



SHORT COMMUNICATION

Experimental Study on *Plasmodium knowlesi* Normocyte Binding Protein Xa Region II (PkNBPXall) for Erythrocyte Binding

Wong, K.C.¹, Lai, M.Y.¹, De Silva, J.R.¹, Cheong, F.W.¹, Fong, M.Y.¹, Lau, Y.L.^{1*}

¹Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

*Corresponding author: lauyeeling@um.edu.my

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ABSTRACT

Normocyte binding protein Xa (NBPXa) has been implied to play a significant role in parasite invasion of human erythrocytes. Previous phylogenetic studies have reported the existence of three types of NBPXa for *Plasmodium knowlesi* (PkNBPXa). PkNBPXa region II (PkNBPXall) of type 1, type 2 and type 3 were expressed on mammalian cell surface and interacted with human and macaque (*Macaca fascicularis*) erythrocytes. The binding activities of PkNBPXall towards human and macaque erythrocytes were evaluated using erythrocyte-binding assay (EBA). Three parameters were evaluated to achieve the optimal protein expression of PkNBPXall and erythrocyte binding activity in EBA: types of mammalian cells, post transfection time and erythrocyte incubation time. COS-7, HEK-293, and CHO-K1 cells showed successful expression of PkNBPXall, despite the protein expression is weak compared to the positive control. COS-7 was used in EBA. All three types of PkNBPXall showed rosette formation with macaque erythrocytes but not with human erythrocytes. Future studies to enhance the PkNBPXall expression on surface of mammalian cells is indeed needed in order to elucidate the specific role of PkNBPXall in erythrocytes invasion.

Keywords: *Plasmodium knowlesi*; normocyte binding protein Xa; cloning; protein expression; erythrocyte binding.

INTRODUCTION

Plasmodium merozoite invasion of the erythrocyte is a complex process, involving steps of initial recognition, attachment, reorientation of the apical end, formation of a tight-junction and subsequent entry into the host cell. These complex processes involve a cascade of molecular interactions between ligands of the parasite and its corresponding receptors present on the erythrocyte surface (Koch & Baum, 2016). The members of the reticulocyte binding-like (RBL) protein family have proven to play a major role in host cell recognition and invasion (Gunalan *et al.*, 2013). For *Plasmodium knowlesi* (*P. knowlesi*), two distinct but related functional RBL genes, normocyte binding protein Xa and Xb (PkNBPXa and PkNBXPb), are described (Meyer *et al.*, 2009). Normocyte binding protein Xa (NBPXa) has been implied to play a significant role in parasite invasion of human erythrocytes. Disruption of PkNBPXa creates a block in parasite invasion into human erythrocytes (Moon *et al.*, 2016). This suggests that PkNBPXa could be a potential malaria vaccine target, the antibodies against this important antigen could potentially break the asexual life cycle and halt the pathogenesis of the parasites.

A phylogenetic study revealed the occurrence of three PkNBPXa types (types 1, 2, and 3) among Peninsular Malaysia and Malaysian Borneo clinical isolates. The study also

indicated PkNBPXa dimorphism in two of the types (type 1 and type 2) throughout Malaysia while PkNBPXa type 3 was found in clinical isolates of Peninsular Malaysia only (Ahmed *et al.*, 2014). PkNBPXa has been identified as a key mediator for transmission of *P. knowlesi* into humans and its polymorphisms were postulated to be the important determinants that lead to high parasitaemia and disease diversity in *P. knowlesi* infection (Ahmed *et al.*, 2014; Moon *et al.*, 2016). In the present study, clinical samples representing these three types were selected for cloning and surface expression of PkNBPXa region II (PkNBPXall) on mammalian cells. The binding activity of expressed PkNBPXall with erythrocytes was assessed using erythrocyte-binding assay (EBA) to investigate the role of PkNBPXa in the invasion of erythrocytes.

MATERIALS AND METHODS

Ethics statement

This study was approved by the University of Malaya Medical Centre Medical Ethics Committee (MEC No. 817.18) and University of Malaya Institutional Animal Care and Use Committee [PAR/6/03/2015/AA(R)], respectively. Written informed consent was obtained from all human blood donors for the study.

