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RESEARCH ARTICLE

Inhibition of *Plasmodium knowlesi* merozoite invasion into human erythrocytes by antibodies raised against the parasite's secreted protein with altered thrombospondin repeat (SPATR)

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ABSTRACT

The *Plasmodium* secreted protein with an altered thrombospondin repeat (SPATR) has been known to play an important role in the malaria parasite's invasion into host erythrocytes. This protein is immunogenic and has been considered as one of the potential vaccine candidates against malaria parasite infection. Thus far, only a handful immunological studies have been carried out on *P. knowlesi* SPATR (PkSPATR), and none of these studies investigated the immunoprotective properties of the protein. In the present study, the ability of anti-PkSPATR antibodies to inhibit invasion of human erythrocytes was assessed in an *in vitro* merozoite invasion inhibition assay. The antibodies were harvested from the serum of a rabbit which was immunised with recombinat PkSPATR. Results from the merozoite invasion inhibition assay revealed significant antibody invasion inhibitory activity in a concentration dependent manner (concentration range: 0.375 – 3.00 mg/ml) with inhibition rate ranging from 20% to 32%. Future studies, such as anti-PkSPATR antibodies inhibitory effect on sporozoite invasion of human liver cells, need to be carried out to assess the potential of PkSPATR as a knowlesi malaria vaccine candidate.

Keywords: *Plasmodium knowlesi*; SPATR; antibody; merozoite invasion inhibition assay.

INTRODUCTION

The simian malaria parasite *Plasmodium knowlesi* is presently the main cause of human malaria infections in Malaysia. The cumulative number of human knowlesi malaria cases in Malaysia in 2017-2021 reached 17,125 with 48 deaths (WHO, 2022). The abundant distribution of the host macaques and *Anopheles* mosquito vectors contribute to this high rate of transmission in the country (Naserrudin *et al*., 2022). Human *P. knowlesi* infections are also reported in other countries in Southeast Asia except Timor Leste. *P. knowlesi* is unique to other *Plasmodium* species as it has a quotidian 24-h erythrocytic cycle that results in rapid increase in blood parasitaemia, and may sometimes produce severe malaria in humans (Eka *et al*., 2022).

The *Plasmodium* merozoite is characterized by the presence of secretory organelles at the apical membrane, namely the micronemes, dense granules and rhoptries. These secretory organelles aid in the parasite invasion and survival inside the host cells (Preiser *et al*., 2000). The proteins expressed by these organelles play important roles in the host-cell recognition, selection of target cells and facilitating parasite invasion into the host cell. Some of these proteins have been studied for their immunological properties and have been proven to be immunogenic at varying degrees.

The secreted protein with altered thrombospondin repeat (SPATR) is a conserved protein in apicomplexan parasites (Naitza *et al*., 1998). The SPATR was initially found in the murine malaria parasite *Plasmodium yoelii*. Subsequently, SPATR orthologues were discovered in other *Plasmodium* species. Basically, SPATR contains an altered version of the thrombospondin repeat (TSR) domain that plays a significant role in sporozoite motility, host-cell attachment and invasion. It also possesses a cysteine-rich region known as Type II EGF-like domain that is involved in host cell recognition (Curtidor *et al*., 2008). SPATR is expressed at multiple stages of the malaria parasite life cycle, and its function at different stages of the life cycle has been characterized (Chattopadhyay *et al*., 2003). Immunofluorescence analysis revealed the localization of this protein on the sporozoite surface (Mahajan *et al*., 2005), around the rhoptries, and on the membrane of infected erythrocytes (Chattopadhyay *et al*., 2003; Mahajan *et al*., 2005).

SPATR has been shown to be immunogenic, and antibodies produced against it have been shown to inhibit malaria parasite sporozoite invasion into the host cell (Chattopadhyay *et al*., 2003). High-activity binding peptides (HABPs) from *Plasmodium falciparum* SPATR (PfSPATR) have been demonstrated to bind to erythrocytes and inhibit *in vitro* invasion of merozoites (Curtidor *et al*., 2008). Studies on *Plasmodium berghei* SPATR (PbSPATR) revealed the importance of the protein in blood stage development. PbSPATR-deficient hepatic merozoites are dispensable from hepatocytes but result in unsuccessful development of blood stage infection (Gupta *et al*., 2020).

