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

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**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 PkNBPXb), 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 (PkNBPXaII) on mammalian cells. The binding activity of PkNBPXaII with mammalian cell surface and interacted with human and macaque (*Macaca fascicularis*) erythrocytes. The binding activities of PkNBPXaII towards human and macaque erythrocytes were evaluated using erythrocyte-binding assay (EBA). Three parameters were evaluated to achieve the optimal protein expression of PkNBPXaII and erythrocyte binding activity in EBA: types of mammalian cells, post transfection incubation time, and erythrocyte incubation time. COS-7, HEK-293, and CHO-K1 cells showed successful expression of PkNBPXaII, despite the protein expression is weak compared to the positive control. COS-7 was used in EBA. All three types of PkNBPXaII showed rosette formation with macaque erythrocytes but not with human erythrocytes. Future studies to enhance the PkNBPXaII expression on surface of mammalian cells is indeed needed in order to elucidate the specific role of PkNBPXaII in erythrocytes invasion.

**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/8/03/2015/AA(R)), respectively. Written informed consent was obtained from all human blood donors for the study.
Construction of recombinant plasmid for PkNBPXaII 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 PkNBPXaII surface expression on mammalian cells was performed according to Lim et al. (2017) with minor modification. Briefly, the gene of interest, PkNBPXaII region was amplified with polymerase chain reaction (PCR) using forward primer: 5' -AGATCTCCCTTCGTAACTTTGTATAGCG-3', both was amplified with polymerase chain reaction (PCR) using reverse primer: 5' -AGATCTCCCTTCGTAACTTTGTATAGCG-3', both containing BglII 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 PkNBPXaII 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 PkNBPXaII of each type was then cloned into expression vector pDisplay™ plasmid (Invitrogen, Waltham, Massachusetts, USA) with addition of Aequorea coeruleans green fluorescent reporter gene (AcGFP1) at the C-terminal. Nucleotide sequences of the recombinant plasmid pDisplay-PkNBPXaII-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 PkNBPXaII (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-PkNBPXaII-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 PkNBPXaII expression was then evaluated, and the most suitable mammalian cell line (COS-7) was chosen for expression for the downstream works (EBA).

Optimisation of PkNBPXaII expression with different post transfection time before EBA

Optimisation of surface expression of PkNBPXaII 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 EBA, 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 PkNBPXaII 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 microscope 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 PkNBPXaII 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 studies (Park et al., 2019). Only COS-7, HEK-293, and CHO-K1 cells were 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 studies (Park et al., 2019). Only COS-7, HEK-293, and CHO-K1 cells showed successful expression of PkNBPXaII protein by showing green fluorescence under FITC-filter (Figure 1). HS27 cell line was not a suitable cell line for PkNBPXaII 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 PkNBXaII 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.

EBA results indicated that no rosette formation was observed for all three types of recombinant PkNBXaII 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 PkNBXaII surface expression on transfected COS-7 cells were carried out on modification of post transfection time before EBA and incubation time...
with erythrocytes during EBA. PkNBPXaII type 1-transfected COS-7 cells were allowed to grow for 24 h, 48 h and 72 h before being used for EBAs. PkNBPXaII 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 PkNBPXaII (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 PkNBPXaII was smaller compared to PkDBPαII H1 strain (positive control; Figure 3B). This phenomenon was also observed by Semenya et al. (2012) where PkNBPXbII showed binding towards both rhesus and macaque erythrocytes but not with human erythrocytes. A previous study has also reported that for PkDBPαII, 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 PkDBPαII binding (Nichols et al., 1987). Hence, the difference in binding of PkNBPXaII towards human and macaque erythrocytes could also be due to the existence of alternative receptors for PkNBPXaII 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 PkNBPXaII on transfected COS-7 mammalian cells by targeting expressed *myc*-epitope of pDisplay™ vector carrying PkNBPXaII recombinant protein. In Figure 4, PkNBPXaII-transfected COS-7 cells showed green and red fluorescence under FITC-filter and TRITC-filter, respectively. Surface expression of PkNBPXaII was found to be lesser when compared to positive control PkDBPαII. The reduced in expression of PkNBPXaII 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 PkNBPXaII 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 (Trösemeier 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 PkNBPXaII may lead to decreased binding and rosette formation in EBA. However, no study has reported successful surface expression of PkNBPXaII to-date. Optimisation of surface expression and trafficking of PkNBPXaII 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 PkNBPXaII on mammalian cells including COS-7, HEK-293 and CHO-K1 cells. PkNBPXaII binds with macaque erythrocytes, but not human erythrocytes. Expression of PkNBPXaII is comparatively lower as compared to PkDBPαII. Although the difference in binding activity between the three types of PkNBPXaII could not be demonstrated in this study via EBA, studies in future using an alternative approach showing the differences among different types of PkNBPXaII would be vital to allow for a deeper understanding of the effects of the different types of PkNBPXaII in *P. knowlesi* infection.

**Figure 3.** Erythrocyte binding assays of COS-7 cells transfected with (A) recombinant PkNBPXaII plasmid and (B) recombinant PkDBPαII 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 PkNBPXaII and positive control wells (red arrow). 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 4. Immunofluorescence assay of COS-7 cells transfected with (A) recombinant PkNBPXaII plasmid and (B) recombinant PkDBPxlII 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×/0.45 aperture and ×20 magnification eye piece.

Figure 5. Kyte-Doolittle hydropathy plot derived from the amino acid sequence of PkNBPXaII. 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).
ACKNOWLEDGEMENTS
This study was supported by the Long Term Research Grant Scheme (LR002A-2018) of the Ministry of Higher Education, Malaysia.

Conflict of interest
The author declares that they have no conflict of interests.

REFERENCES