Construction of recombinant plasmid for PkNBPXall surface expression on mammalian cell

Three types of PkNBPXa were represented by clinical isolate SBH31 (type 1), clinical isolate UM118 (type 2) and clinical isolate HAN (type 3), respectively from the samples used by Ahmed *et al.* (2016). The construction of recombinant plasmids for PkNBPXall surface expression on mammalian cells was performed according to Lim *et al.* (2017) with minor modification. Briefly, the gene of interest, *PkNBPXall* region was amplified with polymerase chain reaction (PCR) using forward primer: 5' -AGATCTTACACGTGCGAAGAAATTGTG-3' and reverse primer: 5' -AGATCTCCCTTCGTAACCTTTGTATAGCG-3'; both containing *Bgl*II restriction enzyme (RE) cut site. PCR was performed with the cycling profile: 95°C for 5 minutes (min), 30 cycles at 95°C for 30 seconds (s), 55°C for 45 s and 72°C for 70 s; and 72°C for 10 min. The amplified PCR product for each type of *PkNBPXall* was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and ligated into PCR vector pGEM[®]-T Easy Vector (Promega Corp., Madison, Wisconsin, USA). Each ligation mixture was transformed into One-Shot[®] TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Waltham, Massachusetts, USA) for propagation and maintenance. The *PkNBPXall* of each type was then cloned into expression vector pDisplay[™] plasmid (Invitrogen, Waltham, Massachusetts, USA) with addition of *Aequorea coerulea* green fluorescent reporter gene (*AcGFP1*) at the C-terminal. Nucleotide sequences of the recombinant plasmid pDisplay-*PkNBPXall*-*AcGFP1* for all three types were confirmed by sequencing services from the commercial laboratory (First BASE Laboratories Sdn. Bhd).

Mammalian cells transfection and expression

COS-7 cells (ATCC[®] CRL-1651[™]), human embryonic kidney cells (HEK-293; ATCC[®] CRL-1573[™]), Chinese hamster ovarian cells (CHO-K1; ATCC[®] CCL-61[™]) and human skin fibroblast cells (Hs27; ATCC[®] CRL-1634[™]) were used to express PkNBPXall (Type 1, 2 and 3) on the cell surface. Mammalian cells were plated into six-well culture plates and allowed to reach a monolayer, 80-90% confluency before transfection. The culture medium supernatant was discarded and replaced with serum-free Dulbecco's modified Eagle's medium (DMEM)-high glucose medium prior to transfection. Mammalian cells were then transfected with pDisplay-*PkNBPXall*-*AcGFP1* plasmid (2.5 µg per well) (Type 1, 2 and 3, each type in all four cell lines) by using Lipofectamine 3000[™] reagent (Invitrogen, Waltham, Massachusetts, USA) with recommended protocols and grown at 37°C in 5% CO₂. After 24 hours (h) of incubation, growth medium with transfection reagents were discarded and replaced with complete DMEM-high glucose culture medium. Transfected cells were incubated for another 24 h for subsequent use. The PkNBPXall expression was then evaluated, and the most suitable mammalian cell line (COS-7) was chosen for expression for the downstream works (EBA).

Optimisation of PkNBPXall expression with different post transfection time before EBA

Optimisation of surface expression of PkNBPXall on transfected COS-7 cells was carried out via modification of post-transfection time (24 h, 48 h and 72 h) before EBA. 48 h post-transfection was then chosen for the downstream works (EBA).

Erythrocyte-binding assay (EBA)

The 48 h post-transfected cells were incubated with erythrocytes (1% haematocrit) for 2 h at 37°C incubator and supplied with 5% CO₂. For EBAs with monkey erythrocytes, *Macaca fascicularis* (*M. fascicularis*) erythrocytes were used. Four

human donors ($n = 4$) were used for EBAs with human erythrocytes.

Optimisation of erythrocyte binding activity with different erythrocyte incubation time during EBA

The erythrocyte binding activity in EBA was optimised by incubating the erythrocytes with cells for different incubation time (2 h, 4 h, 6 h and overnight).

