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Erythrocyte Binding Activity of PkDBPαII of *Plasmodium knowlesi* Isolated from High and Low Parasitemia Cases

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Abstract. Invasion of *Plasmodium knowlesi* merozoite into human erythrocytes involves molecular interaction between the parasite's Duffy binding protein (PkDBPαII) and the Duffy antigen receptor for chemokines on the erythrocytes. This study investigates the binding activity of human erythrocyte with PkDBPαII of *P. knowlesi* isolates from high and low parasitemic patients in an erythrocyte binding assay. The binding activity was determined by counting the number and measuring the size of rosettes formed in the assay. The protein PkDBPαII of *P. knowlesi* isolated from low parasitemia cases produced significantly higher number of rosettes with human erythrocytes than high parasitemia case isolates (65.5 ± 12.9 and 17.2 ± 5.5, respectively). Interestingly, PkDBPαII of isolates from high parasitemia cases formed significantly larger rosettes with human erythrocytes than PkDBPαII of isolates from low parasitemia cases (18,000 ± 13,000 μm^2 and 1,315 ± 623 μm^2, respectively).

Malaria is a life-threatening disease which affects millions of people in Africa, South and Central America, and almost all countries in Southeast Asia. Presently, in Malaysia, the monkey malaria parasite *Plasmodium knowlesi* is the main cause of human malaria cases.[1]

Malaria parasites cause infection in humans through proliferation in erythrocytes. *Plasmodium knowlesi* invades human erythrocytes via interaction between its Duffy binding protein (PkDBP) and the Duffy antigen receptor for chemokines (DARC) on the erythrocytes.[2] Duffy binding protein occurs in three distinct forms: alpha (α), beta (β), and gamma (γ). PkDBPβ and PkDBPγ bind only to macaque erythrocytes, whereas PkDBPα binds to both macaque and human erythrocytes. PkDBPα can be divided into seven different regions (I–VII), and region II contains the critical binding motifs for binding to DARC.[3,4]

Plasmodium knowlesi has a short erythrocytic cycle of approximately 24 hours. This quick replicating pattern can contribute to high parasitemia and possibly severe malaria. According to Daneshvar et al.,[5] the threshold for high parasitemia level of severe knowlesi cases is > 100,000 parasites/μL. It has also been suggested that a knowlesi malaria patient with ≥ 35,000 parasites/μL or 1% parasitemia can be regarded to be at high risk of developing complications.[6] Others have reported that the risk of severe knowlesi malaria could be increased with a parasitemia level of > 20,000 parasites/μL.[7]

It has been postulated that polymorphisms in invasion-related genes may lead to enhanced binding ability of *Plasmodium* parasites to human erythrocytes, thus increasing the virulence and multiplication rate of the parasites.[8] It has been reported that the polymorphisms in the *P. knowlesi* normocyte binding protein are important determinants of high parasitemia and disease severity in *P. knowlesi* infection.[9] Our previous studies have revealed genetic polymorphism in the PkDBPαII of clinical isolates from Malaysia,[10,11] and the PkDBPαII haplotypes displayed differential erythrocyte binding levels to human erythrocytes in erythrocyte-binding assays (EBAs).[12] This has therefore prompted us to investigate whether difference of the parasitemia level in *P. knowlesi* malaria can be associated with binding activity of PkDBPαII to human erythrocytes.

The use of human blood samples in this study was approved by the University of Malaya Medical Centre Medical Ethics Committee (Ref. MEC No. 817.18). *Plasmodium knowlesi* isolates of high parasitemia cases were obtained from human blood samples UM0009 (parasitemia: 27%) and BS2017_0189 (parasitemia: 6.4%), whereas isolates of low parasitemia cases were from samples UM0001 (parasitemia: 0.1%) and SBH610 (parasitemia: 0.1%).

For EBAs, the PkDBPαII of *P. knowlesi* isolates (GenBank accession numbers: MT062874–MT062877) was cloned into expression vector pDisplay-AcGFP1 and heterologously expressed on the surface of CV-1 in Origin Simian-7 (COS-7) mammalian cells, as according to the methods described previously.[12] Full details of the PkDBPαII cloning and COS-7 cell transfection are available in the Supplemental data file. Human erythrocytes were added to the transfected cells, and positive binding was shown by the formation of rosettes, which were aggregates of erythrocytes surrounding transfected cells which expressed the PkDBPαII.[12] For the determination of rosette number, 1,500 transfected cells were observed at ×20 magnification. For determining rosette size, 10 rosettes were randomly chosen from each parasitemia group, and their sizes were measured accordingly using an imaging software (NIS-Elements Basic Research version 3.07, Nikon Corporation, Japan). In the assays, six batches of human erythrocytes from the same donor were collected and used. The PkDBPαII of two different isolates were tested during each assay, with technical triplicates. The negative controls consisted of assays in which the COS-7 cells were transfected with expression vector without the PkDBPαII gene.

