RESEARCH ARTICLE

Cloning, expression and purification of *Plasmodium knowlesi* circumsporozoite protein and immunoblot analysis with *P. knowlesi* strain A1H1 protein extract

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ABSTRACT

Circumsporozoite protein (CSP) is a sporozoite major surface protein of *Plasmodium* species. The protein showed promising protection level as a vaccine candidate against *Plasmodium falciparum* infection. There is a lack of studies on *P. knowlesi* CSP (PkCSP) as a vaccine candidate due to the high polymorphic characteristic of central repeat region. Recent studies showed the protein has a relatively conserved region at the C-terminal, which consists of T- and B-cell epitopes. This could be the target region for vaccine development against the pre-erythrocytic stage of the parasite. In this study, recombinant PkCSP was expressed using *Escherichia coli* system. Recombinant PkCSP was immunized in animal models and the antiserum was evaluated using immunoblot analysis. Results showed that PkCSP can be successfully expressed using the bacterial system. Endpoint titre of the antiserum were ranged up to 1:819200. Immunoblot analysis showed the antiserum recognized recombinant PkCSP but not total protein extract from *P. knowlesi* erythrocytic stage. In conclusion, PkCSP could elicit strong immune response in animal models. However, serum antibodies could not recognize protein from the parasite's erythrocytic stage extract indicating it is not expressed at the erythrocytic stage. Therefore, PkCSP remains as a potential pre-erythrocytic vaccine candidate against *P. knowlesi* infection.

Keywords: Circumsporozoite protein; immunoblot analysis; *Plasmodium knowlesi*; recombinant protein expression.

INTRODUCTION

Plasmodium knowlesi has been the predominant species causing malaria in Malaysia for the past few years. Knowlesi malaria has been reported in several Southeast Asia countries such as Cambodia, Indonesia, Myanmar, the Philippine, Thailand, and Singapore (Luchavez *et al*., 2008; Ng *et al*., 2008; Figtree *et al*., 2010; Jiang *et al*., 2010; Jongwutiwes *et al*., 2011; Khim *et al*., 2011). In addition, the animal hosts, *Macaca fascicularis, Macaca nemestrina,* and *Macaca leonina* as well as the mosquito vector, female *Anopheles* mosquitoes are found in forested and agricultural areas of these regions, thereby increasing the chance of the parasite transmission to human (Moyes *et al*., 2016; Zhang *et al*., 2016; Zaw & Lin, 2019). Knowlesi malaria can develop into potentially fatal complications if not treated immediately (Daneshvar *et al*., 2009).

Circumsporozoite protein (CSP) is a major surface protein of *Plasmodium* sporozoites. The amino acid sequences of CSP across *Plasmodium* species have a well-conserved overall domain structure, with two non-repeat end regions, N- and C-terminal, which flank a central repeat region, which is highly polymorphic among species (Aldrich *et al*., 2012). There are two highly conserved motifs located at the end of each terminal, Region I and II. Region I is a short motif containing 5 amino acids (KLKQP) and it is found in all malaria parasites except for the avian *Plasmodium* species. The motif was suggested to have a role in sporozoite recognition and entry into

hepatocytes. At the C-terminal, region II shares similar sequence to type 1 repeat of thrombospondin. Disruption of this region has shown to impair sporozoite motility and affects sporozoite infectivity to the host (Tewari *et al*., 2002).

CSP plays crucial roles in *Plasmodium* pre-erythrocytic life cycle. It involves in development and motility of the sporozoites in mosquito host, attachment of sporozoites to hepatocytes, and invasion of the hepatocytes (Zhao *et al*., 2016). The protein is one of the main vaccine candidates for *P. falciparum* infection as it is present in large amount on the surface of the sporozoites and has high immunogenicity in human hosts. RTS'S/ AS01 vaccine, commercially known as Mosquirix™ (GlaxoSmithKline Biologicals, United Kingdom) is developed based on *P. falciparum* CSP to prevent invasion of sporozoites into the hepatocytes. The vaccine is currently undergoing phase 4 clinical trial with a few adverse effects being reported (Almeida *et al*., 2021). This vaccine antigen composed of the highly conserved tandem repeat NANP amino acid sequence and the C-terminal region which consists of T- and B-cell epitopes, with hepatitis B surface antigen as the carrier matrix (Almeida *et al*., 2021). Previous clinical trials have proven that this vaccine is effective in reducing severe malaria rates (Laurens, 2020).

Unlike *P. falciparum* CSP, there is no vaccine for *P. knowlesi* infection. Recent studies have identified a few *P. knowlesi* vaccine candidates such as merozoite surface protein 1 (MSP-1), apical membrane antigen 1 (AMA-1), and merozoite surface protein 7D

(MSP7D) (Mahdi Abdel Hamid *et al.*, 2011; Cheong *et al.*, 2013; Ahmed & Quan, 2019). *P. knowlesi* CSP however, was left out of the picture due to its highly polymorphic characteristic at the central repeat region (Lee *et al.*, 2011). Nonetheless, genetic diversity study on PkCSP revealed that the C-terminal non-repeat region of the protein is relatively conserved and hence, it could be beneficial for the development of *P. knowlesi* vaccine (Chong *et al.*, 2020). Studies by Gordon *et al.* (1995) and Young *et al.* (1985) showed that the *P. falciparum* CSP expression in heterologous expression systems could be difficult therefore, it is necessary to know whether PkCSP can be expressed using the common bacterial expression system. In this study, recombinant PkCSP was expressed, and its immunogenic property was confirmed with immunoblot analysis.