Fluorescence microscopy

After 2 h of erythrocytes incubation, cells were washed with 1 × phosphate buffer saline (PBS) to remove non-adherent erythrocytes. The nuclei of mammalian cells were stained with 1 µg/ml Hoechst 33,342 dye (Invitrogen, Waltham, Massachusetts, USA). The cells were observed using DAPI-filter (380 nm excitation wavelength) and FITC-filter (488 nm excitation wavelength) mounted on a Nikon ECLIPSE TE300 inverted fluorescence microscope. Positive rosettes were defined as 50% of the surface of transfected mammalian cells covered with adherent erythrocytes that emitted green fluorescence (Lim *et al.*, 2017). Mammalian cells transfected with pDisplay-*PkDBPall*-*AcGFP1* H1 strain plasmid (Lim *et al.*, 2017) were used as a positive control. Empty pDisplay-*AcGFP1* plasmid and non-transfected mammalian cells were used as negative controls.

Immunofluorescence assay (IFA)

Immunofluorescence assay (IFA) was performed to determine the surface expression of PkNBPXall by targeting the *myc*-epitope carried by the pDisplay[™] expression vector. Firstly, transfected cells were fixed with cold 4% (w/v) paraformaldehyde for 15 min at room temperature. The fixed cells were then incubated with blocking buffer, 3% (w/v) bovine serum albumin (BSA)/ PBS, for 30 min at 37°C. Then, the cells were incubated with mouse anti-*myc* IgG antibody (Invitrogen, Waltham, Massachusetts, USA) diluted to 1:5000 for 1 h at 37°C. Subsequently, COS-7 cells were incubated with Alexa Fluor 594-conjugated anti-mouse IgG antibody (Invitrogen, Waltham, Massachusetts, USA) diluted to 1:200 for 1 h at 37°C. Lastly, COS-7 cells were visualised under fluorescence microscopy with TRITC-filter (540 nm excitation wavelength) and FITC-filter (488 nm excitation wavelength) mounted on a Nikon ECLIPSE TE300 inverted, fluorescence microscope.

RESULTS AND DISCUSSION

Different types of mammalian cell lines, COS-7, HEK-293, CHO-K1 and Hs27 cells, were used to express PkNBPXall on the cell surface. Mammalian cells such as HEK-293 and CHO-K1 cell lines are extensively used as host cells for the expression of recombinant proteins for both structural and functional studies (Ooi *et al.*, 2016). On the other hand, Hs27 cells are human dermal fibroblasts that have demonstrated the capability of collagen (protein) expression for cytotoxicity study (Park *et al.*, 2019). Only COS-7, HEK-293, and CHO-K1 cells showed successful expression of PkNBPXall protein by showing green fluorescence under FITC-filter (Figure 1). Hs27 cell line was not a suitable cell line for PkNBPXall expression as majority of the cells died after transfection. COS-7 mammalian cell line was selected to be used for downstream works (EBA) from the three cell lines with successful transfection as it has unique characteristics of fibroblast-like morphology, rapid division rate and ease of maintenance. Besides, the selection of COS-7 cells was also due to its highly efficiency as a transfection host in EBA as reported in previous studies (Cheng *et al.*, 2013; Lim *et al.*, 2017).

