 0.2% Tween 20 and probed with anti-*P. knowlesi*-positive human serum diluted 1:200 with TBS containing 2.5% skimmed milk for two hours with constant shaking at room temperature. After three additional washes, the membrane was treated for one hour at room temperature with 1:2,500 diluted biotin-labeled goat anti-human IgM plus IgG plus IgA (Kirkegaard and Perry Inc., Gaithersburg, MD), followed by streptavidin-alkaline phosphatase AP (Invitrogen). Finally, the protein band was revealed by the nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate substrate (Sigma Chemical Co., St. Louis, MO) as the chromogenic substrate. The color was allowed to develop at room temperature in dark. The membrane was dried and photographed.

Quantitation of concentration of recombinant pkMSP-1₃₃. The concentration of the purified recombinant pkMSP-1₃₃ was determined by using the Bradford protein assay with bovine serum albumin (BSA) as standard (Bradford Assay Kit; Bio-Rad).

Evaluation of recombinant pkMSP-1₃₃ in Western blot assays. The purified recombinant pkMSP-1₃₃ was tested by Western blotting using serum samples from patients infected with *P. knowlesi* (n = 31) and non-*P. knowlesi* human *Plasmodium* species, which include *P. falciparum* (n = 11), *P. vivax* (n = 16), and *P. ovale* (n = 1). In addition, serum samples from patients with non-malarial parasitic infections, which include filariasis (n = 4), amebiasis (n = 16), cysticercosis (n = 12), toxoplasmosis (n = 17), toxocarosis (n = 3) and healthy donors (n = 65) were also tested. Polyvinylidene difluoride membrane strips containing blotted recombinant pkMSP-1₃₃, with protein amounts ranging from 70 ng to 350 ng, were incubated with different serum samples, followed by incubation with biotin-labeled goat anti-human IgG, streptavidin-alkaline phosphatase, and nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate substrate.

Evaluation of recombinant pkMSP-1₃₃ in ELISA. A checkerboard ELISA was performed to determine the working concentrations of the coating antigen (pkMSP-1₃₃) and dilutions of patient serum. Recombinant pkMSP-1₃₃ was serially diluted starting from the initial protein concentration of 20 µg/mL by using 0.05 M sodium carbonate buffer, pH 9.6. The diluted antigen was coated on 96-well microtiter plates (TPP, Trasadingen, Switzerland) and incubated overnight at 4°C. The wells were washed three times with phosphate-buffered saline containing 0.1% Tween-20. Blocking buffer (1% BSA in PBS) was added into each well and incubated for two hours at 37°C. The wells were then washed three times. Known positive and negative patient serum samples diluted 1:50, 1:80, 1:100, and 1:200 in 1% BSA/PBS were added respectively into each well and incubated for one hour at 37°C. After samples were washed five times, 1:2,500 diluted peroxidase-labeled goat anti-human IgG (Kirkegaard and Perry, Inc.) was added and samples were incubated for one hour at 37°C. The wells were washed five times with PBS containing 0.1% Tween 20 and incubated with 3, 3', 5, 5'-tetramethyl benzidine, (Amresco, Solon, OH) for 30 minutes in dark. The reaction was stopped by adding 2 N H₂SO₄. The OD was read at 450 nm. Samples were run in duplicates. The OD of a sample was determined by subtracting the OD of the blank containing 3, 3', 5, 5'-tetramethyl benzidine from the mean OD of the sample. The

cut-off value was calculated as the $M_N + 2\sigma$ of the healthy donor serum group, where M_N and σ are the mean OD and the SD, respectively. Samples with OD values greater than $M_N + 2\sigma$ were considered positive. Finally, the optimal concentrations of antigens and dilutions of patient serum were determined, and the appropriate ELISA with a specific antigen concentration and patient serum dilution was used with the same serum sample as that used for Western blot assays.

Statistical analysis. The sensitivity and specificity of Western blotting and ELISA for detection of malarial infection were calculated by using the formulas sensitivity = $TP/(TP + FN) \times 100\%$ and specificity = $TN/(TN + FP) \times 100\%$, where TP = true positive, TN = true negative, FP = false positive, and FN = false negative of malarial infection.

RESULTS

Cloning and expression of recombinant pkMSP-1₃₃.

Primers were designed to amplify the *P. knowlesi* MSP-1₃₃ gene from genomic DNA by PCR, which had an expected size of 705 basepairs. The gene was cloned into expression vector pRSET A and transformed into BL21 (DE3) pLysS host cells. The recombinant clone showed high expression of a protein with an approximate size of 28 kDa (Figure 1), which was absent in the non-recombinant plasmid control.