MATERIALS AND METHODS

Construction of recombinant PkCSP plasmid
P. knowlesi genomic DNA was extracted from *P. knowlesi* strain A1H1 culture using blood extraction kit (QIAGEN, Hilden, Germany). Primers for *PkCSP* were designed based on the *PkCSP* gene sequence from *P. knowlesi* strain H genome assembly, chromosome 8 (NCBI reference sequence: NC_011909, Location: 1757461-1758552). DNA encoding for the signal peptide of PkCSP (Amino acid position: 1-19) was excluded during the design of primers. *BamHI* restriction sites were introduced to the primer pair design to facilitate cloning. The *CSP* gene was amplified using single-step polymerase chain reaction (PCR) with the primer pair PkCSP FP: 5'-*GGATCC*ACACACTTCGAACATAATG-3' and PkCSP RP: 5'-*GGATCC*TTAATTGAATAATGCTAGGAC-3'. The PCR conditions were as follows: initial denaturing step at 95°C for 4 minutes; 35 cycles at 95°C for 30 seconds, 51°C for 45 seconds, and 72°C for 80 seconds; final elongation step at 72°C for 10 minutes. The PCR product was cloned into pGEM®-T plasmid vector (Promega Corporation, USA). Then, the recombinant plasmid was digested using BamHI restriction enzyme (New England Biolabs, Inc., USA) and subsequently cloned into Novagen® pET-30a(+) (Merck KGaA, Germany, USA) which allows the expression of polyhistidine (His)-tagged recombinant proteins in *E. coli*. Subsequently, the recombinant plasmid was transformed into *Escherichia coli* expression host T7 Express *lysY/Iq* (New England Biolabs, Inc., USA). All the recombinant plasmids constructed were sent for sequencing to verify the gene identity as well as confirming the integrity and orientation of the *PkCSP* gene before expression.

Expression of PkCSP
Single colony T7 host cell containing recombinant *PkCSP* plasmid was inoculated and propagated overnight in Luria-Bertani (LB) broth containing kanamycin (30 µg/ml) and chloramphenicol (35 µg/ml) at 37°C with constant shaking at 250 rpm. Next day, the overnight culture was sub-cultured in LB broth until the optical density at 600 nm (OD_{600}) reached 0.4-0.6. The culture was induced with 1 mM isopropyl β-D-1-thiogalactopyanoside (IPTG). One millilitre of cell fraction was obtained at 2-, 4- and 6-hour post IPTG induction. Cell pellets were harvested by centrifugation at 6500 rpm for 10 minutes and stored at -20°C for SDS-PAGE analysis. After determining the optimal expression time for PkCSP post-IPTG induction, large scale protein expression was carried out and the cell pellets were stored at -80°C for purification purpose.

Purification of recombinant PkCSP
Recombinant PkCSP (rPkCSP) was affinity purified under hybrid condition of the ProBond™ purification system (Invitrogen, USA). Fifty millilitre of cell pellet was resuspended in 8 mL of 6 M guanidinium lysis buffer (pH 7.8) and incubated on ice for 10 min to lyse the cell pellet. Then, the pellet was further lysed by sonication to break the cells until clear lysate was obtained. Purification column containing nickel-NTA (Ni-NTA) agarose resin was prepared under denaturing condition. The His-tagged protein was then allowed to

bind to the Ni-NTA resin on ice for 2 hours. The column was moved onto a stand and the resin was allowed to settle to the bottom of the column. The remaining lysate supernatant was removed, and the resin was washed with denaturing binding buffer (pH 7.8), denaturing wash buffer (pH 6.0) and native wash buffer (pH 8.0) according to the manufacturer's instruction to obtain refolded rPkCSP protein. The purified recombinant PkCSP was eluted with native elution buffer (pH 8.0) which consists of 250 mM imidazole. The purity of recombinant PkCSP was determined via SDS-PAGE and western blot analysis.

Dialysis of PkCSP
The PkCSP protein was dialysed in phosphate buffered saline (PBS). Briefly, the purified recombinant protein was transferred into dialysis tubing with a molecular weight cut-off point of 10 kDa and dialysed against 1X PBS for up to 16 hours at 4°C with 2 buffer changes during the duration of dialysis. The buffer-to-sample volume ratio used was 300:1 to maintain the concentration gradient. Finally, the concentration of the dialysed recombinant PkCSP protein was determined using Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, USA).

SDS-PAGE, Coomassie brilliant blue staining and Western blot
Crude protein lysate and purified recombinant PkCSP were resolved in 12% SDS-PAGE under reducing conditions using β-mercaptoethanol as the reducing agent. The gels were stained with coomassie brilliant blue to reveal the protein bands. Purified recombinant PkCSP was excised from SDS-PAGE gel and sent for protein identification using MALDI/TOF.

Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane and blocked overnight in 5% blocking buffer (1X tris-buffered saline (TBS) containing 5% skimmed milk) at 4°C. The membrane was probed with His-Tag® monoclonal antibody (EMD Millipore Corp., USA) diluted with 2.5% blocking buffer (1:2500 dilution) for one hour at room temperature. The membrane was washed three times with 0.2% TBS-T (1X TBS containing 0.2% Tween-20) and incubated with biotin-labelled goat anti-mouse IgG (1:2500 dilution) for one hour, followed by alkaline phosphatase-conjugated streptavidin (1:2500 dilution) for one hour. Finally, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) was added in the dark to develop colour.

Animal ethics
The animal use protocol was reviewed and approved by the Faculty of Medicine, Institutional Animal Care and Use Committee, Universiti Malaya (2019-201010/PARA/R/TJH).

Mice immunization
Six- to eight-week-old female BALB/c mice were used for immunization (n=3). Purified PkCSP, 30 µg, was mixed with complete freund's adjuvant at 1:1 ratio and the mixture were injected into mice as primary immunization (day 0). For booster injections, 30 µg of purified PkCSP mixed with incomplete freund's adjuvant and administered on day 14 and 21. All injections were given subcutaneously. Serum of each mouse was collected on day 0, 14, 21 and 31.

Determination of endpoint titre of mice sera
Indirect ELISA was carried out to determine the endpoint titre of day 31 post-immunization mice antisera. The mice antisera were serially diluted by 2-fold starting from 1:400 dilution to 1:819200 dilution in 1% BSA/PBS. Cut-off value was set to be mean+2SD of the pre-immune serum of each mouse. Horseradish peroxidase-labelled anti-mouse IgG + IgM (1:2500 dilution) was used as the enzyme-conjugated secondary antibodies. Antiserum from the most reactive mouse was used in immunoblot analysis of the *P. knowlesi* total protein extract and the expressed recombinant PkCSP.

P. knowlesi total protein extract preparation and immunoblot analysis

Erythrocytic stage *P. knowlesi* total protein extract was extracted from *P. knowlesi* strain A1H1 culture (5% haematocrit, 5% parasitaemia) according to Cooper (2002). Briefly, 5 ml parasite culture was washed with 45 ml PBS for three times at 800 × *g*, 5 mins at 4°C. Red blood cells were lysed with 0.01% saponin in PBS and rocked on ice for 10 mins before washing with PBS. Finally, the parasite extract was pelleted at 1800 x *g* for 10 mins and resuspended in 1X SDS sample buffer for SDS-PAGE.

PkA1H1 total protein extract was loaded to the wells of SDS-PAGE gel along with pET-30a(+) lysate, crude recombinant PkCSP lysate, and purified recombinant PkCSP. The resolved proteins were stained with coomassie brilliant blue for visualisation of the protein bands. The separated proteins were transblotted onto PVDF membrane and the western blot assay was carried out using recombinant PkCSP mouse antiserum (1:250 dilution) to probe the blocked membrane. Biotin-labelled goat anti-mouse IgG + IgM (1:2500 dilution) was used as a secondary antibody. Following this, the membrane was incubated in alkaline phosphatase-conjugated streptavidin (1:2500 dilution) for one hour. Lastly, the chromogenic substrate NBT/BCIP was added to the membrane. The colour was allowed to develop at room temperature in dark.

RESULTS

Cloning and sequencing of the recombinant PkCSP

The amplified *PkCSP* fragment from *P. knowlesi* strain A1H1 genomic DNA has a size of 1047 bp (including RE cutting site *GGATCC*). The sequenced gene was analysed using Standard Nucleotide BLAST (BLASTn) and showed a percentage identity of 99% when compared to the reference gene, confirming the identity of the amplified gene sequence as *P. knowlesi CSP* gene. Nucleotide sequence alignment with the reference gene was shown in Supplementary file 1. Amino acid sequence of the recombinant PkCSP was deduced from the nucleotide sequence using BioEdit software. Region I and II motifs, central repeat region, and the T-cell epitopes in the C-terminal region were identified from the deduced amino acid sequence (see Supplementary file 2).