The increasing number of human knowlesi cases is a worrying trend. This should prompt the development of efficient rapid diagnostic tools and ultimately a possible competent vaccine capable of providing immunity against the parasite infection. While there have been many immunological studies demonstrating the importance of SPATR *Plasmodium* orthologues, not many have been reported for *P. knowlesi* SPATR (PkSPATR). The only studies thus far, described the binding of anti-PkSPATR antibodies from pooled infected *Rhesus* monkey sera to recombinant PkSPATR in an enzyme-linked immunosorbent assay (Mahajan *et al*., 2005), and the use of recombinant PkSPATR as a biomarker for serodiagnosis of human knowlesi malaria (Palaeya *et al*., 2013). Our present study went one step further, in determining the immune-protectivity property of anti-PkSPATR antibodies in an *in vitro* merozoite invasion inhibition assay.

MATERIALS AND METHODS

Ethic approval

Ethical approval for human blood collection was granted by the Medical Research Committee of the Ministry of Health, Malaysia (NMRR-15-67223975). The experimental protocol involving the use of animal was approved by the Faculty of Medicine Institutional Animal Care and Use Committee, Universiti Malaya (2019-220903/PARA/R/UWA).

Construction of recombinant PkSPATR exon II [(PkSPATR(II)] plasmid

Firstly, genomic DNA was extracted from 100 µl of a *P. knowlesi*-infected human blood sample using DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany). The exon II of PkSPATR gene was PCR amplified by employing the primer set PkSPATRII F: 5'-TGCTGGTGCCCTAG-3' and PkSPATRII R: 5'-GCGACCAATCAGAATTAA-3' (GenBank Accession AY952327.1:233-785). The thermal cycler condition was performed at initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 1 min and extension at 72°C for 1 min. Lastly, the reaction was terminated at final extension 72°C for 8 min. The amplified PCR products were purified using QIAquick PCR purification kit (QIAGEN, Germany) according to the manufacturer's instructions. Purified PCR products were cloned into the cloning vector pGEM®-T (Promega Corp., USA) and plasmid of positive recombinant clones were digested using the restriction enzyme *Bam*HI. Then, the digested plasmid were ligated into the expression vector pET-30a(+) (Merck Millipore, USA) and transformed into the *Escherichia coli* expression host T7 Express *lysY*/Iq (New England Biolabs, Inc., USA).

Expression and purification of recombinant PkSPATR(II) protein

A positive recombinant T7 Express *lysY*/Iq clone containing the PkSPATR(II) was propagated in Luria-Bertani (LB) medium supplemented with chloramphenicol (final concentration: 34 µg/ml) and kanamycin (final concentration: 30 µg/ml) overnight at 250 rpm and 37°C. Non-recombinant clone containing empty pET30a(+) vector was used as control for expression study. The overnight cultures were diluted until optical density at 600 nm (OD_{600}) was 0.1 and the prepared cultures were propagated until OD_{600} reached 0.4-0.6. Then, expression was induced by adding 100 µl of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution and the cultures were incubated at 37°C and 250 rpm for 0 to 4 h. After 4 h, the cells were pelleted and harvested by centrifugation at 6500 rpm for 10 min.

Expressed recombinant PkSPATR(II) was purified using the nickel-NTA agarose resin in hybrid condition following the protocol from Probond™ Purification System (QIAGEN, Germany). Prior to purification, the bacterial cell lysate was prepared by resuspending the pelleted cells in a pre-warmed 8 mL guanidine lysis buffer (pH 7.8). The cell suspension was incubated for 10 min at room temperature. Then, the protein lysate was sonicated at 75% amplitude, with 15 s pulse and 30 s interval rest until the lysate turned into clear suspension. The cell suspension was centrifuged at 5000 rpm for 15 min at 4°C and supernatant was harvested. The supernatant was added into the prepared propylene purification column (Qiagen, Germany) containing the nickel-NTA agarose resin and incubated for 1 h. The resin was washed with a denaturing binding buffer (pH 7.8) twice, followed by twice washing with denaturing wash buffer (pH 6.0) containing 8 M urea. Next, the resin was washed for 4 times with 8 ml of native wash buffer (pH 8.0) and lastly, the purified recombinant PkSPATR(II) was eluted with native elution buffer (pH 8.0) containing 250 mM imidazole.

