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Two Genetically Distinct *Plasmodium knowlesi* Duffy Binding Protein Alpha Region II (PkDBPαII) Haplotypes Demonstrate Higher Binding Level to Fy(a+b+) Erythrocytes than Fy(a+b–) Erythrocytes

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Abstract. Invasion of human erythrocytes by merozoites of *Plasmodium knowlesi* involves interaction between the *P. knowlesi* Duffy binding protein alpha region II (PkDBPαII) and Duffy antigen receptor for chemokines (DARCs) on the erythrocytes. Information is scarce on the binding level of PkDBPαII to different Duffy antigens, Fy^a and Fy^b. This study aims to measure the binding level of two genetically distinct PkDBPαII haplotypes to Fy(a+b–) and Fy(a+b+) human erythrocytes using erythrocyte-binding assay. The binding level of PkDBPαII of Peninsular Malaysian and Malaysian Borneon haplotypes to erythrocytes was determined by counting the number of rosettes formed in the assay. Overall, the Peninsular Malaysian haplotype displayed higher binding activity than the Malaysian Borneon haplotype. Both haplotypes exhibit the same preference to Fy(a+b+) compared with Fy(a+b–), hence justifying the vital role of Fy^b in the binding to PkDBPαII. Further studies are needed to investigate the *P. knowlesi* susceptibility on individuals with different Duffy blood groups.

Plasmodium knowlesi, the fifth human malaria parasite, has been reported to cause infections in many of the Southeast Asian countries in recent years. In Malaysia, the burden of *P. knowlesi* cases has surpassed those of *Plasmodium vivax*, thus acknowledged as the main cause of human malaria in the country.[1]

The asexual multiplication of *P. knowlesi* relies on the invasion of its merozoites into human erythrocytes involving a complex molecular interaction between *P. knowlesi* Duffy binding protein alpha (PkDBPα) and the Duffy antigen receptor for chemokines (DARCs) on human erythrocytes, which serve as the ligand and the receptor, respectively.[2] *P. knowlesi* Duffy binding protein alpha, a large protein that consists of seven regions (I–VII), has its binding domains found in the cysteine-rich region II;[3] therefore, the binding domain is termed as *P. knowlesi* Duffy binding protein alpha region II (PkDBPαII).

Plasmodium knowlesi Duffy binding protein alpha region II isolates from Malaysia were found to be genetically distinct based on geographical separation (Peninsular Malaysia and Malaysian Borneo), following the discovery of genetic diversity among PkDBPαII isolates.[4,5] The inability of *P. knowlesi* to invade human erythrocytes without Duffy determinants affirms the role of DARCs as the obligate receptor for *P. knowlesi*.[6] The two immunologically distinct human erythrocyte Duffy antigens, Fy^a and Fy^b, are encoded by alleles that follow codominant inheritance. This gives rise to four Duffy phenotypes, Fy(a+b–), Fy(a–b+), Fy(a+b+), and Fy(a–b–).[7] Generally, Fy(a+b–) is primarily found in Peninsular Malaysia, whereas Fy(a+b+) predominates in indigenous population originating from Malaysian Borneo.[8]

Apart from *P. knowlesi*, *P. vivax* also uses the Duffy receptor on erythrocytes to mediate invasion. It has been noticed that the Duffy binding protein region II of *P. vivax* (PvDBPII) has a higher binding level to Fy^b than Fy^a. Moreover, the lower binding level to Fy^a correlates with a lower risk to acquire clinical vivax malaria.[9] This had stimulated the interest in PkDBPαII, which strictly relies on Duffy antigens for merozoite invasion into human erythrocytes. To date, comparative studies on the binding of PkDBPαII to different Duffy blood phenotypes are lacking.

Although *P. knowlesi* infection is widespread across all Southeast Asian countries, its exceptionally high burden in Malaysian Borneo is rather puzzling. With the ubiquity of Fy(a+b+) blood group in Malaysian Borneo, the Duffy antigen difference may have a significant role in influencing the binding of PkDBPαII to erythrocytes. The aim of this study was to measure and compare the binding level of human Fy(a+b–) and Fy(a+b+) erythrocytes to two genetically distinct PkDBPαII haplotypes.

