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Evaluation of codon optimized recombinant *Plasmodium knowlesi* Merozoite Surface Protein-1_{19} (pkMSP-1_{19}) expressed in *Pichia pastoris*

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Abstract. Malaria causes high global mortality and morbidity annually. *Plasmodium knowlesi* has been recognised as the fifth human *Plasmodium* sp. and its infection is widely distributed in Southeast Asia. Merozoite surface protein-1_{19} (MSP-1_{19}) appears as a potential candidate for malaria blood stage vaccine as it could induce protective immunity. In this study, codon optimized *P. knowlesi* MSP-1_{19} (pkMSP-1_{19}) was expressed and purified in yeast *Pichia pastoris* expression system. The purified recombinant protein was further evaluated using Western blot assay using knowlesi malaria, non-knowlesi human malaria, non-malarial parasitic infections and healthy serum samples (n = 50). The sensitivity of purified pkMSP-1_{19} towards detection of knowlesi infection was as 28.6% (2/7). pkMSP-1_{19} did not react with all non-malarial parasitic infections and healthy donor sera, yet reacted with some non-knowlesi human malaria sera, therefore lead to a specificity of 86.0% (37/43).

INTRODUCTION

Malaria is a devastating infectious disease resulting in high global mortality and morbidity. According to World Malaria Report (2013), there are approximately 207 million of malaria clinical cases resulting to 627000 deaths each year. The common four human *Plasmodium* sp. are *Plasmodium falciparum*, *P. vivax*, *P. malariae and P. ovale*. Recently, *P. knowlesi* has been recognised as the fifth malaria species known to cause human infection (White, 2008). Its natural hosts are long-tailed (*Macaca fascicularis*) and pig-tailed (*M. nemestrina*) macaques that are commonly found in forested areas of Southeast Asia (Singh *et al.*, 2004; Bronner *et al.*, 2009). There are numerous reports of *P. knowlesi* infecting humans in the Southeast Asia region including in Borneo (Singh *et al.*, 2004), Singapore (Ng *et al.*, 2008), Philippines (Luchavez *et al.*, 2008), Malaysia (Lee *et al.*, 2009), Myanmar (Jiang *et al.*, 2010) and Thailand (Jongwutiwes *et al.*, 2004). Imported knowlesi malaria cases have been reported in European countries due to eco-tourism or work in the forested areas of Southeast Asia countries (Bronner *et al.*, 2009; Ta *et al.*, 2010; Orth *et al.*, 2013).

Merozoite surface protein 1 (MSP-1) is a high molecular mass protein found on merozoite suface of all *Plasmodium* sp. and MSP-1 is proposed to have essential functions for the pathogenicity of the parasites. During schizonts rupture, MSP-1 undergoes proteolytic processing and is cleaved into four fragments MSP-1_{83}, MSP-1_{30}, MSP-1_{38}, and MSP-1_{42} (Holder & Freeman, 1982). During maturation and invasion of the merozoites into erythrocytes, the MSP-1_{42} fragment is further cleaved into soluble MSP-1_{33} which is shed from the merozoite (Blackman *et al.*, 1991b), and membrane

bound MSP-1_{19} which is remained on the merozoite surface and is carried into new erythrocyte (Blackman *et al.*, 1991a).

MSP-1_{19} is one of the most-studied MSP-1 fragments and has long been considered as a leading vaccine candidate against malaria blood stage parasites. Study showed that antibodies directed against MSP-1_{19} can interrupt merozoite invasion *in vitro* (Blackman *et al.*, 1990; Egan *et al.*, 1999; O'Donnell *et al.*, 2001), inhibit the secondary proteolytic processing of MSP-1 (Blackman *et al.*, 1994) and delay the intracellular parasite development (Moss *et al.*, 2012). On the other hand, immunization studies using MSP-1_{19} in animal models such as rodents, mice and primates found that protective immune response is elicited during challenge with life *Plasmodium* parasites (Daly & Long, 1993; Collins *et al.*, 1999; Yang *et al.*, 1999; Stowers *et al.*, 2001; Mehrizi *et al.*, 2011). By using recombinant *Plasmodium* sp. MSP-1_{19}, Muerhoff *et al.* (2010) showed that anti-MSP-1_{19} antibodies were detected in all malaria patients infected with *P. falciparum*, *P. vivax*, *P. ovale*, or *P. malariae*, indicating that anti-MSP-1_{19} antibodies could also be detected in human knowlesi infections. Hence, this suggests that *P. knowlesi* MSP-1_{19} could be used as a suitable antigen for serodiagnosis of knowlesi malaria.

