Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading

Genetic diversity of PfEMP1-DBL 1-α and its association with severe malaria in a hyperendemic state of India

Ronnaly Rout[1], Gunanidhi Dhangadamajhi[1], Biranchi N Mohapatra[2], Shantanu K Kar[1], Manoranjan Ranjit[1*]

[1]*Regional Medical Research Centre (ICMR), Bhubaneswar-751023, Orissa, India*
[2]*SCB Medical College & Hospital, Cuttack-753007, Orissa, India*

ARTICLE INFO

Article history:
Received 31 May 2010
Received in revised form 15 June 2010
Accepted 29 June 2010
Available online 20 July 2010

Keywords:
Plasmodium falciparum
Rosetting,
Parasite density
DBL 1-α
Severe malaria

ABSTRACT

Objective: To find out the extent of duffy-binding-like (DBL) α gene diversity and the rosetting potential of the parasite population in association with severe malaria. **Methods:** Genotyping of DBL α domain was done by PCR using three sets of primers (FR, F1R2 and F2R2) and the rosetting frequency was assessed by parasite culture followed by ethidium bromide staining and visualization under a fluorescent microscope. **Results:** The significant association of high parasite density with severe malaria and the positive correlation between rosetting frequency and parasite density *in vivo* ($\rho = 0.613$, $P < 0.0001$) were observed. Moreover, the parasite strains having multiple fragments of F2R2 region and 'b' variant of FR region of DBL 1-α showed increased rosetting frequency and supported the strain specific association of disease severity. **Conclusions:** The findings suggest that rosetting mediated higher parasitemia might have contributed to the development of severe disease. As the rosetting domain of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), the DBL α binds to multiple host receptors; the significant association of multiple fragments of F2R2 region with severe malaria suggests several receptor-ligand interactions as the possible mechanisms of pathogenesis. Alternatively, the high percentage distribution of smaller fragments with mild malaria suggests the lack of adequate rosetting epitopes that might have contributed to low rosetting frequency in mild malaria.

1. Introduction

The symptoms of malaria occur during the blood stage of infection and display a spectrum of disease severity ranging from asymptomatic to severe disease, including severe anemia, cerebral malaria and eventually death[1]. The various clinical features of malaria are thought to occur because of a combination of a high parasite burden and the ability of parasitized erythrocytes to adhere to vascular endothelial cells (cytoadherence), uninfected erythrocytes (rosetting) and platelets (clumping or autoagglutination[2]. All these phenomena have been linked to interactions between parasite-encoded, clonally variant antigen *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) on the surface of parasitized erythrocytes and host receptors[3]. Cell adhesive properties of infected erythrocytes (IEs) cause microvascular obstruction, reduced oxygen supply and impaired tissue perfusion and likely to develop severe disease and death of the host[4]. However, which phenotype (s) contribute to life-threatening malaria has not been proved. There is strong evidence that rosetting contributes to severe malaria in sub-Saharan Africa and not in Papua New Guinea (PNG) and South East Asia[5] suggesting the possibility of geographic variation in rosetting phenotypes causing severe malaria. However, the identity of other parasite adhesion phenotypes that are implicated in disease pathogenesis remains unclear. The PfEMP1 is encoded by repertoire of around 60 *var* genes family but only one is expressed at a time giving rise to an antigenically distinct PfEMP1 variant[6]. The switching of *var* gene expression in each new asexual blood stage cycle gives rise to antigenic variation in malaria that helps in evading the immune responses and may result in new adhesion phenotype[7]. The PfEMP1 variants contain extracellular regions consisting of tandemly arranged cysteine-rich domains called duffy-binding-like (DBL), cysteine-rich interdomain regions (CIDR) and C2 domains. The number,

*Corresponding author: Dr Manoranjan Ranjit, Scientist D, Regional Medical Research Centre, Indian Council of Medical Research, Chandrasekharpur, Bhubaneswar, Orissa, India-751023.
Tel: +91-9437488192
Fax: +91-674-2301351
E-mail: ranjit62@gmail.com

location and type of DBL and CIDR domains vary among PfEMP1 variants[8]. Of the six different types of DBL domains (α, β, γ, δ, ε and X), rosetting is mediated by DBL 1-α domain of PfEMP1 by means of several specific receptors, which include complement receptor 1 (CR1), the blood group antigen ABO and heparan sulfate like glucosoaminoglycan[9]. Although DBL1-α is a hyper variable protein domain, some studies have shown specific DBL 1-α sequence in relation to rosette formation[10].