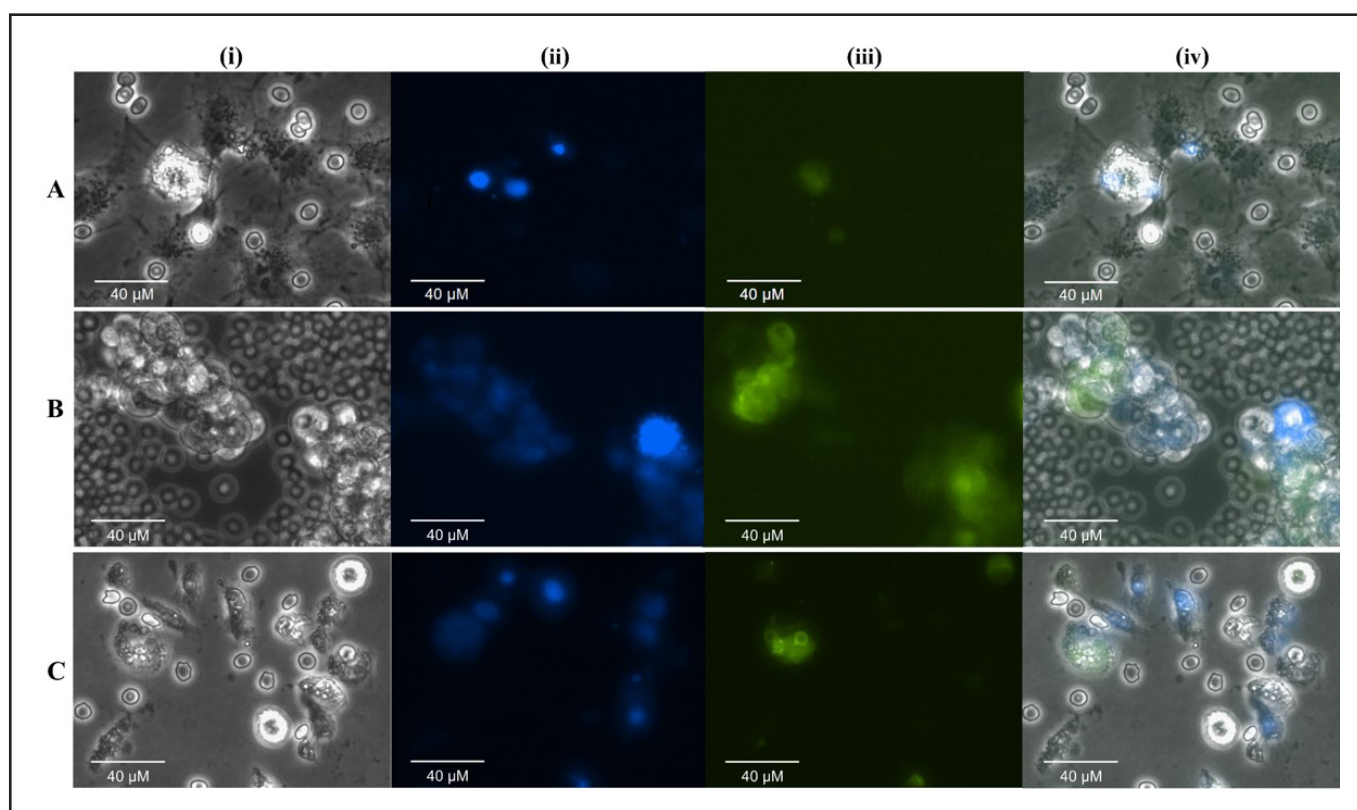


Figure 1. Erythrocyte binding assays of (A) COS-7 cells (B) HEK-293 cells (C) CHO-K1 cells transfected with recombinant PkNBPAII plasmid using human erythrocytes. (i) Bright field image, (ii) Nuclei of cell show blue fluorescence under DAPI-filter, (iii) GFP shows green fluorescence under FITC-filter, (iv) Merge image of bright field, DAPI and FITC-filter. Images were captured by a Nikon ECLIPSE TE300 inverted fluorescence microscope using the Plan Fluor ELWD 40×/0.45 aperture and ×20 magnification eye piece.