Expression and purification of recombinant PkCSP

With the optimal expression condition, the culture lysate at 4 hours post-IPTG induction showed thicker band at approximately 55 kDa when compared to the non-recombinant plasmid control (Figure 1A).
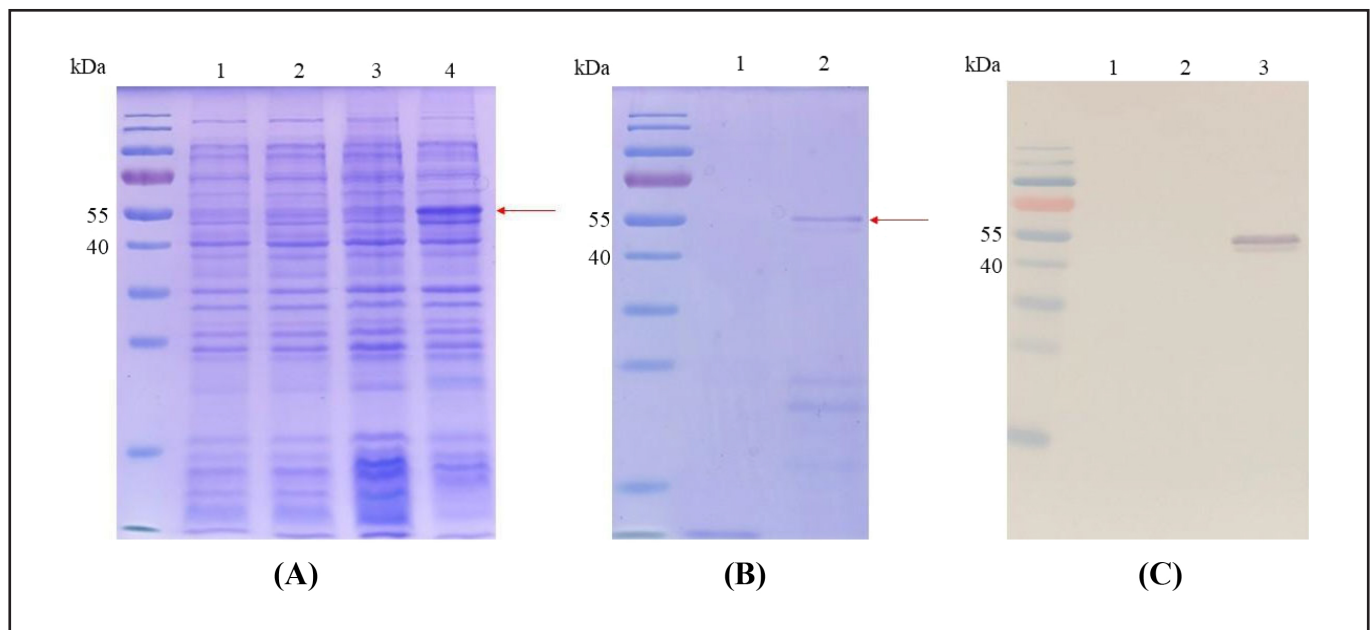
Recombinant PkCSP was purified under hybrid condition using Ni-NTA column. The purified protein was resolved in SDS-PAGE. Western blot assay using His-Tag® monoclonal antibody revealed the recombinant PkCSP protein band at ~55 kDa whereas the non-recombinant plasmid control did not show any band (Figure 1B). The recombinant PkCSP protein band excised from the SDS-PAGE was confirmed to be *P. knowlesi* protein by MALDI/TOF (see Supplementary file 3). Quantification of recombinant PkCSP indicated a concentration of protein that is ranged from 0.1 mg/ml to 0.3 mg/ml for a 200 mL culture.

#### Determination of endpoint titre of mice antisera

Endpoint titre is the reciprocal of the highest sample dilution that gives a positive reaction, which is indicated by an absorbance reading above the cut-off value (Frey *et al.*, 1998). In this study, endpoint titres of the recombinant PkCSP mice antisera were determined by indirect ELISA assay. Overall, high antibody response towards PkCSP was observed, with the endpoint titre ranging between 1:204800 and 1:819200 dilution (Figure 2). Mouse 3 (M3) had the highest endpoint titre (1:819200 dilution), hence the antiserum from M3 was used for immunoblot analysis.

Immunoblot analysis of recombinant PkCSP and *P. knowlesi* total protein extract using recombinant PkCSP mouse antiserum

The antiserum collected from the mouse immunized with recombinant PkCSP protein was probed against recombinant PkCSP protein and *P. knowlesi* total protein extract. The results of the assay showed that the antiserum recognized recombinant PkCSP protein at ~55 kDa (Figure 3B, lane 2 and 3, arrows). Low cross-reactivity with *E. coli* proteins was observed (Figure 3B, lane 1) with minimal nonspecific bands observed. There was no band seen in the *P. knowlesi* total protein extract.