The protein concentration was measured using Bradford assay (Bio-Rad, USA). The purified protein was observed in a SDS-PAGE gel and sent for matrix-assisted MALDI-TOF to confirm the protein identity. Prior to immunisation, purified proteins were subjected to dialysis in 1X PBS.

Rabbit immunisation

New Zealand White rabbits, aged 14-16 weeks old, were used in the immunization. The rabbits was separately immunized with recombinant PkSPATR(II) and purified nonrecombinant pET-30a(+) protein as the negative control. The protein, 100 µg, prepared in complete Freund's adjuvant (1:1, v/v), was administered into the rabbit via subcutaneous injection. In the subsequent boosters (weeks 4, 8, and 12), the protein was prepared in incomplete Freund's adjuvant (1:1, v/v). Post week 14 of the immunization, the rabbit was anaesthetized using ketamine and xylazine. Cardiac puncture was performed to obtain large volume of blood. The rabbit was euthanized by administering pentobarbital through the cardiac puncture. Serum was recovered from the blood by centrifugation at 2000 r.p.m. for 10 min.

Immunofluorescence assay (IFA)

An IFA was performed to demonstrate specific interaction between anti-PkSPATR(II) antibodies in the immunised rabbit serum with *P. knowlesi* (strain A1H1) merozoites in infected erythrocytes (schizont stage). Briefly, a thin blood smear of a *P. knowlesi*-infected erythrocyte culture was prepared on a microscope slide. The smear was fixed with cold acetone-methanol (9:1, v/v) for 15 min at -20°C. The slide was air-dried, rehydrated with 1X PBS and washed three times with 1X PBS. The slide was blocked with a blocking buffer (3% BSA in PBS) for 1 h at room temperature. The blocking buffer was removed from the slide, and rabbit serum (1:100 dilution with 3% BSA in PBS) was added to the slide and incubated for 1 h at 37°C in a humidified incubator. The slide was washed three times with 1X PBS, followed by incubation with FITC-labeled anti-rabbit IgG (KPL Inc., USA) (1:100 dilution in 3% BSA in PBS) for 1 h at 37°C in a dark humidified incubator. The slide was then washed three times. Lastly, the smear was incubated with DAPI Antifade solution (Merck Millipore Corp., USA) (1:100 dilution) for 15 min at room temperature under dark conditions. The slide was washed three times with 1X PBS and let dry. The dried slide was examined under a fluorescence microscope at 100X magnification using a BX51-FL-CCD microscope (Olympus, Japan).

Affinity purification of anti-PkSPATR(II) antibodies from rabbit serum

The anti-PkSPATR(II) antibodies were purified using the plate-based affinity purification method. Purified recombinant PkSPATR(II) protein [50 µg/ml in 0.05 M sodium bicarbonate (pH 9.6)] was used to coat the purification plate well. The pre-coated plate was blocked with 100 µl of 1% BSA in TBS for 1 h at 4°C. The blocking buffer was then removed, and the plate was washed for 5 times using 0.2% TBS-T. Then, 100 µl of rabbit serum was added into the well and

incubated for 2 h at room temperature. Next, the plate was washed for 8 times before proceeding to elution with 0.2 M glycine-HCl in 1 mg/ml of BSA (pH 2.2). The elution mixture was incubated for 20 min before 15 µl of neutralization buffer [1 M Tris-HCl (pH 9.1)] was added into the well. The harvested purified antibody was dialyzed against 1X PBS. The antibody concentration was measured using a NanoDrop spectrophotometer at 280 nm wavelength (Thermo ScientificTM, USA). Antibodies from the serum of rabbit immunized with nonrecombinant pET-30a(+) were similarly purified and were used as negative control in the subsequent assay.