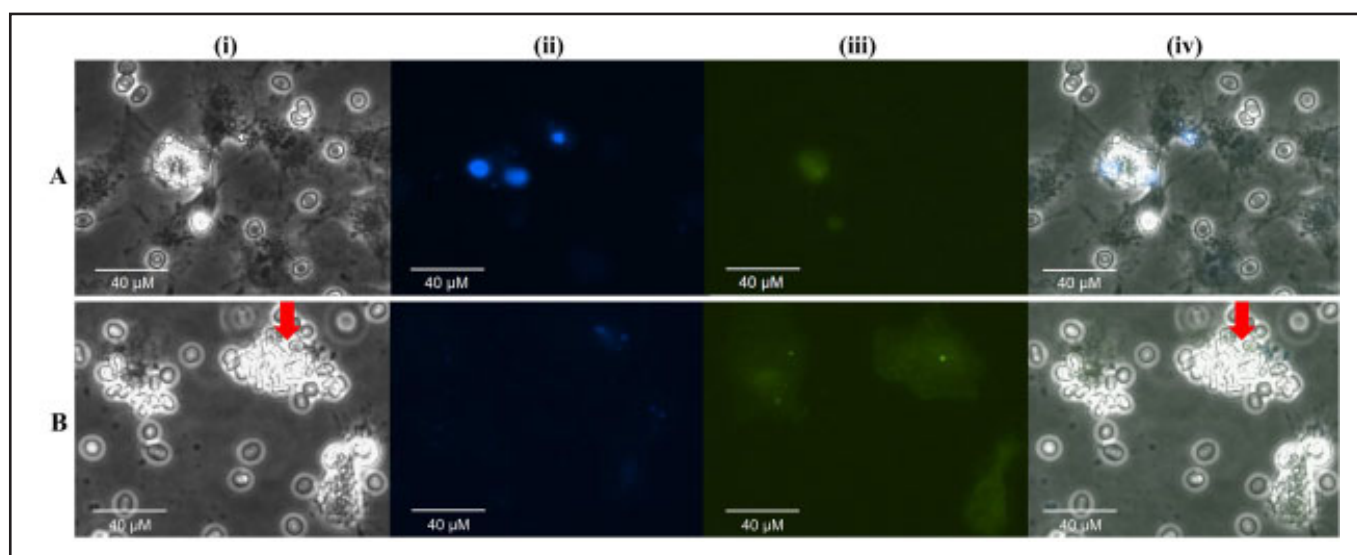


Figure 2. Erythrocyte binding assays of COS-7 cells transfected with (A) recombinant PkNBPAII plasmid and (B) recombinant PkDBPAII H1 strain plasmid (positive control) using human erythrocytes. (i) Bright field image, (ii) Nuclei of COS-7 cells show blue fluorescence under DAPI-filter, (iii) GFP shows green fluorescence under FITC-filter, (iv) Merge image of bright field, DAPI and FITC-filter. Formation of rosette was observed in positive control well (red arrow), but not in PkNBPAII well. Images were captured by a Nikon ECLIPSE TE300 inverted, fluorescence microscope using the Plan Fluor ELWD 40×/0.45 aperture and ×20 magnification eye piece.

EBA results indicated that no rosette formation was observed for all three types of recombinant PkNBPAII with human erythrocytes (Figure 2A). Positive control in these experiments formed rosettes with both human (Figure 2B)

and macaque erythrocytes (Figure 3B). Similar results were observed when optimisations of PkNBPAII surface expression on transfected COS-7 cells were carried out on modification of post transfection time before EBA and incubation time