The use of human blood samples in the present study was approved by the University of Malaya Medical Centre Medical Ethics Committee (MEC Ref. No.: 817.17). Individuals with blood group phenotypes Fy(a+b–) and Fy(a+b+) were recruited to participate in this study ($n = 4$ for each Duffy blood group). These individuals consented to participate in this study. Duffy group genotyping was conducted using allele-specific PCR based on the method described previously with the primers in Table 1.[10]

Two PkDBPαII recombinant protein constructs representing the most prevalent haplotype in Peninsular Malaysia and Malaysian Borneo, respectively, were obtained from a previous study by Lim et al.[11] Haplotypes H2 and H47 were selected as representative for Peninsular Malaysia and Malaysian Borneo, respectively. Haplotype H2 has the highest frequency (19/60) among PkDBPαII sequences from Peninsular Malaysian isolates,[5] whereas haplotype H47 has the highest frequency (10/49) among PkDBPαII sequences from Malaysian Borneon isolates.[4] The PkDBPαII recombinant proteins were expressed on the surface of COS-7 (ATCC® CRL-1651™) mammalian cells. Protein expression and erythrocyte-binding assays were performed as previously described.[11,12] The binding level of PkDBPαII haplotypes to erythrocytes was determined by counting the number of rosettes formed in the assay. COS-7 cells transfected with

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TABLE 1
Primers for Duffy group genotyping

Name of the primer	Primer sequence (5'→3')
FY_forward	CCCTCATTAGTCCTTGGCTCTTCT
FY*A_reverse	CAGCTGCTTCCAGGTTGGCTC
FY*B_reverse	CAGCTGCTTCCAGGTTGGCTT
FY*BES_forward	CCCTCATTAGTCCTTGGCTCTTTC

pDisplay-*AcGFP1* without *PkDBPαII* gene (empty vector), and non-transfected COS-7 cells were used as negative controls. All erythrocyte-binding assays were technically duplicated.

Statistical analysis was performed using SPSS (ver. 20) statistical software (IBM Corp., Chicago, IL). Separate independent *t*-test analysis was applied to the two PkDBPαII haplotypes to compare the mean difference between the binding level (rosettes formation) of Fy(a+b–) and Fy(a+b+) erythrocytes to PkDBPαII. A *P*-value less than 0.05 indicates a significant difference.

In this study, a higher binding level was observed in Fy(a+b+) than Fy(a+b–) erythrocytes in both haplotypes (Figure 1). The numbers of rosettes formed in the Peninsular Malaysian haplotype (with both Fy[a+b–] and Fy[a+b+]) were higher than those in the Malaysian Borneon haplotype (with both Fy[a+b–] and Fy[a+b+]). For the Peninsular Malaysian haplotype, the number of rosettes formed (mean ± SD) with Fy(a+b–) and Fy(a+b+) erythrocytes was 27.00 ± 4.97 and 65.00 ± 17.32, respectively ($P = 0.006$). When assayed with the Malaysian Borneon haplotype, 3.50 ± 1.91 and 26.00 ± 10.42 rosettes were obtained for Fy(a+b–) and Fy(a+b+) erythrocytes, respectively ($P = 0.021$) (Table 2). The rosette number difference observed for Fy(a+b–) and Fy(a+b+) erythrocytes was significant for both haplotypes. For the Peninsular Malaysian haplotype, the number of rosettes formed in Fy(a+b+) was 2.4-fold higher than that formed in Fy(a+b–), whereas for the Malaysian Borneon haplotype, the number of rosettes observed in Fy(a+b+) was 7.4-fold higher than that observed in Fy(a+b–) erythrocytes. Meanwhile, no rosettes were observed in negative controls.