Since, Odisha (formerly known as Orissa, an eastern India state) is hyperendemic for malaria, more than 85% of malaria cases are due to *Plasmodium falciparum* (*P. falciparum*) and contribute maximum number of malaria deaths of the country[11]. Better understanding of rosetting in the pathology of severe malaria is essential in this region. Thus, in the present study, effort has been made to examine the extent of DBL1-α gene diversity and rosetting potential of the parasite population in association with severe malaria.

2. Materials and methods

2.1. Study site and field isolates

The study was conducted between April 2008 to October 2009 in SCB Medical College and Hospital, Cuttack of Odisha. Blood samples were collected from clinically suspected malaria patients and were screened for *P. falciparum* infection using both thick and thin film methods. Severity of malaria was classified according to the definitions and associated characters as described by the WHO[12]. The inclusion criteria for the uncomplicated malaria cases were axillary temperature >37.5 ℃ or symptoms of headache, fever and myalgia, no schizontaemia, no intake of antimalarial drugs within the preceding week and no history of hospitalization (to exclude those who already had a severe malarial attack). For cerebral malaria cases were unarousable coma for > 6 hrs after severe convulsions and negative for other causes of cerebral involvement, axillary temperature > 37.5 ℃. Exclusion criteria were (Ⅰ) confirmed diagnosis of co-infection with other *Plasmodium* species, (Ⅱ) symptoms of mild or severe malaria with other acute infections including intestinal geohelminthic infections, (Ⅲ) chronic diseases like tuberculosis, leprosy and malnutrition. Each patient was treated according to the local guidelines and care was provided until discharge from the hospital. The study was approved by the Ethical committee of the Regional Medical Research Centre, Bhubaneswar.

2.2. Isolation of DNA and genotyping of PfEMP1-DBL1-α

DNA was isolated from 100 μL of blood following the standard protocol[13]. In brief, erythrocytes were lysed with lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, Ribonuclease (RNase) 20 μg/mL, 0.5% sodium dodecyl sulfate (SDS) and proteinase K 100 μg/mL) at 55 ℃ for 16 hrs. DNA was obtained by phenol extraction or ethanol precipitation and resuspended in 50 μL of DNase free water. PCR amplification of *var* DBL1-α sequences was obtained using three sets of primers (DBL1-α-FR, DBL 1-α-F1R2 and DBL 1-α-F2R2) as described by Ozarkar *et al*[14]. The amplified products were resolved by polyacrylamide gel electrophoresis.

2.3. Parasite culture and rosetting assessment

For parasite culture about 1 mL of *P. falciparum* infected blood were collected in Acid-Citrate-Dextrase (ACD) vials and transported to the laboratory for immediate culture following the protocol of Rowe *et al*[15]. Briefly, after removal of lymphocytes, samples were washed and cultured with Roswell Park Memorial Institute (RPMI) medium supplemented with (25 mM HEPES, 2 mM glutamine, 25 mM glucose, 25 μg/mL gentamicin, and 10% human AB serum) at 1-2% haematocrit at 37 ℃ and gassed with 5% CO_2. Parasites were grown for at least 16 hrs *in vitro*, and maturity was assessed with Geimsa stained thin films. No additional erythrocytes were added during this short culture step. Only those that matured to the pigmented trophozoite stage were assessed for rosetting during the first cycle of *in vitro* growth. An aliquot of culture suspension was stained with 25 μg/mL ethidium bromide for 5 minutes at 37 ℃. A wet preparation of the culture suspension at 2% haematocrit was viewed under fluorescent microscope at 40× objective lens. At least 200 infected cells were counted and the mature infected RBCs binding to two or more uninfected RBCs were scored as a rosette. The rosetting frequency was expressed as the percentage of rosettes in 200 infected cells.