For determination of optimal expression time and conditions, a time-course experiment was conducted. The recombinant pkMSP-1₃₃ could be expressed by induction with all three concentrations of IPTG tested (0.1 mM, 0.5 mM and 1.0 mM). Expression of pkMSP-1₃₃ could be observed as early as one hour after induction with IPTG. However, the quantity of protein expressed at one hour after induction was lower compared with the quantity of protein expressed at two hours after induction. Thus, we concluded that recombinant pkMSP-1₃₃ could be expressed optimally after two hours of induction with 0.1 mM IPTG at 37°C with constant shaking (250 rpm).

Purification of recombinant pkMSP-1₃₃. Purification of recombinant pkMSP-1₃₃ was performed by using the MagneHis (Promega) system and ProBond™ (Invitrogen) purification systems (Figure 1). For larger-scale purification,

FIGURE 1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of expression and purification of recombinant *Plasmodium knowlesi* merozoite surface protein-1₃₃ (pkMSP-1₃₃) in *Escherichia coli* BL21 (DE3) pLysS. **A**, Coomassie brilliant blue stained. **B**, Western blot probed with *P. knowlesi*-infected patient serum. Lanes 1 and 2, negative control pRSET A clone at 0 and 2 hours after induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG); lane 3, purification of 1 mL negative control pRSET A culture with nickel resin using the MagneHis purification system (Promega, Madison, WI); lane 4, Bio-Rad (Hercules, CA) Prestained Broad Range Protein Marker; lanes 5 and 6, recombinant pkMSP-1₃₃ at 0 and 2 hours after induction with 0.1 mM IPTG; lane 7, purification of 1 mL recombinant pkMSP-1₃₃ with the MagneHis purification system (Promega). Lanes 8 and 9, 50 mL of culture of negative control pRSET A and recombinant pkMSP-1₃₃ purified by using the ProBond™ purification system (Invitrogen, Carlsbad, CA), respectively. The purified approximately 28-kDa recombinant pkMSP-1₃₃ was detected (arrows).

the OD₆₀₀ of culture after two hours of IPTG induction was 0.756, which contained 3.78×10^8 cells/mL. Lysozyme (8 mg) was used during native cell lysate preparation, and 8 mL of 6 M guanidinium lysis buffer (pH 7.8) was used during hybrid cell lysate preparation. The concentration of the purified pkMSP-1₃₃ was then measured by using the Bradford assay. The concentration of purified pkMSP-1₃₃ was 0.8 mg/mL.

Western blot analysis of recombinant pkMSP-1₃₃ with human serum samples. The purified pkMSP-1₃₃ was evaluated by Western blotting using serum samples from persons with *P. knowlesi* human malaria, non-*P. knowlesi* human malaria, non-malarial parasitic infections, and healthy donors (Figure 2). The recombinant pkMSP-1₃₃ detected all *P. knowlesi* infections and showed 100% sensitivity (31 of 31) for *P. knowlesi* infections. Results indicated that 96.43% of the 28 samples of *P. vivax*, *P. falciparum*, and *P. ovale* (non-*P. knowlesi* malarial infections) reacted with recombinant pkMSP-1₃₃. The overall sensitivity of the recombinant pkMSP-1₃₃ for detection of malarial infection was 98.31% (58 of 59). Only three serum samples among 117 non-*Plasmodium* and healthy donor serum samples reacted with recombinant pkMSP-1₃₃. The specificity of Western blotting was 97.44% (114 of 117).

ELISA of recombinant pkMSP-1₃₃ with human serum samples. Based on results from a checkerboard ELISA with protein concentration of 5, 10, and 20 µg/mL, no significant difference was observed between concentrations of 10 µg/mL and 20 µg/mL. Serum samples used in Western blot assays were used in ELISAs with 10 µg/mL of recombinant pkMSP-1₃₃ coated on each well and a 1:80 dilution of serum samples. The sensitivity of the recombinant pkMSP-1₃₃ for *P. knowlesi* infection and non-*P. knowlesi* malarial infection was 80.65% (25 of 31) and 71.43% (20 of 28) respectively, and the overall sensitivity for malarial detection was 76.27% (45 of 59). Six samples of persons with non-*Plasmodium* parasitic infections and healthy donor serum samples reacted with recombinant pkMSP-1₃₃, which indicated a specificity of 94.87% (111 of 117). Results of detection of recombinant pkMSP-1₃₃ with serum from persons infected with *P. knowlesi* and other para-