The interaction between PkDBPαII and Duffy antigen on the surface of erythrocytes is a crucial step in the invasion of *P. knowlesi* merozoites for asexual multiplication. In the present study, a significant difference was observed between the binding of PkDBPαII to different Duffy groups. A higher binding level was observed between PkDBPαII and Fy(a+b+) erythrocytes compared with Fy(a+b–). This binding pattern was found to be consistent in both Peninsular Malaysian and Malaysian Borneon haplotypes. This phenomenon is similar to that observed between PvDBPII and human erythrocytes.

The entry of *P. vivax* into host erythrocytes is almost solely dependent on the Duffy pathway,[13] although recent studies reported some *vivax* malaria cases in Duffy-negative individuals.[14–16] Nonetheless, PvDBPII still remains as one of the most important binding proteins for *P. vivax* invasion. In a comparative study, PvDBPII was shown to exhibit higher binding to Fy^b than Fy^a. The researchers also found that individuals with Fy(a+b–) erythrocytes correlate to 30–80% reduced risk of acquiring uncomplicated clinical vivax malaria, as compared with those with Fy(a–b+).[9]

Plasmodium knowlesi Duffy binding protein alpha region II and PvDBPII are orthologous binding proteins with identical amino acid residues in the binding domain (Tyr94, Asn95,

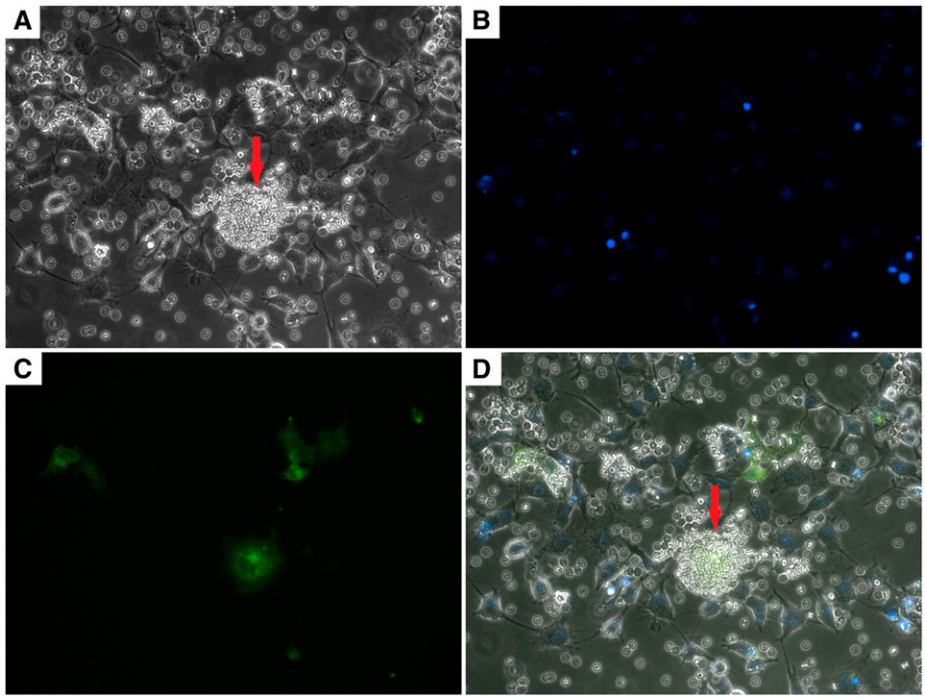

FIGURE 1. Erythrocyte-binding assay to assess the binding level of *Plasmodium knowlesi* Duffy binding protein alpha region II (PkDBPαII) to erythrocytes. (**A**) Formation of rosette showing erythrocytes covering more than 50% surface of COS-7 cell transfected with recombinant PkDBPαII (red arrow). (**B**) Cell nuclei stained with Hoechst dye were seen as blue fluorescence under 4′,6-diamidino-2-phenylindole filter. (**C**) Transfected COS-7 cells emitted green fluorescence under FITC filter, indicating expression of GFP tag cloned to C-terminal of PkDBPαII. (**D**) Merged image of **A–C** showing the location of rosette, stained nuclei, and transfected cells. This figure appears in color at www.ajtmh.org.