Merozoite invasion inhibition assay

The *P. knowlesi* (strain A1H1) culture was grown in human erythrocytes and RPMI-1640 complete medium supplemented with 10% heat-inactivated horse serum (Thermo Scientific™, USA) at 37°C and humidified atmosphere of 90% N_2, 5% CO_2, and 5% O_2. The culture was grown until it was abundant with late-stage parasites at approximately 3-5% parasitaemia. Prior to the assay, synchronization was performed using Histodenz to obtain late stage schizonts. The synchronized culture was plated into 96-well plates at 1% parasitaemia and 2% haematocrit (180 µl/well). Then, 20 µl of serially diluted purified antibodies (0.0234 mg/ml to 3.000 mg/ml) in complete RPMI1640 medium, were added into the culture. Complete medium (20 µl) were added as negative control. Duplicates were performed for every antibody concentration. The culture was maintained at 37°C, 90% N_2, 5% CO_2, and 5% O_2 for 8-10 h. Then, the culture was harvested and Giemsa-stained thin smears were prepared for the determination of ring parasitaemia. The slides were observed under a compound microscope at 100X magnification. The ring stage parasites were counted and the parasitaemia was calculated to determine the invasion percentage (%). The invasion inhibition rate the anti-PkSPATR antibodies was determined as follows:

Invasion inhibition rate:

$$\frac{\text{(parasitaemia of negative control culture – parasitaemia of antibody treated culture)}}{\text{parasitaemia of negative control culture}} \times 100\%$$

where parasitaemia is:

$$\frac{\text{number of parasite-infected erythrocytes in the culture}}{\text{total number of erythrocytes}} \times 100\%$$

The assay was repeated five times. Normality test was performed to determine for the statistical analysis. The statistical significance of inhibition between different concentrations of antibodies and control (0 mg/ml) was determined using Dunn's multiple comparisons test in Graph pad prism. Statistical difference values of $P \leq 0.05$ were considered significant.

RESULTS

Expression and purification of recombinant PkSPATR(II)

The PkSPATR(II) was successfully cloned and expressed. SDS-PAGE analysis of the purified recombinant PkSPATR(II) showed a major band with the expected molecular mass of ~25 kDa (Figure 1A). The protein was affinity-purified to produce a clear single ~25 kDa protein (Figure 1B). No band was obtained for the control pET-30a(+) vector.