Figure 1. Recombinant PkCSP expressed using T7 expression system and purified with ProBond™ purification system. (A) Recombinant PkCSP was observed at ~55 kDa 4 hours post-IPTG induction (lane 4, arrow) under coomassie brilliant blue stained SDS-PAGE gel. Lanes 1 and 3 are pET-30a(+) at 0 and 4 hours respectively; lanes 2 and 4 are recombinant PkCSP at 0 and 4 hours respectively. (B) Coomassie brilliant blue stained SDS-PAGE gel showing purified pET-30a(+) (lane 1) and purified recombinant PkCSP (lane 2, arrow). (C) Western blot assay showing recombinant PkCSP was probed with His-Tag monoclonal antibodies at ~55 kDa. Lane 1: empty lane; lane 2: purified pET-30a(+); lane 3: purified recombinant PkCSP.
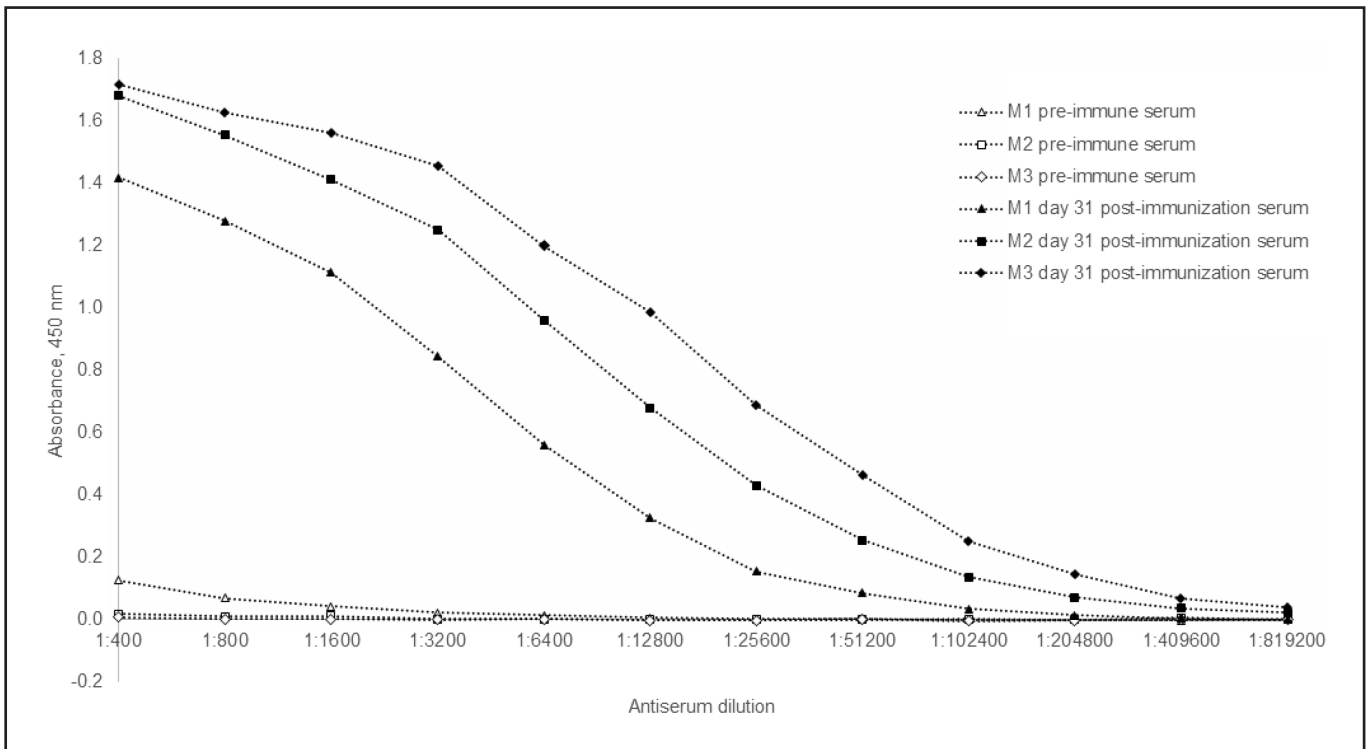
Figure 2. Endpoint titre assay for day 31 post-immunization mice antisera. High endpoint titres were observed for all three mice ranging between 1:204800 and 1:819200 dilution, with mouse 3 (M3) having the highest endpoint titre. Note: M = mouse.

(A)   **(B)**
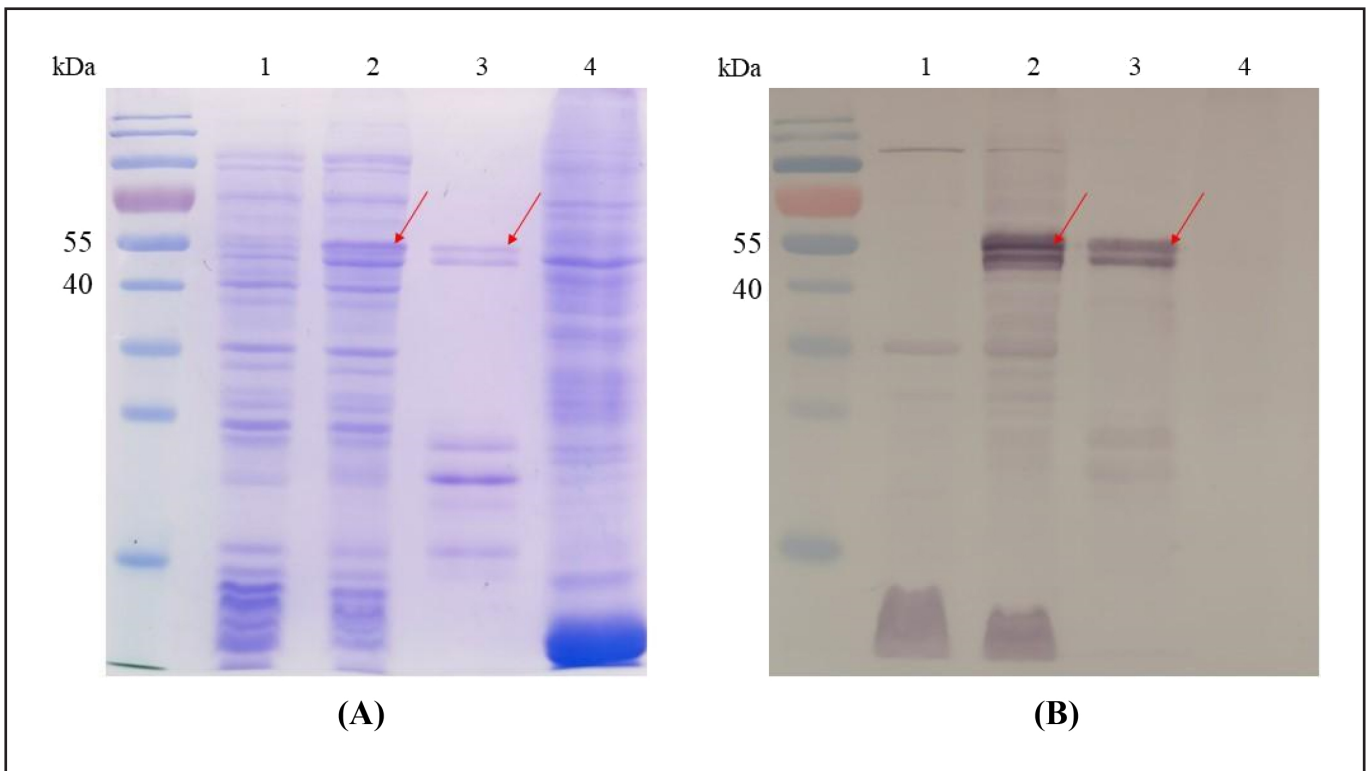
Figure 3. Immunoblot analysis of recombinant PkCSP and *P. knowlesi* total protein extract using recombinant PkCSP mouse antiserum. (A) Proteins were resolved in 12% SDS-PAGE and stained with coomassie brilliant blue to reveal the protein bands. (B) Western blot assay showing bands that were probed with recombinant PkCSP mouse antiserum (1:250 dilution). In both A and B, lanes 1-4 represent pET-30a(+) lysate, crude recombinant PkCSP lysate, purified recombinant PkCSP and *P. knowlesi* total protein extract. Red arrows indicate the recombinant PkCSP protein bands.