Statistical analysis was carried out using IBM SPSS Statistics 21 software (IBM Corp., Chicago, IL). Rosette number and rosette size difference were analyzed using independent sample *t*-test. In the test, a *P*-value of < 0.05 was considered as statistically significant.

In the EBAs, the transfected COS-7 cells were tested with human erythrocytes. Figure 1 shows the expression of PkDBPαII on COS-7 cells and adherence of human erythrocytes to the COS-7 cells to form rosettes. Results from the assays showed that the mean number of rosettes formed

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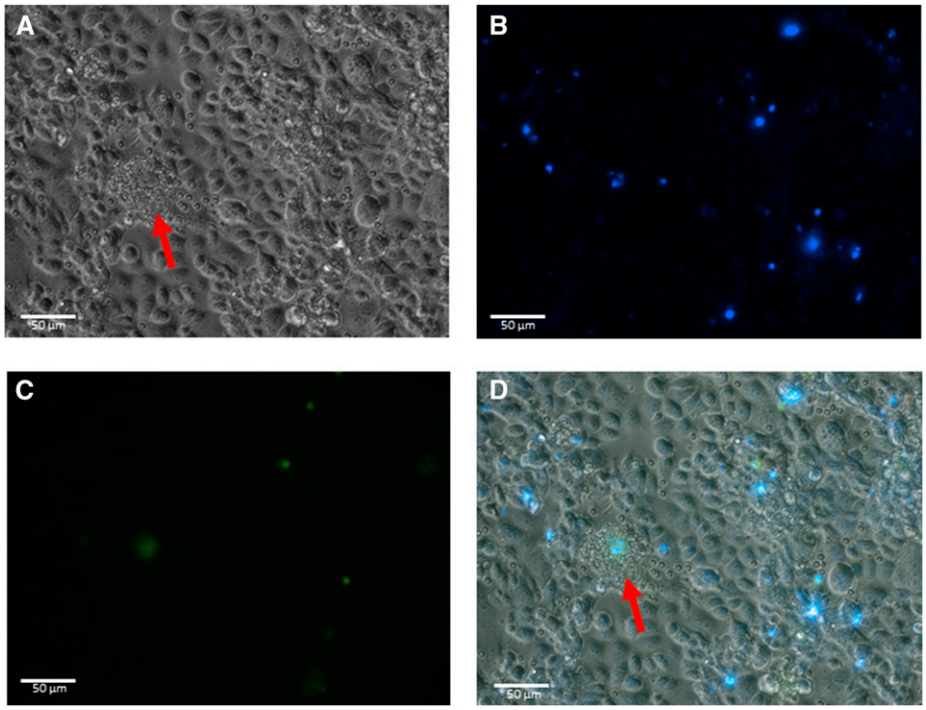

FIGURE 1. Binding activity of Duffy binding protein (PkDBPαII) to human erythrocytes in erythrocyte-binding assay. (**A**) Rosette formation (red arrow) on COS-7 cells that express PkDBPαII, more than 50% of the cell surface covered by adherent human erythrocytes. (**B**) Nuclei of COS-7 cells are stained blue with Hoechst dye. (**C**) COS-7 cells which are transfected emit green fluorescence, indicating co-expression of PkDBPαII and green fluorescent protein tag (which serves as a reporter gene in the expression system). (**D**) Merged images of (**A**, **B**, and **C**) showing the location of rosettes, transfected cells, and their nuclei. This figure appears in color at www.ajtmh.org.

between PkDBPαII of low parasitemia isolates with human erythrocytes was significantly ($P < 0.05$) higher than that of rosettes formed between PkDBPαII of high parasitemia isolates (Table 1). For the PkDBPαII of low parasitemia isolates, the mean number of rosettes formed was 65.5 ± 12.9, whereas for the PkDBPαII of high parasitemia isolates, the mean number was 17.2 ± 5.5.