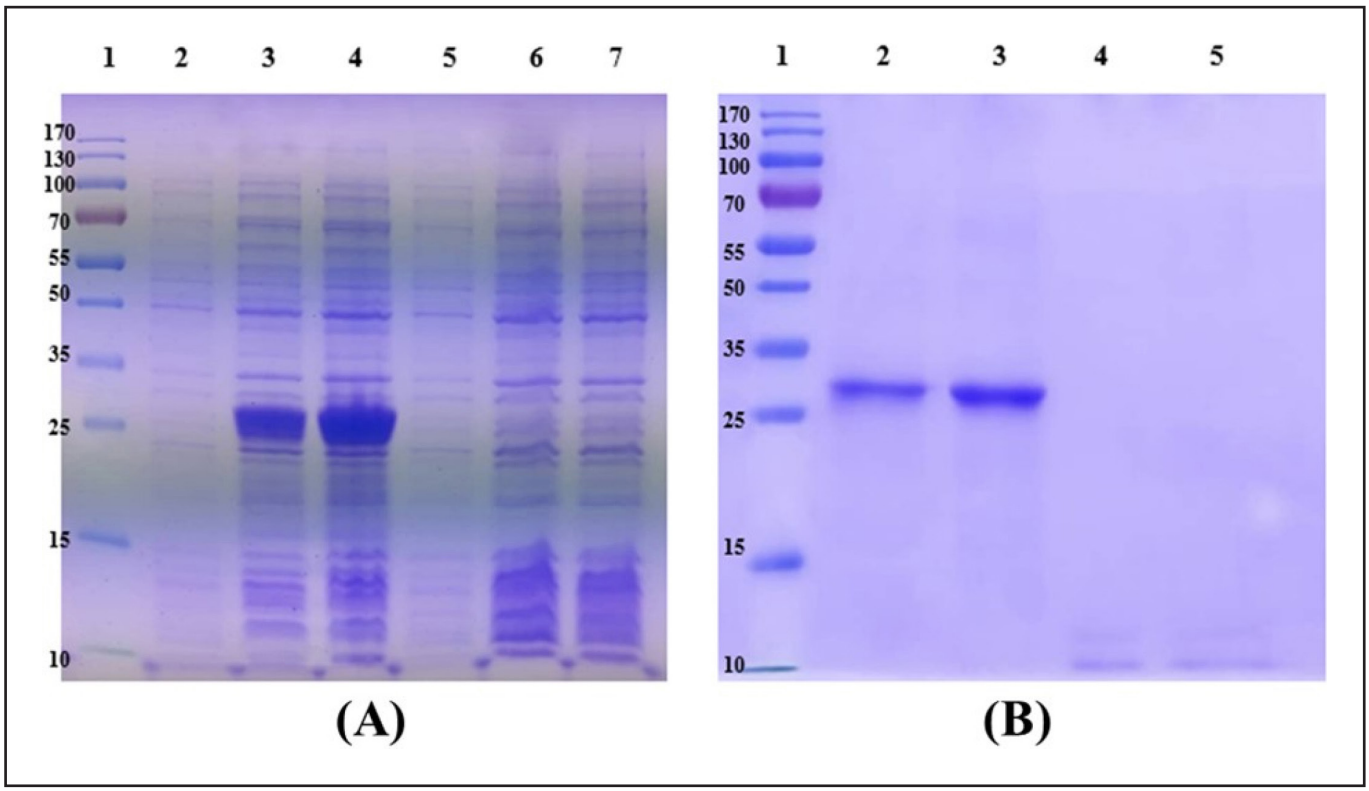

Figure 1. SDS-PAGE analysis of expressed protein **(A)** Protein expression of ~25 kDa recombinant PkSPATR(II). Lane 1: Pageruler pre-stained protein ladder (kDa); lanes 2, 3 and 4: expression of recombinant PkSPATR(II) at hours 0, 2 and 4 post IPTG induction; lanes 5, 6 and 7: expression of pET-30a(+) vector without PkSPATR(II) gene at hours 0, 2 and 4 post IPTG induction. **(B)** Purified proteins. Lane 1: Pageruler pre-stained protein ladder (kDa); lanes 2 and 3: ~25 kDa recombinant PkSPATR; lanes 4 and 5: negative control pET-30a(+) vector.

IFA showing interaction of intraerythrocytic *P. knowlesi* merozoites with anti-PkSPATR(II) antibodies in rabbit serum

Figure 2 shows the immunofluorescence images of smear from *P. knowlesi* culture treated with immunised rabbit serum. Bright-field (BF) represents the slide observation under a light microscope, and the *P. knowlesi* nuclei are seen as blue fluorescence under a DAPI filter. Interaction of the rabbit serum anti-PkSPATR(II) antibodies to FITC-labelled anti-rabbit IgG antibody shows green fluorescence under FITC filter. The immunised rabbit serum produced green fluorescencing of schizonts under the FITC filter, indicating interaction between anti-PkSPATR(II) antibodies with *P. knowlesi* merozoites in the infected erythrocytes. No green fluorescence was produced by the non-recombinant pET-30a(+)-immunised rabbit serum (negative control).

Merozoite invasion inhibition assay

The merozoite invasion inhibition assay was performed to evaluate the ability of anti-PkSPATR(II) antibodies to inhibit *P. knowlesi* invasion into human erythrocytes. The anti-PkSPATR(II) antibodies showed inhibitory activities to parasite invasion at concentrations above 0.188 mg/ml, and in a concentration dependent manner (Figure 3). Significant inhibition, as compared to the non-treated culture (0.00 mg/ml), was observed at antibody concentrations 3.00 mg/ml ($P = 0.0001$), 1.50 mg/ml ($P = 0.0008$), 0.75 mg/ml ($P = 0.0032$) and 0.375 mg/ml ($P = 0.0150$). The invasion inhibitory rate ranged from 20% to approximately 32%. No effect on the parasite erythrocyte invasion was observed for the control antibodies (data not shown).