DISCUSSION

In this study, *E. coli* expression system was chosen to express PkCSP as the method is simple, cost-effective, and able to express high yield of recombinant protein. This study showed that PkCSP, which consists of twelve 12-amino acid repeat units can be expressed using the *E. coli* expression system. Signal peptide of PkCSP was removed in the construction of recombinant *PkCSP* plasmids to facilitate the expression of the protein (Vedadi *et al.*, 2007). Purification of protein was performed in hybrid condition, thereby exposing the protein to denaturing conditions and then to native conditions to refold the PkCSP protein. The expressed recombinant PkCSP appeared to be ~15 kDa larger than its theoretical mass when resolved in SDS-PAGE. This may be due to the presence of protein complexes that are not fully disrupted even in the presence of reducing agents. A similar scenario was observed by Plassmeyer *et al.* (2009) in which the recombinant *P. falciparum* CSP expressed had a molecular mass larger than the theoretical mass due to the incomplete separation of the protein complexes.

The purified recombinant PkCSP was used to immunized mice. Endpoint titre assay showed that all three mice immunized with the recombinant PkCSP produced high level of immune response against the recombinant PkCSP. The endpoint titres of the mice antisera were ranged from 1:204800 to 1:819200 dilution. This indicates that PkCSP is highly immunogenic, hence, the protein elicited strong immune response in animal models.

Immunoblot analysis using the most reactive PkCSP mouse antiserum showed that the antiserum recognized the PkCSP protein at 55 kDa, validating that the antibodies in the serum can bind to the epitopes of the linearized PkCSP protein. The immunoblot analysis also showed that the antibodies in the PkCSP mouse antiserum do not recognize any protein from the resolved *P. knowlesi* total protein extract, which contains *Plasmodium* parasites of different stages except for pre-erythrocytic stages. This is in line with the nature of the protein in which it is found expressed only in sporozoite surface.

In conclusion, recombinant PkCSP was successfully expressed using *E. coli* expression system. Antiserum produced against the recombinant protein was able to recognize the protein under ELISA and western blot assay. Further immunoblot analysis against *P. knowlesi* strain A1H1 extract showed that the PkCSP mouse antiserum could not recognize any protein from *P. knowlesi* strain A1H1 erythrocyte protein extract indicating that it is not expressed at the erythrocytic stage. The limitation in the study is that immunoblot analysis could not be performed on native PkCSP of the sporozoites. Current available *P. knowlesi* culture-adapted line (A1H1) was unable to produce gametocytes (Moon *et al.*, 2013; Zeeman *et al.*, 2013). UM01, a *P. knowlesi* culture-adapted line was able to produce gametocytes however, failed to infect mosquito (Amir, 2016). Therefore, it is difficult to obtain sporozoites for this study. Besides, future study could also examine T cell response of PkCSP immunized mice which was not included in this study due to limited number of mice. Nonetheless, it is worthwhile to evaluate the species-specific protective capacity of PkCSP antibodies against *P. knowlesi* infection as immunity response induced with CSP of other species may not confer similar protection against *P. knowlesi* infection and vice versa (Nussenzweig & Nussenzweig, 1984).

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Competing interests

The authors declare that they have no competing interests.