FIGURE 2. Detection of recombinant *Plasmodium knowlesi* merozoite surface protein-1₃₃ (pkMSP-1₃₃) with patient serum samples infected with *P. knowlesi* and other parasite species. Each Western blot strip, which contained 70 ng of the purified recombinant pkMSP-1₃₃, was tested with serum from each category. Selected samples from each category are shown. Lane 1, Bio-Rad (Hercules, CA) Prestained Broad Range Protein Marker; lanes 2 and 3, serum of patients infected with *P. knowlesi*; lane 4, serum of a patient infected with *P. falciparum*; lane 5, serum of a patient infected with *P. vivax*; lanes 6–10, serum of patients infected with non-malarial parasitic diseases (6 = filariasis, 7 = amebiasis, 8 = toxoplasmosis, 9 = cysticercosis, 10 = toxocariasis), lane 11, healthy donor serum, which served as a negative control.

TABLE 1

Detection of recombinant pkMSP-1₃₃ with patient serum infected with *Plasmodium knowlesi* and other parasite species in Western blot assay and ELISA*

Human serum group	No. serum samples tested	Western Blot		ELISA	
		No. positive	No. negative	No. positive	No. negative
<i>P. knowlesi</i>	31	31	0	25	6
Non- <i>P. knowlesi</i> human malaria					
<i>P. vivax</i>	16	16	0	11	5
<i>P. falciparum</i>	11	10	1	9	2
<i>P. ovale</i>	1	1	0	0	1
Non-malarial parasitic infection					
Filariasis	4	1	3	1	3
Amebiasis	16	1	15	3	13
Cysticercosis	12	0	12	0	12
Toxoplasmosis	17	1	16	2	15
Toxocariasis	3	0	3	0	3
Healthy donor	65	0	65	0	65

*pkMSP-1₃₃ = recombinant *Plasmodium knowlesi* merozoite surface protein-1₃₃. ELISA = enzyme-linked immunosorbent assay.

site species in Western blot assays and ELISA are shown in Table 1. Sensitivity and specificity of Western blot assays and ELISA using recombinant pkMSP-1₃₃ are shown in Table 2.

DISCUSSION

Plasmodium knowlesi replicates and completes its blood stage cycle in 24 hours, the shortest of all human *Plasmodium* species. Thus, it can lead to quotidian fever and rapid hyperparasitemia, which is potentially severe and fatal if not treated.¹² A previous study showed that protective T cell responses can be induced by epitopes within the dimorphic regions of MSP-1₃₃, instead of MSP-1₄₂, in *P. falciparum* because of the low prevalence of T cell responses to MSP-1₁₉.²⁹ The availability of *P. knowlesi* MSP-1₃₃ protein is a prerequisite for its biochemical and immunologic characterization. Thus, in this study, *P. knowlesi* MSP-1₃₃ was chosen as the target protein for recombinant expression in an *E. coli* system.

Recombinant protein has some advantages over native protein. Expression of recombinant protein is rapid, simple, and convenient. Difficulties in long-term *in vitro* cultivation of *P. knowlesi* have led to a limited supply of live *P. knowlesi*. Thus, sustainable recovery of sufficient quantities of pure native *P. knowlesi* MSP-1₃₃ is a limiting factor. The *E. coli* expression system was used chosen in this study because of its safety, simplicity, known genetic properties, and efficiency. Foreign DNA can easily be transformed into *E. coli* with well-established genetic manipulation methods. Also, the *E. coli* system can express large amounts of protein within a few hours because its growth rate is high. Large amounts of native, non-glycosylated recombinant pkMSP-1₃₃ can be

TABLE 2

Sensitivity and specificity of Western blot assay and ELISA using recombinant pkMSP-1₃₃*

Assay	Sensitivity (%)		Overall	Specificity (%)
	<i>Plasmodium knowlesi</i> infection	Non- <i>P. knowlesi</i> malarial infection		
Western blot	100.00	96.43	98.31	97.44
ELISA	80.65	71.43	76.27	94.87

*ELISA = enzyme-linked immunosorbent assay; pkMSP-1₃₃ = recombinant *Plasmodium knowlesi* merozoite surface protein-1₃₃.

produced with this efficient and inexpensive system compared with other expression systems using virus, yeast, or mammalian cells.^{30,31} Soluble and stable recombinant pkMSP-1₃₃ could be easily expressed and obtained by induction with 0.1 mM IPTG and constant shaking (250 rpm) for two hours at 37°C.

For larger-scale protein purification, Probond™ (Invitrogen) native and hybrid conditions were chosen to preserve the protein structure. In the hybrid condition, the protein lysate and column were prepared under denaturing conditions and native buffers were used during the washing and elution steps to refold the protein.