DISCUSSION

The SPATR of several *Plasmodium* species has been demonstrated in previous studies to be immunogenic. In our present study, the exon II of PkSPATR was targeted for recombinant expression and tested for immunoprotective property against *P. knowlesi* infection *in vitro*. This exon contains a thrombospondin (TSR) functional domain at the C-terminus. This domain is cysteine rich and plays a key role in cell adhesion of the protein. In addition, exon II has also been associated with parasite attachment and invasion into the host cell (Mahajan *et al*., 2005).

Antibody response is an important element of naturally acquired immunity against malaria (Boyle *et al*., 2019). Antibodies acquired naturally or through immunisation can confer protection against malarial infection by distinguishing specific antigens on the parasite or infected erythrocyte by blocking the parasite adhesion and invasion process (Teo *et al*., 2016). This is the likely mode of action of anti-PkSPATR(II) antibodies in the merozoite invasion inhibition assay, as PkSPATR has been shown to be expressed on the surface of *P. knowlesi* merozoites (Mahajan *et al*., 2005). Immunofluorescence analysis has also revealed the localisation of PkSPATR on the surface of *P. knowlesi* sporozoites (Mahajan *et al*., 2005). Therefore, it would be worthwhile in future studies to examine the inhibitory effect of anti-PkSPATR(II) antibodies on the invasion of *P. knowlesi* sporozoites into liver cells.