In routine serodiagnosis assays for parasitic infections, the antigens used are usually harvested from the parasites which are grown *in vitro* or *in vivo*. Growing and maintaining parasites is laborious, hazardous and expensive. An alternative and safer way to produce antigens is via recombinant DNA technology. Many studies on the production and use of recombinant antigens have been reported, particularly using the *Escherichia coli* bacterial expression system. However, one of the major obstacles for optimal expression in *E. coli* is the absence of post-translational modification (e.g., folding, glycosylation, phosphorylation, and acylation) systems. This problem could be overcome by using eukaryotic expression system such as the methylotrophic yeast *Pichia pastoris* (Cereghino & Cregg, 2000). Recombinant proteins expressed in *P. pastoris* are subjected to post-translational modifications that may be crucial for proper structure and functioning of the proteins. Previous studies have shown that recombinant antigens produced by *P. pastoris* were antigenically adequate for serodetection of parasitic infections (Biemans *et al.*, 1998; Fong & Lau, 2004; Lau & Fong, 2008; Vicentin *et al.*, 2014).

Gene expression levels depend on many factors such as promoter sequences and regulatory elements. Usually taxonomically-closely related organisms will use similar codons for their protein synthesis whereas taxonomically-distant organisms utilise another set of codons (Ikemura, 1982). In order to improve the expression level of recombinant genes, several strategies have been used. One of the strategies is codon optimization, which is the alteration of the codon of the recombinant gene to the typical codon usage of the host used in the expression (Lithwick & Margalit, 2005). Studies also indicated that the level and yield of codon optimized recombinant protein is higher compared to native recombinant protein, and the codon optimized protein could be purified more readily than the native version (Burgess-Brown *et al.*, 2008; Tokuoka *et al.*, 2008).

In this paper, *P. knowlesi* MSP-1_{19} was expressed and purified in *P. pastoris* expression system (pkMSP-1_{19}). The pkMSP-1_{19} was evaluated in Western blot assay by using sera of patient infected with knowlesi malaria, non-knowlesi human malaria, non-malarial parasitic infections and healthy donor.

MATERIALS AND METHODS

Codon optimization of *P. knowlesi MSP-1_{19}*

The codon optimized *P. knowlesi MSP-1_{19}* gene for *P. pastoris* expression system was designed based on the published sequence (GenBank accession number XM_002258546.1) with preferred codon usage of *P. pastoris* (Figure 1). The codon optimized *MSP-1_{19}* gene was synthesized by GENEART, Germany. Restriction enzyme (RE) *Eco*RI cutting site was incorporated into