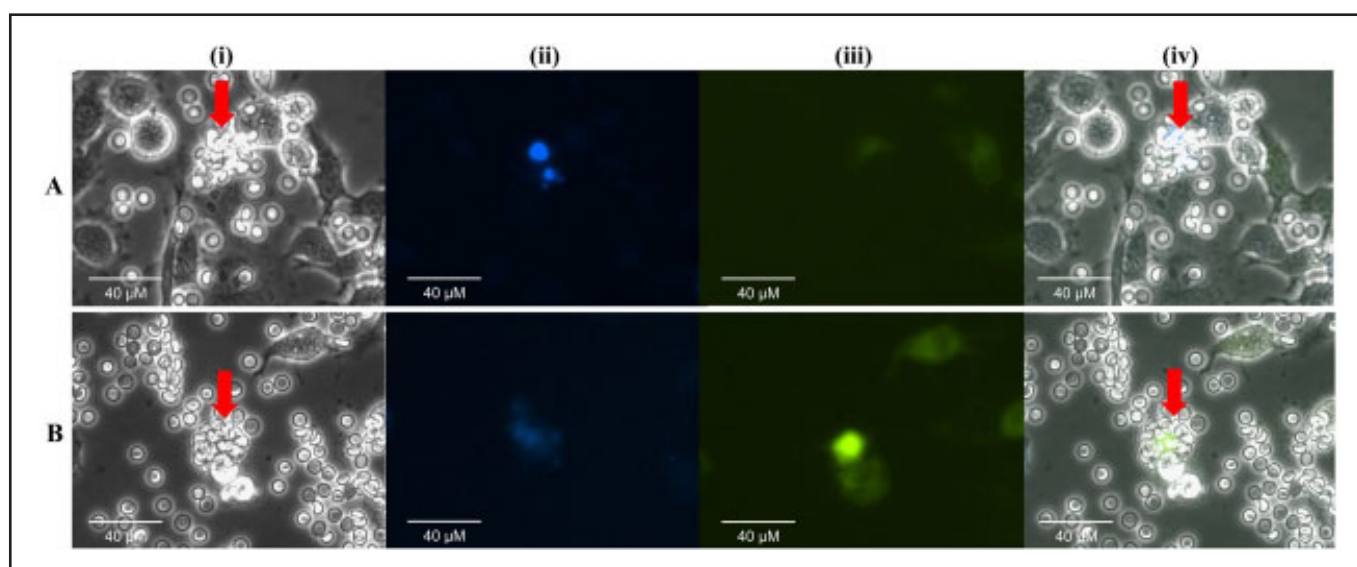


Figure 3. Erythrocyte binding assays of COS-7 cells transfected with (A) recombinant PkNBPAII plasmid and (B) recombinant PkDBPAII H1 strain plasmid (positive control) using macaque erythrocytes. (i) Bright field image with formation of rosette (red arrows), (ii) Nuclei of COS-7 cells show blue fluorescence under DAPI-filter, (iii) GFP shows green fluorescence under FITC-filter, (iv) Merge image of bright field, DAPI and FITC-filter. Formation of rosette was observed in both PkNBPAII and positive control wells (red arrow). Images were captured by a Nikon ECLIPSE TE300 inverted fluorescence microscope using the Plan Fluor ELWD 40 \times /0.45 aperture and \times 20 magnification eye piece.

with erythrocytes during EBA. PkNBPAII type 1-transfected COS-7 cells were allowed to grow for 24 h, 48 h and 72 h before being used for EBAs. PkNBPAII type 1 48 h-post transfected COS-7 cells were used for optimisation of incubation time with erythrocytes for 2 h, 4 h, 6 h and overnight during EBAs. However, no rosette formation was observed in all the EBAs with all optimised parameters using human erythrocytes. On the other hand, when *M. fascicularis* erythrocytes were incubated with all the three recombinant PkNBPAII (Type 1, 2 and 3) expressed on the transfected COS-7 cell surface, rosettes formation was observed (Figure 3A). However, the rosette formation of macaque erythrocytes with PkNBPAII was smaller compared to PkDBPAII H1 strain (positive control; Figure 3B). This phenomenon was also observed by Semenya *et al.* (2012) where PkNBPAII showed binding towards both rhesus and macaque erythrocytes but not with human erythrocytes. A previous study has also reported that for PkDBPAII, EBA using human erythrocytes showed lesser number of rosettes as compared to macaque erythrocytes (Lim *et al.*, 2017). It was postulated that these results pointed to the existence of alternative receptor determinants for PkDBPAII binding (Nichols *et al.*, 1987). Hence, the difference in binding of PkNBPAII towards human and macaque erythrocytes could also be due to the existence of alternative receptors for PkNBPAII on macaque erythrocytes which are absent in human erythrocytes.

In addition to GFP fluorescence signal, IFA was performed to further determine the surface expression of PkNBPAII on transfected COS-7 mammalian cells by targeting expressed *myc*-epitope of pDisplay™ vector carrying PkNBPAII recombinant protein. In Figure 4, PkNBPAII-transfected COS-7 cells showed green and red fluorescence under FITC-filter and TRITC-filter, respectively. Surface expression of PkNBPAII was found to be lesser when compared to positive control PkDBPAII. The reduced in expression of PkNBPAII could be attributed to multiple underlying factors. One of the factors could be protein hydrophobicity. Protein fragment that

contains many hydrophobic regions tends to have inward protein folding and lead to protein aggregations. These protein aggregations could then affect the protein function and subsequent proper protein expression (Stefani, 2004). In the present study, Kyte-Doolittle hydropathy plot result revealed that PkNBPAII consists of many hydrophobic regions (Figure 5), with majority of the amino acid sequences distributed over the positive values (hydrophobic) within the plot (Kyte & Doolittle, 1982). Besides, the recombinant protein expression may also be affected by the rate of protein translation from mRNA (Trösemeyer *et al.*, 2019), presence of charged residues within protein sequences for the regulation of protein translation efficiency (Requiao *et al.*, 2017), and post-translational events such as phosphorylation and ubiquitination for proper protein folding and function (Tokmakov *et al.*, 2012). The weak surface expression of PkNBPAII may lead to decreased binding and rosette formation in EBA. However, no study has reported successful surface expression of PkNBPAII to-date. Optimisation of surface expression and trafficking of PkNBPAII was also suggested by Semenya *et al.* (2012) with adjustment on boundaries of targeted region or change in expression vector, expression host and expression culture condition that could be considered for future research to address the unanswered aspects of this present study.