TABLE 2

Erythrocyte-binding assays of Peninsular Malaysian and Malaysian Borneon *Plasmodium knowlesi* Duffy binding protein alpha region II (PkDBPαII) haplotypes using Fy(a+b–) (n = 4) and Fy(a+b+) (n = 4) erythrocytes

	No. of rosettes (mean ± SD)		
Haplotypes of PkDBPαII	Blood phenotype Fy(a+b–)	Blood phenotype Fy(a+b+)	P-value*
Peninsular Malaysia	27.00 ± 4.97	65.00 ± 17.32	0.006
Malaysian Borneo	3.50 ± 1.91	26.00 ± 10.42	0.021

* $P < 0.05$ is considered statistically significant.

Lys96, Arg103, Leu168, and Ile175).[17] These binding residues are essential in the binding to Duffy determinants on erythrocytes as domain deletion abolishes PkDBPαII–DARC interaction.[18] Hence, PkDBPαII and PvDBPII demonstrate a similar preference to erythrocytes that express Fy^b antigens.

The higher binding level of PkDBPαII to Fy(a+b+) than Fy(a+b–) erythrocytes is likely attributed to the pressure of Fy^b antigen on the Fy(a+b+) erythrocyte surfaces. The amino acid substitution of Asp42 in Fy^b by Gly42 in Fy^a as a result of single nucleotide polymorphism gives rise to the immunological difference between the two Duffy antigens.[19] Because the binding of PkDBPαII to the Duffy receptor is mediated by electrostatic interactions,[20] the single amino acid difference between Fy^a and Fy^b may confer some changes in the binding ability of antigens, as Asp42 (pI = 3.2) in Fy^b is more negatively charged than the isoelectrically neutral Gly42 (pI = 6) in Fy^a.[9]

Recently, a similar study investigating the binding activity of Fy(a+b–) and Fy(a+b+) erythrocytes to PkDBPαII using erythrocyte-binding assays was reported.[12] Likewise, a higher binding level to PkDBPαII was demonstrated by Fy(a+b+) than Fy(a+b–). However, the study used only one PkDBPαII haplotype.[12] In the present study, both Peninsular Malaysian and Malaysian Borneon haplotypes of PkDBPαII were used.[4] The two geographically distinct haplotypes were observed to display different binding levels and activity to human erythrocytes.[11] Among the six amino acid residues (Y94, N95, K96, R103, L168, and I175) that are crucial for PkDBPαII–Duffy binding, all but one was conserved. The amino acid substitution occurs at position 95, in which asparagine (N) was substituted with aspartate (D) in the Malaysian Borneon haplotype. Other than N95D, a total of 11 other amino acid differences between Peninsular Malaysian and Malaysian Borneon PkDBPαII were present. It is plausible that the amino acid variations confer some changes on the 3D-conformation structure on the binding protein, thus affecting the binding ability of Malaysian Borneon PkDBPαII to Duffy antigens.[11] Regardless of their difference in terms of binding ability to the Duffy antigen, PkDBPαII of both haplotypes demonstrated a higher binding level to Fy(a+b+) than Fy(a+b–) in the present study. With the same preference to Fy(a+b+) observed in both the haplotypes, the vital role of Fy^b in the binding to PkDBPαII is justified.

In conclusion, Fy(a+b+) had a significantly higher binding level to PkDBPαII than Fy(a+b–). The same binding trend to Fy(a+b+) erythrocytes was observed for both the Peninsular Malaysian and Malaysian Borneon haplotypes of PkDBPαII. Hence, it was deduced that amino acid difference between Fy^a and Fy^b has a role in causing the different binding levels to PkDBPαII. Further studies should be performed to investigate the knowlesi infection susceptibility on individuals with different Duffy blood groups.

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