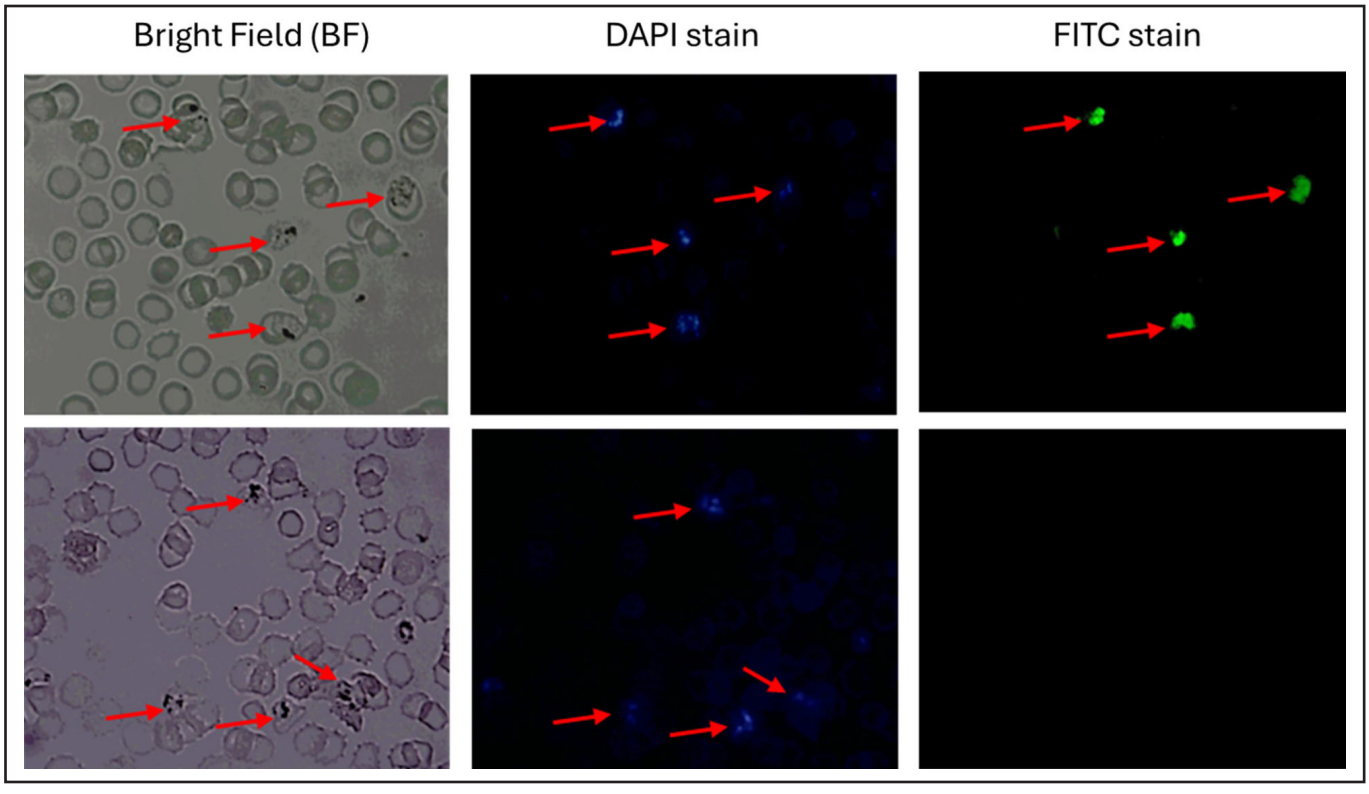

Figure 2. Immunofluorescence images of PkSPATR (II)-immunised rabbit serum against schizonts stage of *P. knowlesi* parasite. **Top panel**: Infected erythrocytes treated with PkSPATR(II)-immunised rabbit serum. Schizonts containing merozoites are indicated by red arrows. Nuclei of the merozoites are stained blue under the DAPI filter. Green fluorescence observed under the FITC filter indicates interaction between merozoites and anti-PkSPATR(II) antibodies which is detected with FITC-labeled anti-rabbit IgG antibody. **Bottom panel**: Infected erythrocytes treated with non-recombinant pET-30a(+)-immunised rabbit serum (negative control). No green fluorescence is observed under the FITC filter.