In conclusion, the present study clearly threw light on the surface expression of PkNBPAII on mammalian cells including COS-7, HEK-293 and CHO-K1 cells. PkNBPAII binds with macaque erythrocytes, but not human erythrocytes. Expression of PkNBPAII is comparatively lower as compared to PkDBPAII. Although the difference in binding activity between the three types of PkNBPAII could not be demonstrated in this study via EBA, studies in future using an alternative approach showing the differences among different types of PkNBPAII would be vital to allow for a deeper understanding of the effects of the different types of PkNBPAII in *P. knowlesi* infection.

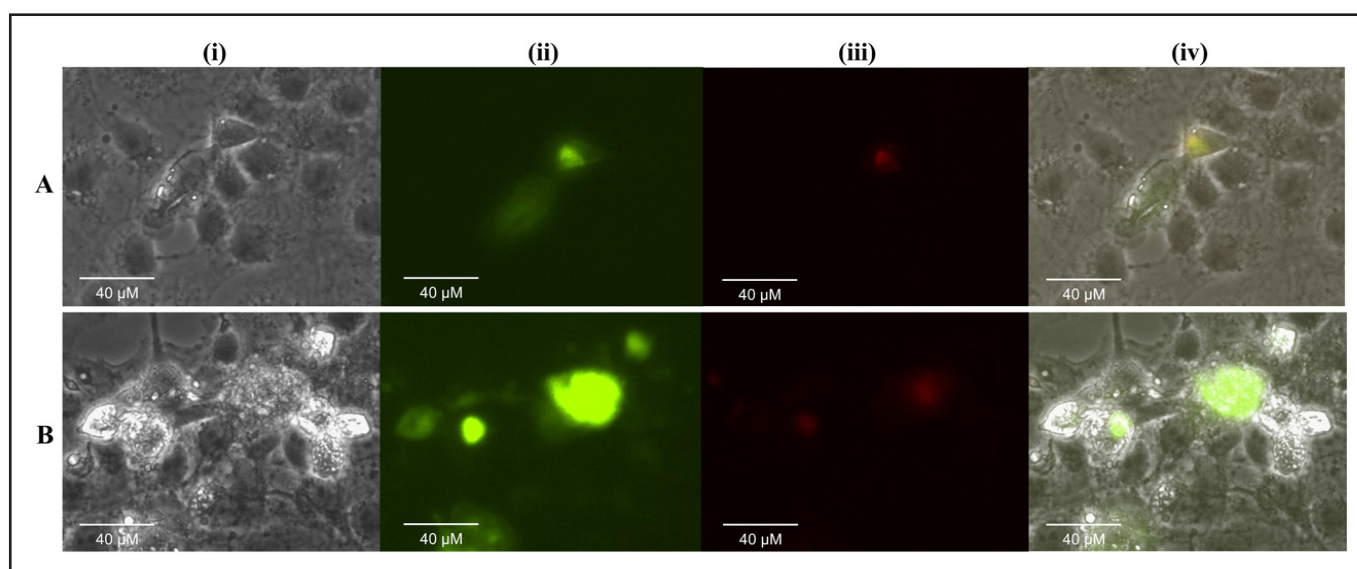


Figure 4. Immunofluorescence assay of COS-7 cells transfected with (A) recombinant PkNBPX α II plasmid and (B) recombinant PkDBP α II H1 strain plasmid (positive control). (i) Bright field image of transfected cells, (ii) Transfected cells emitted green fluorescence under FITC-filter indicating the expression of GFP, (iii) Red fluorescence was observed under TRITC-filter, indicating the presence of *myc*-epitope, (iv) Merge image of bright field, FITC and TRITC-filter. Images were captured by a Nikon ECLIPSE TE300 inverted, fluorescence microscope using the Plan Fluor ELWD 40 \times /0.45 aperture and \times 20 magnification eye piece.