Rosetting is the spontaneous binding of *Plasmodium*-infected erythrocytes to uninfected erythrocytes to form clusters of cells, and it has been observed to occur in all human malaria. Although much information on rosetting has been generated, especially in *Plasmodium falciparum*, only one study has been specifically conducted to investigate the relationship between rosetting and parasitemia level. Rowe et al.[13] assessed *P. falciparum* clinical isolates from different groups of children, and a consistent positive correlation was found between rosetting frequency and parasitemia level. In their study, rosette frequency was assessed directly on a parasite culture and by determining the percentage of parasite-infected erythrocytes that formed rosettes. This is unlike the EBA performed in our study, in which the rosettes formed were between noninfected human erythrocytes with COS-7 cells that surface-expressed the parasite ligand PkDBPαII. Therefore, this may be the possible explanation for the finding in our study, which saw an opposite trend between rosetting and parasitemia as compared with the positive correlation seen by Rowe et al.[13]

Interestingly, a positive association was observed when comparing the mean size of rosettes formed in the EBA (Table 2). The size of rosettes formed by the PkDBPαII of high parasitemia isolates was very much larger ($P < 0.05$) than that of rosettes formed by the PkDBPαII of low parasitemia isolates, with the former having mean size of 18,000 ± 13,000 µm^2 and the latter having mean size of 1,315 ± 623 µm^2. This almost 14-fold difference in rosette size in the EBA may highlight a possible association between parasitemia level and binding strength among erythrocytes within the rosettes.

It was rather unfortunate that in our study, information on severity of malaria was not recorded at the time of sample collection because of limited access to the clinical data.

TABLE 1

Mean number of rosettes formed in erythrocyte-binding assays using PkDBPαII of *Plasmodium knowlesi* isolates from low and high parasitemia cases

PkDBPαII origin	Sample	Number of rosettes*	Mean ± SD†	SE mean	*P*-value
Low parasitemia	UM0001	74	65.5 ± 12.9	5.2	< 0.05*
	SBH610	57			
High parasitemia	UM0009	17	17.2 ± 5.5	2.2	
	BS2017_0189	19			

PkDBPαII = Duffy binding protein.
* Indicates the number of rosettes which were observed in 1,500 transfected cells at ×20 magnification.
† Data shown are the means of technical triplicate data points (for each sample) which are represented by two different samples (biological replicates).

TABLE 2

Mean size of rosettes formed in erythrocyte binding assays using PkDBPαII of *Plasmodium knowlesi* isolates from low and high parasitemia cases

PkDBPαII origin	Mean ± SD (μm^2)	SE mean	*P*-value
Low parasitemia	1,315 ± 623	199	< 0.05*
High parasitemia	18,000 ± 13,000	4,108	

PkDBPαII = Duffy binding protein.
*Indicates significant statistical difference (< 5% probabality) between the mean size of rosettes formed by the PkDBPαII of low and high parasitemia isolates.

Nonetheless, by looking at parasitemia alone, the rosette size produced by the PkDBPαII of isolates from high parasitemia level was significantly high. It has been argued that rosette formation in natural malaria infection is a factor in determining clinical outcome, with large rosettes or those which are particularly resistant to physiological shear forces being more likely to result in severe disease.[14] Furthermore, it has been suggested that rosetting could intensify the parasite growth and survival by facilitating invasion or promoting immune evasion. This eventually may allow hyperparasitemia to develop and increase the likelihood of severe malaria.[13]

In this study, the number of rosettes and size of rosettes are not positively correlated, and the reason of this remains unclear. Further investigation regarding the relationship between these two variables is deemed necessary. Rosetting in the binding assay may not be a simple direct interaction, but instead may involve a complex mechanism that involves multiple receptor–ligand interactions. It has been reported that rosettes in severe malaria patients bound to more receptors than those in mild disease patients, and this resulted in larger and tighter cell aggregates.[15]

The molecular mechanisms and functions of rosetting in natural *P. falciparum* and *Plasmodium vivax* infections have been deeply investigated. However, this cannot be said for *P. knowlesi*. Until today, rosetting and its clinical implications in human knowlesi malaria have yet to be reported or explored. It is hoped that the findings of our study will trigger rigorous research on rosetting in human knowlesi malaria, particularly in cases from Malaysia Borneo where severe malaria and hyperparasitemia are frequently encountered.[6,7]

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