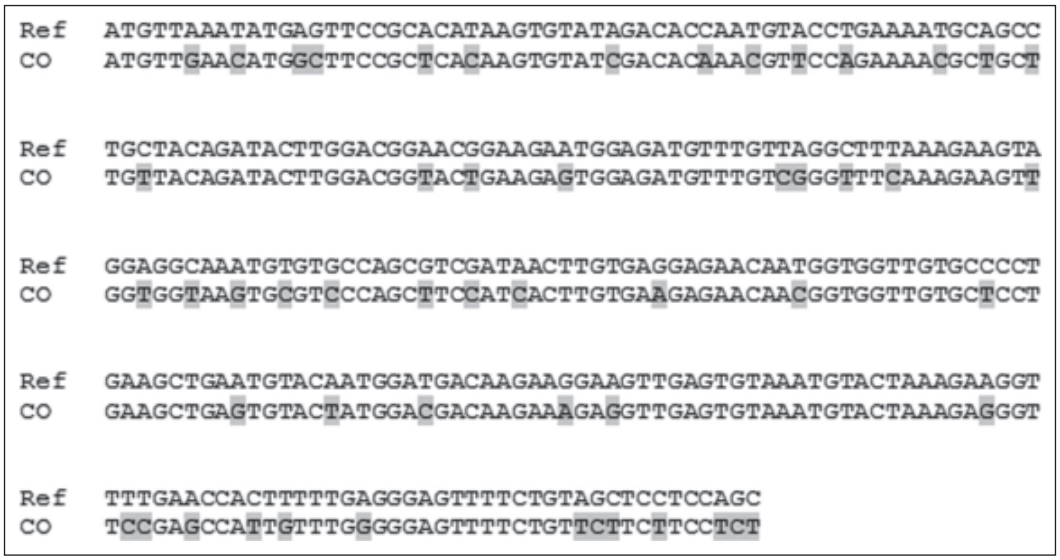

Figure 1. *Plasmodium knowlesi* reference gene and codon optimized *pkMSP-1*$_{19}$ gene. Note: Ref, *P. knowlesi MSP-1*$_{19}$ reference gene XM_002258546.1; CO, codon optimized *pkMSP-1*$_{19}$ sequence. Letters highlighted in grey indicating the altered nucleotides in *pkMSP-1*$_{19}$ sequence.

the synthesized gene for insertion of the gene sequence into expression vector pPICZα A.

Construction of recombinant plasmid

The codon optimized *MSP-1*$_{19}$ fragment and pPICZα A vector were digested by RE *Eco*RI. Dephosphorylation of the digested pPICZα A vector was carried out using CIAP (calf intestinal alkaline phosphatase) (New England Biolabs, UK) to prevent plasmid self-ligation. Ligation was performed at 16°C for two hours followed by transformation into *E. coli*. The positive recombinant MSP-1$_{19}$-pPICZα A plasmid was isolated and linearized by RE *Sac*I. Transformation into *P. pastoris* was carried out according to the instruction manual of the EasySelect™ *Pichia* Expression kit (Invitrogen Corp., USA). Positive recombinant clones were selected for expression.

Expression of pkMSP-1$_{19}$ in *P. pastoris* expression system

A single colony of the recombinant clone was picked and inoculated in 10 ml buffered complex medium containing glycerol (BMGY, 0.1 M potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 0.0004% biotin and 1% glycerol). The culture was grown overnight at 28°C. The cells were harvested and resuspended in 50 ml buffered complex medium containing methanol (BMMY, 0.1 M potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 0.0004% biotin and 1% methanol) (optical density 1.0 at λ = 600 nm). The culture was allowed to grow for 72 hours. Methanol was added every 24 hours to a final concentration of 1% (v/v) to induce expression. Cells were collected every 24 hours after methanol induction for protein extraction and analysis. *Pichia pastoris* (X-33 strain) carrying pPICZα A vector only (without insert) was similarly treated and used as negative control.

Purification of pkMSP-1$_{19}$

The *P. pastoris* cells were removed from the culture medium by centrifugation at 5,000 rpm for 5 min. The total protein from culture medium was precipitated by ammonium sulfate method. Solid ammonium sulfate was added into the culture medium to get an 80% saturated buffer. The mixture was stirred at 4°C until the ammonium sulfate had completely dissolved. The total protein was pelleted out by centrifugation at 10,000 g for 30 min. The pellet was then resuspended in 4 ml native binding buffer. ProBond™ Nickel-

chelating resin (Invitrogen Corp., USA) was used for single-step purification of pkMSP-1_{19}. The native protein purification method was used as described by the manufacturer. Quantification of protein was carried out using the Bradford Assay Kit (Bio-Rad, USA).