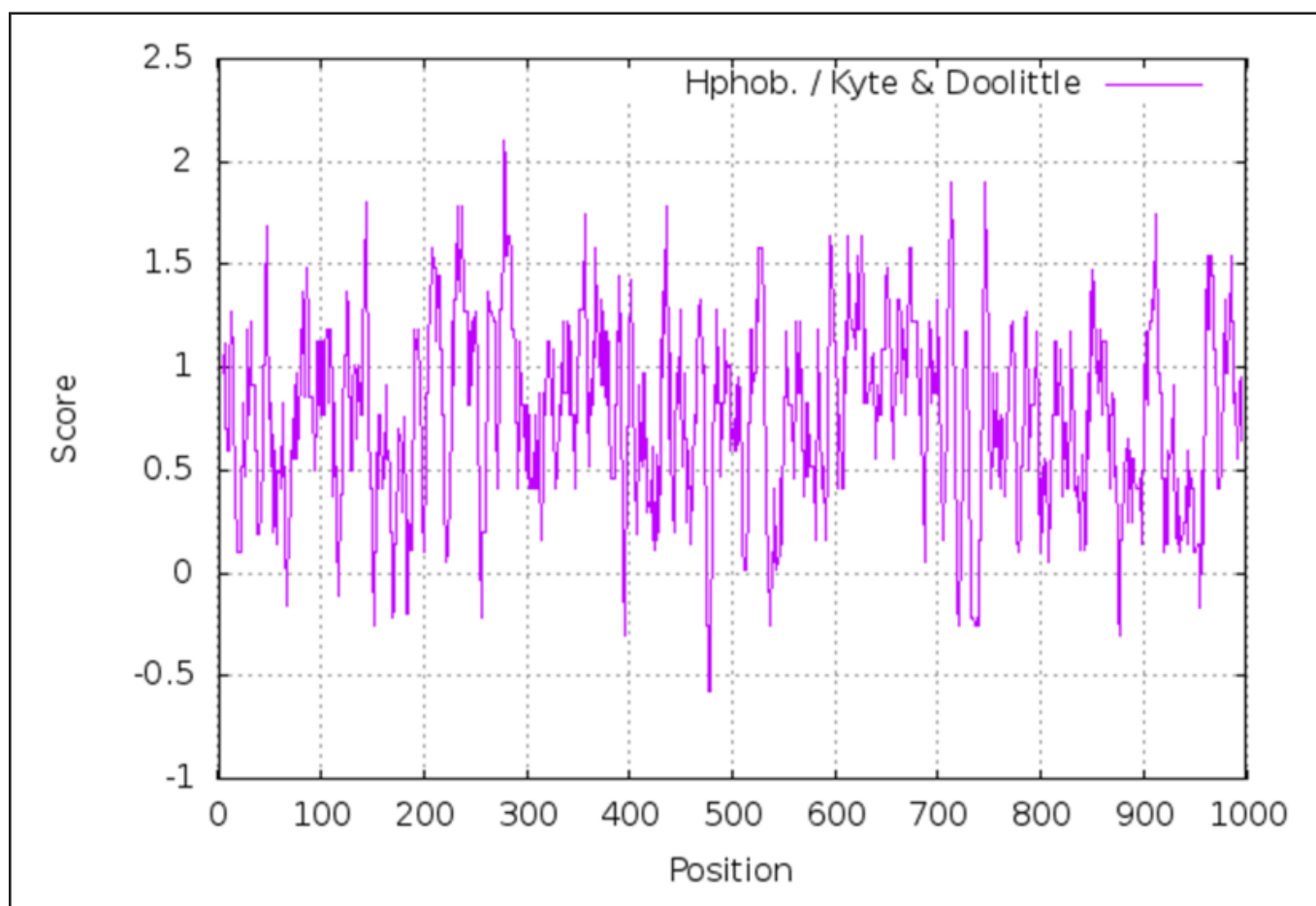


Figure 5. Kyte-Doolittle hydropathy plot derived from the amino acid sequence of PkNBPX α II. A window size of 7 amino acids was used to search for hydrophobic region in this protein. The hydrophobic residues are shown above zero (positive value), whereas the hydrophilic residues are below zero (negative value).

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Conflict of interest

The author declares that they have no conflict of interests.

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