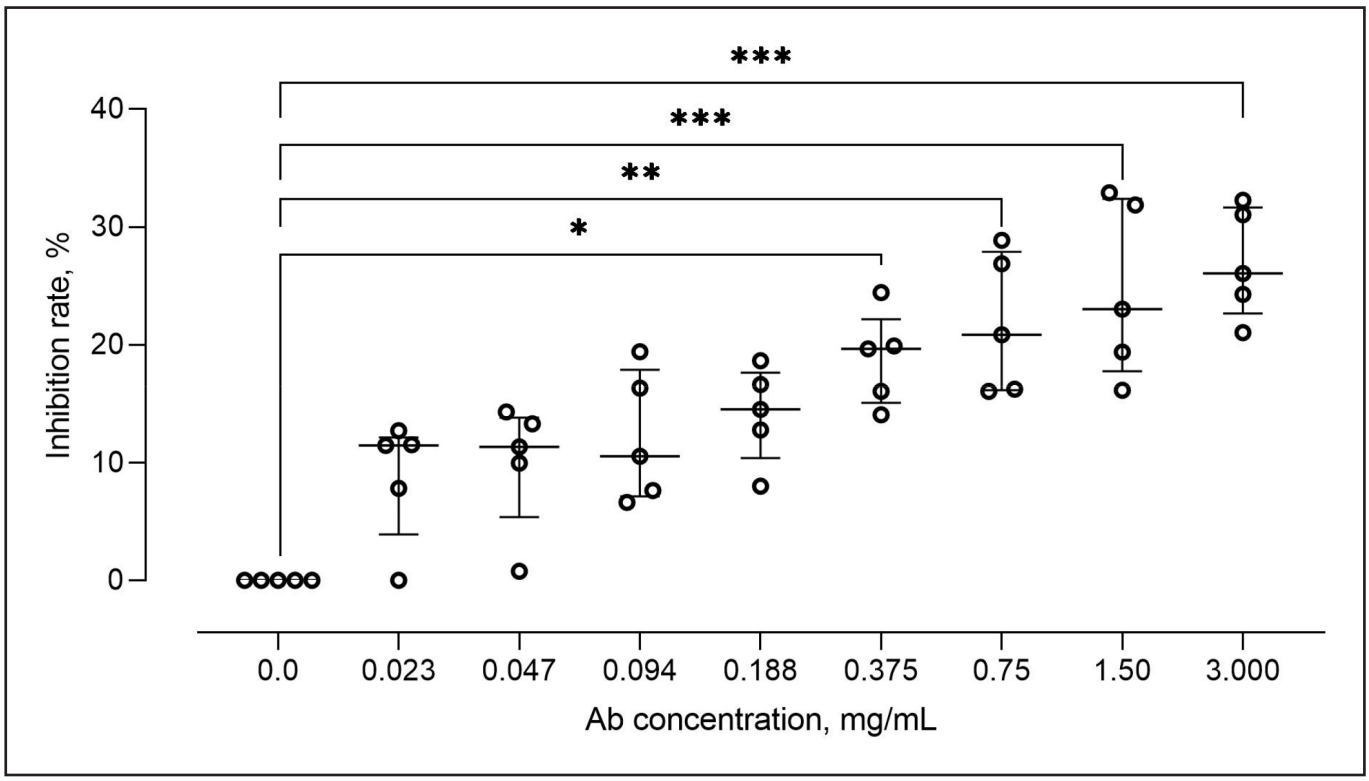

Figure 3. Merozoite invasion inhibition rate of anti-PkSPATR(II) antibodies. Each data point represents the mean of duplicate readings in each biological replicate. The effect of anti-PkSPATR(II) in merozoite invasion inhibition was determined using Kruskal-Wallis and Dunn's multiple comparison test in Graph pad prism. Asterisks indicate significant difference, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

The merozoite invasion inhibition rate of anti-PkSPATR(II) antibodies ranged from 20% – 32%, which is not far from the range obtained for antibodies against *P. knowlesi* apical membrane protein (PkAMA-1) (14% – 40%) (Ng *et al*., 2023) and Duffy binding protein alpha region II (PkDBPaII) (22% – 46%) (Azlan *et al*., 2022). PkAMA-1 and PkDBPaII are two proteins that play important roles in the invasion process of *P. knowlesi* into human erythrocytes. It appears that antibodies against PkSPATR, PkAMA-1 and PkDBPaII could only confer partial invasion inhibition on the parasite. This non-absolute inhibition may be due to the existence of alternative invasion pathways of *P. knowlesi* into human merozoites, as has been seen in *P. falciparum* (Ord *et al*., 2012) and *Plasmodium vivax* (Popovici *et al*., 2020).

As mentioned above, the inhibitory effect of anti-PkSPATR(II) antibodies on *P. knowlesi* sporozoite invasion is worth investigating. Studies on antiserum against *P. falciparum* SPATR (PfSPATR) reported strong reactivity against sporozoites even at high dilutions of 1:6400. More importantly, *in vitro* inhibition assay showed that the antiserum at a final dilution of 1:50 could inhibit sporozoite invasion into human liver cells by > 80%, and the inhibition was antigen specific (Chattopadhyay *et al*., 2003).

Another potential advantage of PkSPATR as a vaccine candidate lies in the presence of asparagine-rich motifs in the protein. PkSPATR has a high abundance of asparagine residues (≈ 7%) that can potentially initiate production of opsonizing antibodies and trigger phagocytosis of *P. knowlesi* sporozoites and merozoites by the immune cells (Mahajan *et al*., 2005; Hill *et al*., 2017).

CONCLUSIONS

In summary, this study demonstrated the ability of PkSPATR to elicit production of antibodies that have inhibitory effect against *P. knowesi* invasion into human erythrocytes. Further immuno-characterisation studies, particularly on anti-PkSPATR antibodies inhibitory effect on sporozoite invasion of human liver cells, need to be carried out to assess the potential of PkSPATR as a knowlesi malaria vaccine candidate.

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Conflict of Interest

The authors declare no conflict of interest.

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