SDS-PAGE, Coomassie Brilliant Blue and Western blot

The expressed pkMSP-1_{19} was separated by SDS–PAGE and stained with Coomassie brilliant blue (Bio-Rad, USA). The separated protein was transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) for Western Blot analysis. The membrane was blocked with 5% skimmed milk overnight and incubated with *P. knowlesi*-positive patient serum (1: 250 dilution) for two hours at room temperature. After three washings with 0.2% Tween-20 in tris buffered saline (TBST), the membrane was incubated with biotin-labelled goat anti-human IgM + IgG + IgA (KPL Inc., USA, 1: 2500 dilution) for one hour, followed by streptavidin-alkaline phosphatase (KPL Inc., USA, 1: 2500 dilution) for one hour at room temperature. Finally the membrane was incubated with BCIP/NBT substrate (Sigma Chemical Co., USA) and the membrane was rinsed with distill water once the protein bands had developed.

Matrix-assisted laser desorption/ ionization (MALDI) mass spectrometry

The expected protein band was excised from the Coomassie blue-stained SDS-PAGE gel. Destaining of the gel piece was carried out by using 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate (NH_4HCO_3) with constant shaking until the gel piece was clear. Reduction was performed by adding 10 mM dithiothreitol in 100 mM NH_4HCO_3 to the gel piece and incubated for 30 min at 60°C, followed by alkylation with addition of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and incubation for 20 min in the dark. After three washes with 50% ACN in 100 mM NH_4HCO_3, dehydration was done with 100% ACN and the gel piece was dried in a speed vacuum for 15 min to remove the ACN completely. Digestion of protein was carried out with trypsin in 50 mM NH_4HCO_3 and incubated overnight at 37°C. Protein was extracted by using 100% ACN with shaking for 15 min. The solution which contained the digested sample was transferred to a new micro-centrifuge tube and dried in a speed vacuum. The lyophilized sample was reconstituted with 0.1% formic acid and desalted by using ZipTip (Millipore, Billerica, MA). MALDI-time-of-flight (TOF) analysis on the eluted protein was carried out in UM Proteomics Research Centre (Kuala Lumpur, Malaysia).

Sensitivity and specificity of the recombinant MSP1_{19}

The purified pkMSP-1_{19} was evaluated using Western blot assays with 50 sera of patient infected with knowlesi malaria (n = 7); non-knowlesi human malaria which include *P. falciparum* (n = 8), *P. vivax* (n = 11), and *P. ovale* (n = 1); non-malarial parasitic infections (n = 10); and healthy donor (n = 13). Patient samples infected with malaria were confirmed by microscopic examination and polymerase chain reaction, while patient samples with non-malarial parasitic infections were confirmed by commercial ELISA tests. The PVDF membrane blotted with purified pkMSP-1_{19} was cut into strips and incubated with different serum samples for two hours, followed by biotin-labelled anti-human antibodies and streptavidin-alkaline phosphatase. Sensitivity of pkMSP-1_{19} for detection of malarial infection in Western blot assay was calculated as = (number of true positive)/ (number of true positive + number of false negative), while specificity was calculated as = (number of true negative)/ (number of true negative + number of false positive).

RESULTS

Cloning and expression of pkMSP-1_{19} in *P. pastoris* expression system

The codon optimized fragment of MSP-1_{19} was cloned in frame into pPICZα A vector. pkMSP-1_{19} was expressed extracellulary in *P. pastoris*. Total protein was harvested from the cell culture every 24 hours after methanol induction. A novel band of approximately 19 kDa representing pkMSP-1_{19} protein was

observed in the recombinant clone after 24 hours, which was absent in the negative control (culture medium of *P. pastoris* X33 carrying pPICZα A only) (Figure 2). The purified recombinant protein was identified as *P. knowlesi* MSP-1_{19} by MALDI-TOF mass spectrometry analysis.