Purified recombinant pkMSP-1₃₃ was analyzed and probed with serum from patients infected with *P. knowlesi* and other parasite species. A total of 176 serum samples were used to test the purified recombinant pkMSP-1₃₃ in Western blot assays and ELISA. The sensitivity and specificity of Western blot assays were relatively higher than that of the ELISA. This discrepancy might be caused by borderline activity of patient serum samples in the ELISA. In a study comparing ELISA and Western blotting for detection of antibody against human papillomavirus type 16 E7, Suchankova and others reported positive results in an ELISA but negative results by Western blotting, and they suggested that this result was apparently associated with the borderline activity of serum samples, for which some of OD values were just below or above the cutoff value.³² A similar situation occurred in our study. Some serum samples from persons infected with *P. knowlesi* and from persons infected with non-*P. knowlesi* *Plasmodium* species had OD values just below the cutoff value, and serum samples from persons infected with non-*Plasmodium* parasites had OD values just above the cutoff value. However, there are several advantages of the ELISA over Western blot assays. An optimized ELISA requires lower volumes of serum for each reaction compared with Western blot assays, and this factor could be crucial for small volume samples. Also, the ELISA can quantitatively determine the antibody titer for serum, as the OD value of the sera directly resembles the titer of antibody.

The reactivity of recombinant pkMSP-1₃₃ with serum from persons with non-*P. knowlesi* human malaria could be explained by cross-reactivity caused by their high identities and similarities in MSP-1₃₃ DNA and amino acid sequences (Table 3). Valderrama-Aguirre and others showed that antibodies generated in mice against *P. vivax* MSP-1 fragment known as Pv200L recognized and cross-reacted with *P. falciparum* recombinant MSP-1 Pf190L fragment, which is the homologous region to Pv200L, indicating the sharing of B cell epitopes by these two MSP-1 proteins.³³ The *P. vivax* MSP-1₄₂ (pvMSP-1₄₂) protein, which shows 71% homology with *P. cynomolgi* MSP-1₄₂ (pcMSP-1₄₂), is able to induce similar level of protection during *P. cynomolgi* challenge in

rhesus monkeys because of the immunologic and structural relatedness of these proteins.³⁴ Antibodies against several antigens of *P. cynomolgi* and *P. vivax* have been shown to cross-react in different studies.^{35,36} Thus, *P. knowlesi* MSP-1₃₃ which has amino acid similarity of 82%, 60%, and 70% with that of *P. vivax*, *P. falciparum* and *P. ovale*, respectively, could be sharing some common epitopes with these human *Plasmodium* species.

Another explanation for the reactivity of recombinant pkMSP-1₃₃ with serum from patients with non-*P. knowlesi* human malaria and non-*Plasmodium* parasitic infections is the possibility of previous exposures or infections with *P. knowlesi*.³⁷ Wipasa and others have demonstrated that antibodies against malaria infection were stably maintained over periods of more than five years after the last known malaria infection.³⁸ Thus, antibodies against *P. knowlesi* could still be detected by Western blotting or ELISA after many years of acute infection. It was noted that one of malaria serum samples did not react with the recombinant pkMSP-1₃₃. Although the reason for this observation is unclear, it was likely that the serum sample was collected before the production of antibodies against the malaria parasite.³⁹

Based on these results, pkMSP-1₃₃ may be useful in general serodiagnosis of malarial infections, such as determination of prevalence and endemicity of malarial infection in a population and blood donor screening. It will be particularly useful in seroepidemiologic screening in human and macaque populations in malaria-endemic areas such as Sabah and Sarawak, Malaysia.

In conclusion, recombinant pkMSP-1₃₃ was successfully expressed by using the *E. coli* system under optimal conditions. Large amounts of protein could be obtained to facilitate work on the application of *P. knowlesi* MSP-1₃₃ in immunodiagnosis and vaccination designs for malarial infections. There is a possibility that pkMSP-1₃₃ could cross-react with other *Plasmodium* species. Therefore, further evaluation with immune serum samples against MSP-1₄₂ and/or MSP-1₃₃ of non-*P. knowlesi* *Plasmodium* species should be performed.

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TABLE 3

Plasmodium knowlesi nucleotide and amino acid identities and similarities compared with other human *Plasmodium* species*

<i>P. knowlesi</i>	Nucleotide identities (%)	Amino acid identities (%)	Amino acid similarities (%)
<i>P. vivax</i>	74	70	82
<i>P. falciparum</i>	48	41	60
<i>P. malariae</i>	55	48	71
<i>P. ovale</i>	58	49	70

* Amino acid similarity was calculated based on their characteristics, which include charge and hydrophobicity.

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