REFERENCES

Ahmed, M.A. & Quan, F-S. (2019). *Plasmodium knowlesi* clinical isolates from Malaysia show extensive diversity and strong differential selection pressure at the merozoite surface protein 7D (MSP7D). *Malaria Journal* **18**: 1-11. https://doi.org/10.1186/s12936-019-2782-2

Aldrich, C., Magini, A., Emiliani, C., Dottorini, T., Bistoni, F., Crisanti, A. & Spaccapelo, R. (2012). Roles of the amino terminal region and repeat region of the *Plasmodium berghei* circumsporozoite protein in parasite infectivity. *PLoS ONE* **7**: e32524. https://doi.org/10.1371/journal.pone.0032524

Almeida, M.E.M.d., Vasconcelos, M.G.S.d., Tarragô, A.M. & Mariúba, L.A.M. (2021). Circumsporozoite Surface Protein-based malaria vaccines: a review. *Revista do Instituto de Medicina Tropical de São Paulo* **63**: e11. https://doi.org/10.1590/S1678-9946202163011

Amir, A. (2016). Dissertation: Establishment of a new line of Plasmodium knowlesi. University Malaya.

Cheong, F.W., Fong, M.Y., Lau, Y.L. & Mahmud, R. (2013). Immunogenicity of bacterial-expressed recombinant *Plasmodium knowlesi* merozoite surface protein-1$_{42}$ (MSP-1$_{42}$). *Malaria Journal* **12**: 454. https://doi.org/10.1186/1475-2875-12-454

Chong, E.T.J., Neoh, J.W.F., Lau, T.Y., Lim, Y.A-L., Chai, H.C., Chua, K.H. & Lee, P-C. (2020). Genetic diversity of circumsporozoite protein in *Plasmodium knowlesi* isolates from Malaysian Borneo and Peninsular Malaysia. *Malaria Journal* **19**: 377. https://doi.org/10.1186/s12936-020-03451-x

Cooper, R.A. (2002). SDS-PAGE and western blotting of *Plasmodium falciparum* proteins. *Methods in Molecular Medicine* **72**: 177-188. https://doi.org/10.1385/1-59259-271-6:177

Daneshvar, C., Davis, T.M., Cox-Singh, J., Rafa'ee, M.Z., Zakaria, S.K., Divis, P.C.S. & Singh, B. (2009). Clinical and laboratory features of human *Plasmodium knowlesi* infection. *Clinical Infectious Diseases* **49**: 852-860. https://doi.org/10.1086/605439

Figtree, M., Lee, R., Bain, L., Kennedy, T., Mackertich, S., Urban, M., Cheng, Q. & Hudson, B.J. (2010). *Plasmodium knowlesi* in human, Indonesian Borneo. *Emerging Infectious Diseases* **16**: 672-674. https://doi.org/10.3201/eid1604.091624

Frey, A., Di Canzio, J. & Zurakowski, D. (1998). A statistically defined endpoint titer determination method for immunoassays. *Journal of Immunological Methods* **221**: 35-41. https://doi.org/10.1016/S0022-1759(98)00170-7

Gordon, D.M., McGovern, T.W., Krzych, U., Cohen, J.C., Scheneider, I., LaChance, R., Heppner, D.G., Yuan, G., Hollingdale, M., Slaoui, M. *et al.* (1995). Safety, immunogenicity, and efficacy of a recombinantly produced *Plasmodium falciparum* circumsporozoite protein-hepatitis B surface antigen subunit vaccine. *The Journal of Infectious Diseases* **171**: 1576-1585. https://doi.org/10.1093/infdis/171.6.1576

Jiang, N., Chang, Q., Sun, X., Lu, H., Yin, J., Zhang, Z., Wahlgren, M. & Chen, Q. (2010). Co-infections with *Plasmodium knowlesi* and other malaria parasites, Myanmar. *Emerging Infectious Diseases* **16**: 1476-1478. https://doi.org/10.3201/eid1609.100339

Jongwutiwes, S., Buppan, P., Kosuvin, R. Seethamchai, S., Pattanawong, U., Sirichaisinthop, J. & Putaporntip, C. (2011). *Plasmodium knowlesi* malaria in humans and macaques, Thailand. *Emerging Infectious Diseases* **17**: 1799-1806. https://doi.org/10.3201/eid1710.110349

Khim, N., Siv, S., Kim, S., Mueller, T., Fleischmann, E., Singh, B., Divis, P.C.S., Steenkeste, N., Duval, L., Bouchier, C. *et al.* (2011). *Plasmodium knowlesi* infection in humans, Cambodia, 2007–2010. *Emerging Infectious Diseases* **17**: 1900-1902. https://doi.org/10.3201/eid1710.110355

Laurens, M.B. (2020). RTS, S/AS01 vaccine (Mosquirix™): an overview. *Human Vaccines & Immunotherapeutics* **16**: 480-489. https://doi.org/10.1080/21645515.2019.1669415

Lee, K-S., Divis, P.C., Zakaria, S.K., Matusop, A., Julin, R.A., Conway, D.J., Cox-Singh, J. & Singh, B. (2011). *Plasmodium knowlesi*: reservoir hosts and tracking the emergence in humans and macaques. *PLoS Pathogens* **7**: e1002015. https://doi.org/10.1371/journal.ppat.1002015

Luchavez, J., Espino, F., Curameng, P., Espina, R., Bell, D., Chiodini, P., Nolder, D., Sutherland, C., Lee, K-S. & Singh, B. (2008). Human infections with *Plasmodium knowlesi*, the Philippines. *Emerging Infectious Diseases* **14**: 811-813. https://doi.org/10.3201/eid1405.071407