Quantification of purified pkMSP-1_{19}

Concentration of purified pkMSP-1_{19} was measured with Bradford assay and BSA was used as the standard (Bio-Rad, USA). Based on the standard curve of BSA, the concentration of pkMSP-1_{19} was 653 mg/l.

Evaluation of purified pkMSP-1_{19} in Western blot using patient sera

The purified pkMSP-1_{19} was analysed by Western blot with 50 patient serum samples (Figure 3). Western blot results showed that purified pkMSP-1_{19} reacted with two of seven of the knowlesi malaria samples, which lead to a sensitivity of 28.6% (2/7). On the other hand, pkMSP-1_{19} did not react with all non-malarial parasitic infections and healthy donor serum samples, yet reacted with six of 20 of non-knowlesi human malaria samples (which all the reactivity was observed in *P. vivax*-infected sera). Therefore, the specificity of pkMSP-1_{19} was 86.0% (37/43).

DISCUSSION

In the present study, recombinant pkMSP-1_{19} was expressed using methylotrophic yeast *P. pastoris* expression system. One of the advantages of yeast expression system is *P. pastoris* can be grown in simple and reasonably priced media, and it utilises methanol as its sole carbon source, which is considerably cheaper compared to bacterial and mammalian cell expression systems which require expensive expression inducer

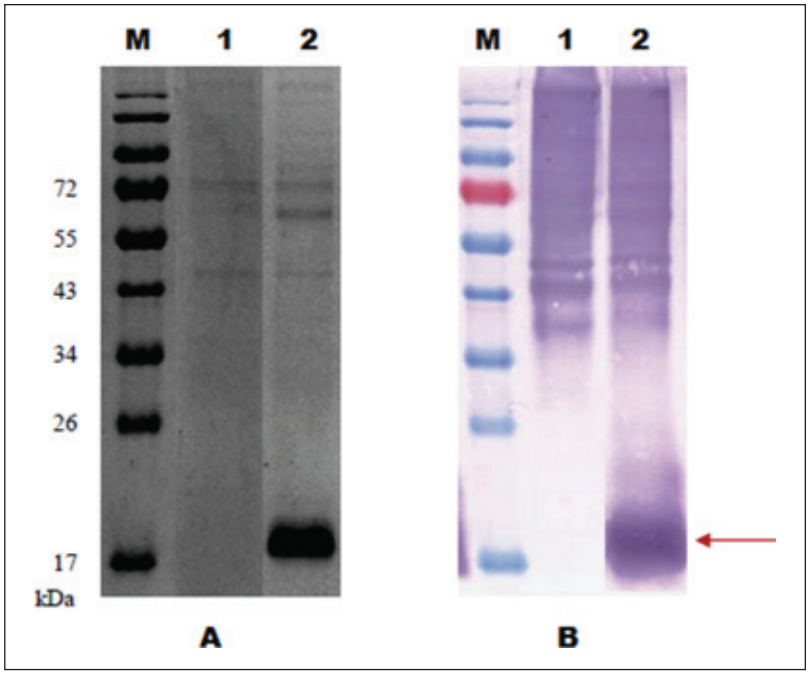

Figure 2. (A) SDS-PAGE and (B) Western blot of pkMSP-1_{19} expression in *P. pastoris* expression system. Equal volume of precipitated culture medium of negative control (Lane 1) and pkMSP-1_{19} (Lane 2) was loaded. Protein was verified by Western blot with *P. knowlesi*-positive patient serum. A novel band with size of approximately 19 kDa was detected in both SDS-PAGE and Western blot assay (arrow) in the recombinant clone, which was absent in the negative control clone.

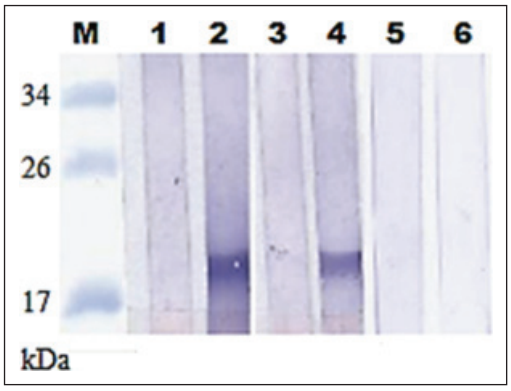

Figure 3. Western blot analysis of purified pkMSP-1_{19} with patient sera. The purified pkMSP-1_{19} was tested with patient sera infected with knowlesi malaria (Lane 1 and 2), non-knowlesi human malaria (Lane 3 and 4), non-malarial parasitic infections (Lane 5) and healthy donor serum (Lane 6). Reactivity was observed in pkMSP-1_{19} with knowlesi malaria and non-knowlesi human malaria serum samples, but not with non-malarial parasitic infections and healthy donor serum samples.

or culture media. In the *P. pastoris* expression system, it is important to keep methanol levels within a relatively narrow range for protein production because methanol overload can result in cell death (Minning *et al.*, 2001). Notably, *P. pastoris* is capable of *N*- and *O*-linked glycosylation as well as other post-translational protein modifications like to those found in mammalian cells (Faber *et al.*, 1995). However, the recombinant pkMSP-1_{19} obtained from *P. pastoris* in this study remained unglycosylated, retaining the same molecular weight of approximately 19 kDa as recombinant *P. knowlesi* MSP-1_{19} expressed in *E. coli* expression system (data not shown).

Eukaryotic proteins expressed intracellularly in *E. coli* prokaryotic expression system are frequently sequestered into insoluble inclusion bodies which require more protein purification steps to remove the contaminating intracellular proteins and obtain an acceptably pure product. Besides, the expressed proteins have to be purified under denaturing condition. Lengthy and effort-consuming procedures are needed for the renaturation and recovery of the purified protein, and to remove the unwanted denaturants. However, recombinant pkMSP-1_{19} was expressed extracellularly in *P. pastoris*. The expressed protein was secreted into the medium, which significantly reduced the amount of contaminating intracellular proteins (Carrio & Villaverde, 2002). Additionally, the secreted recombinant pkMSP-1_{19} was highly soluble and allowed the protein to be purified easily under native condition. Furthermore, studies also demonstrated that *P. pastoris* expression system could produce higher amounts and yield of recombinant protein than *E. coli* expression system (Majerle *et al.*, 1999; Watelet *et al.*, 2002).

Besides, yeast expression system would accumulate the foreign gene products inside peroxisomes. This could be a crucial advantage especially when the protein product is toxic for the host cell and the protein product could be protected from undesired side effects such as proteolysis and aggregation (Faber *et al.*, 1995). Therefore, this could possibly explain the difficulties that we encountered in the on-going experiments on expression of *P. knowlesi* MSP-1_{19} in *E. coli* expression system. Large numbers of clones were screened and expressed in order to find a clone that can express the *P. knowlesi* recombinant MSP-1_{19} easily. On top of that, the expression efficiency of the selected clone decreased drastically after few expressions and subsequently the clone lost its viability after few days. Biased codon usage, toxicity of gene product, solubility and stability of the expressed protein could be the factors that affect the efficient production of *P. knowlesi* MSP-1_{19} in *E. coli* expression system (Wu *et al.*, 2004).