Mahdi Abdel Hamid, M., Remarque, E.J., van Duivenvoorde, L.M., van der Werff, N., Walraven, V., Faber, B.W., Kocken, C.H.M. & Thomas, A.W. (2011). Vaccination with *Plasmodium knowlesi* AMA1 formulated in the novel adjuvant co-vaccine HT™ protects against blood-stage challenge in rhesus macaques. *PLoS ONE* **6**: e20547. https://doi.org/10.1371/journal.pone.0020547

Moon, R.W., Hall, J., Rangkuti, F., Ho, Y.S., Almond, N., Mitchell, G.H., Pain, A., Holder, A. A. & Blackman, M.J. (2013). Adaptation of the genetically tractable malaria pathogen *Plasmodium knowlesi* to continuous culture in human erythrocytes. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 531-536. https://doi.org/10.1073/pnas.1216457110

Moyes, C.L., Shearer, F.M., Huang, Z., Wiebe, A., Gibson, H.S., Nijman, V., Mohd-Azlan, J., Brodie, J.F., Malaivijitnond, S., Linkie, M. *et al*. (2016). Predicting the geographical distributions of the macaque hosts and mosquito vectors of *Plasmodium knowlesi* malaria in forested and non-forested areas. *Parasites & Vectors* **9**: 242. https://doi.org/10.1186/s13071-016-1527-0

Ng, O.T., Ooi, E.E., Lee, C.C., Lee, P.J., Ng, L.C., Pei, S.W., Tu, T.M., Loh, J.P. & Leo, Y.S. (2008). Naturally acquired human *Plasmodium knowlesi* infection, Singapore. *Emerging Infectious Diseases* **14**: 814-816. https://doi.org/10.3201/eid1405.070863

Nussenzweig, R.S. & Nussenzweig, V. (1984). Development of sporozoite vaccines. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences.* **307**: 117-128. https://doi.org/10.1098/rstb.1984.0113

Plassmeyer, M.L., Reiter, K., Shimp Jr, R.L., Kotova, S., Smith, P.D., Hurt, D.E., House, B., Zou, X., Zhang Y, Hickman, M. *et al*. (2009). Structure of the *Plasmodium falciparum* circumsporozoite protein, a leading malaria vaccine candidate. *Journal of Biological Chemistry* **284**: 26951-26963. https://doi.org/10.1074/jbc.M109.013706

Tewari, R., Spaccapelo, R., Bistoni, F., Holder, A.A. & Crisanti, A. (2002). Function of region I and II adhesive motifs of *Plasmodium falciparum* circumsporozoite protein in sporozoite motility and infectivity. *Journal of Biological Chemistry* **277**: 47613-47618. https://doi.org/10.1074/jbc.M208453200

Vedadi, M., Lew, J., Artz, J., Amani, M., Zhao, Y., Dong, A., Wasney, G.A., Gao, M., Hills, T., Brokx, S. *et al*. (2007). Genome-scale protein expression and structural biology of *Plasmodium falciparum* and related Apicomplexan organisms. *Molecular and Biochemical Parasitology* **151**: 100-110. https://doi.org/10.1016/j.molbiopara.2006.10.011

Young, J.F., Hockmeyer, W.T., Gross, M., Ballou, W.R., Wirtz, R.A., Trosper, J.H., Beaudoin, R.L., Hollingdale, M.R., Miller, L.H., Diggs, C.L. *et al*. (1985). Expression of *Plasmodium falciparum* circumsporozoite proteins in *Escherichia coli* for potential use in a human malaria vaccine. *Science* **228**: 958-962. https://doi.org/10.1126/science.2988125

Zaw, M.T. & Lin, Z. (2019). Human *Plasmodium knowlesi* infections in South-East Asian countries. *Journal of Microbiology, Immunology and Infection* **52**: 679-684. https://doi.org/10.1016/j.jmii.2019.05.012

Zeeman, A.M., der Wel, A.V. & Kocken, C.H.M. (2013). Ex vivo culture of *Plasmodium vivax* and *Plasmodium cynomolgi* and in vitro culture of *Plasmodium knowlesi* blood stages. *Methods in Molecular Biology*. **923**: 35-49. https://doi.org/10.1007/978-1-62703-026-7_4

Zhang, X., Kadir, K.A., Quintanilla-Zariñan, L.F., Villano, J., Houghton, P., Du, H., Singh, B. & Smith, D.G. (2016). Distribution and prevalence of malaria parasites among long-tailed macaques (*Macaca fascicularis*) in regional populations across Southeast Asia. *Malaria Journal* **15**: 450. https://doi.org/10.1186/s12936-016-1494-0

Zhao, J., Bhanot, P., Hu, J. & Wang, Q. (2016). A comprehensive analysis of *Plasmodium* circumsporozoite protein binding to hepatocytes. *PLoS ONE* **11**: e0161607. https://doi.org/10.1371/journal.pone.0161607

SUPPLEMENTARY DATA

https://msptm.org/files/Vol39No2/tb-39-2-004-Tan-J-H-supplementary-data.pdf