Previous studies have clearly demonstrated that codon optimization could improve the efficiency and yield of recombinant proteins. Norazmi *et al.* (1999) found that cloning of the native *P. falciparum MSP-1_{19}* fragment into *Mycobacterium smegmatis* resulted in delayed growth, decrease in transformation efficiency and low level expression of recombinant protein. The synthetic homologue of *P. falciparum MSP-1_{19}* fragment using *Mycobacterium* codon bias was generated and this codon optimized fragment lead to normal cell

growth, increased transformation efficiency and substantial high protein expression. Yadava and Ockenhouse (2003) showed that level of protein expression had dramatically improved in the codon optimized-*P. falciparum* F2 domain of erythrocyte binding antigen (*EBA-175*) gene compared to the native gene in both *E. coli* and *P. pastoris* expression system. Similar results were obtained by Yazdani *et al.* (2006) when comparing the codon optimized and native versions of the gene for *P. vivax* Duffy binding protein. Besides, Bai *et al.* (2011) also demonstrated that the yield of codon optimized-P-glycoprotein (an ATP-dependent drug efflux pump) in *P. pastoris* expression system was three-fold higher compared to the native wild-type protein, together with higher purity and improved function. In the present study, the codon optimized-*P. knowlesi MSP-1$_{19}$* gene could be expressed easily and optimally in *P. pastoris* expression system with considerably high yield of purified protein.

The purified pkMSP-1_{19} was evaluated using 50 serum samples from different categories. The sensitivity of pkMSP-1_{19} was unexpectedly low and this could be due to the position of tags were fused to *MSP-1$_{19}$* in the expression vector. pPICZα A expression vector used in the *P. pastoris* system utilized dual tagging (c-myc epitope and His_6-tag) at the C-terminus of the recombinant protein, and Halliwell *et al.* (2001) reported that C-terminally tagged enzymes displayed lower activity compared to N-terminally tagged and native enzymes. Generally, it is unavoidable that addition of a His_6-tag might interfere with protein activity and alter the biochemical properties of recombinant proteins (Wu & Filutowicz, 1999; Terpe, 2003). Taken together, this suggests that adding a His_6-tag at the C-terminus of *MSP-1$_{19}$* may have induced misfolding, which subsequently reduced the immunogenicity of the *P. pastoris*-produced pkMSP-1_{19}.

pkMSP-1_{19} also reacted with non-knowlesi human malaria sera, specifically *P. vivax*-infected sera at certain level. This might be explained by serological cross-reactivity due to high amino acid sequence similarity between *P. knowlesi* MSP-1_{19} and *P. vivax* MSP-1_{19}. Numerous studies had indicated that serum cross-reactivity could occur among malaria patients infected with different human *Plasmodium* sp. (Diggs & Sadun, 1965; Miller *et al.*, 1980; Kumar *et al.*, 1992; Kim *et al.*, 2004). In fact, the amino acid sequence of *P. knowlesi* MSP-1_{19} has a similarity of 88% with MSP-1_{19} of *P. vivax*. Therefore, *P. knowlesi* MSP-1_{19} may share several common immunodominant epitopes with *P. vivax* and lead to cross-reactivity in Western blot assay. In this case, cross-reactivity of MSP-1_{19} would most likely be due to the double epidermal growth factor (EGF)-like modules at the C-terminus of MSP-1_{19}, which is highly conserved among the *Plasmodium* sp. (Drew *et al.*, 2004). The results in the present study were consistent with our previous findings, which cross-reactivity had also been observed in *P. knowlesi* MSP-1_{33} and MSP-1_{42} with non-knowlesi *Plasmodium* sera (Cheong *et al.*, 2013a; Cheong *et al.*, 2013b). No reactivity was observed with non-malarial parasitic infection and healthy donor serum samples, indicating that the pkMSP-1_{19} was highly specific.

As conclusion, pkMSP-1_{19} was successfully expressed and purified in *P. pastoris* expression system. However, sensitivity of purified pkMSP-1_{19} for detection of knowlesi infections in Western blot assay was not up to satisfactory level. Several strategies such as alteration of the position of affinity tags, removal of the affinity tags from the recombinant protein, use of other expression vectors or use of other expression systems should be carried out in the future study to order to evaluate whether the sensitivity of pkMSP-1_{19} could be improved. Nonetheless, pkMSP-1_{19} could still be remained as a valuable candidate in seroprevalence studies and sero-epidemiological screening for knowlesi infections in malaria endemic areas.

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