2.4. Statistical analysis

All statistical analysis was performed with Graphpad Prism (version 4.0). Data were analyzed by Mann-Whitney U test, spearman's rank correlation coefficient and chi-square test.

3. Results

A total of 109 patients from clinically mild malaria group and 115 patients from severe malaria group were enrolled. Amongst the total cases, 82 samples were freshly drawn and subjected to immediate culture, of which 14 failed to grow and 12 were excluded because the parasitaemia was too low to assess rosetting (<0.5%). Only 56 samples which grew to maturity were assessed for resetting, 18 samples were from severe cases and 38 samples were from mild cases. The characteristics of the recruited patients are shown in Table 1. To explore the hypothesis that DBL 1-α polymorphisms could have some role in rosetting leading to severe malaria, three primer sets DBL 1-α - F1R2, F2R2 and FR were included (Figure 1) for DBL 1-α polymorphisms which amplified overall two (a, b) three (a, b, c) and four (a, b, c, d) types of variants respectively and varied among mild and severe malaria cases. The rosetting frequency was found to be significantly high (Mann-Whitney U test, P= 0.0053) in severe malaria cases (median, 33.0%; interquartile range, 20.0-52.0%) compared to mild malaria (median, 15.0%; interquartile range, 10.0-32.0%) (Figure 2A). A moderately significant positive correlation of parasite density with rosetting frequency (spearman's correlation $\rho = 0.613$, P <0.0001) (Figure 2B) was observed. The variants were

Table 1
Clinical and laboratory parameters in patients with severe and mild malaria.

Variable	Subjects with mild malaria(n=109)	Subjects with severe malaria (n=115)
Male/Female (n)	62/47	76/39
Mean age (years)	34.6	36.8
Hemoglobin (Mean±SD) (g/dL)	10.52±1.65	9.79±1.92
Unarousable state* (n)	0	115
Convulsions* (n)	0	47
Mean parasite density *(counts/ μ L)	3 827	45 601

*:P<0.05.

named according to ascending order of their sizes with 'a' fragment being the smallest in each set of primers (Table 2). The results showed that parasite strains having the single fragment of F2R2 region ('a' and 'b') were associated with mild malaria whereas multiple fragments (ab and bc) of F2R2 (Figure 3C) were associated with severe malaria (χ^2 = 21.78; OR= 3.8, P< 0.0001). Although, no such relations were obtained for FR region, the percentage distribution of 'a' fragment appeared to be more in mild malaria cases, whether it was alone (18.4% in mild vs. 10.2% in severe malaria) or with other fragments (Figure 3A). The DBL 1– α –F1R2 did not show any difference of variants in mild and severe malaria cases (Figure 3B). Furthermore, when the rosette frequency were correlated with different size variants of DBL1– α region, the 'b' variant of FR region whose percentage distribution is more in severe malaria cases compared to mild and the multiple fragments (ab and bc) of F2R2 were observed to have high rosette frequency indicating strain specificity of higher rosetting leading to disease severity. The variants of DBL 1– α –F1R2 did not show any observed difference in rosetting frequency.

Table 2
Amplified product size for FR, F1R2 and F2R2 region of DBL1– α .

Approximate fragment size(bp)	FR	F1R2	F2R2
350	+	–	–
380	+	–	–
400	+	–	–
430	+	–	–
550	–	–	+
590	–	+	+
620	–	+	+

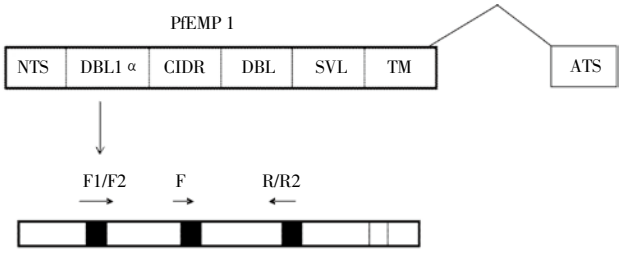

Figure 1. Schematic diagram of the DBL1 α region amplified using the three primers sets. The primer sets are: DBL α – F1R2, DBL α – F2R2 and DBL α – FR.

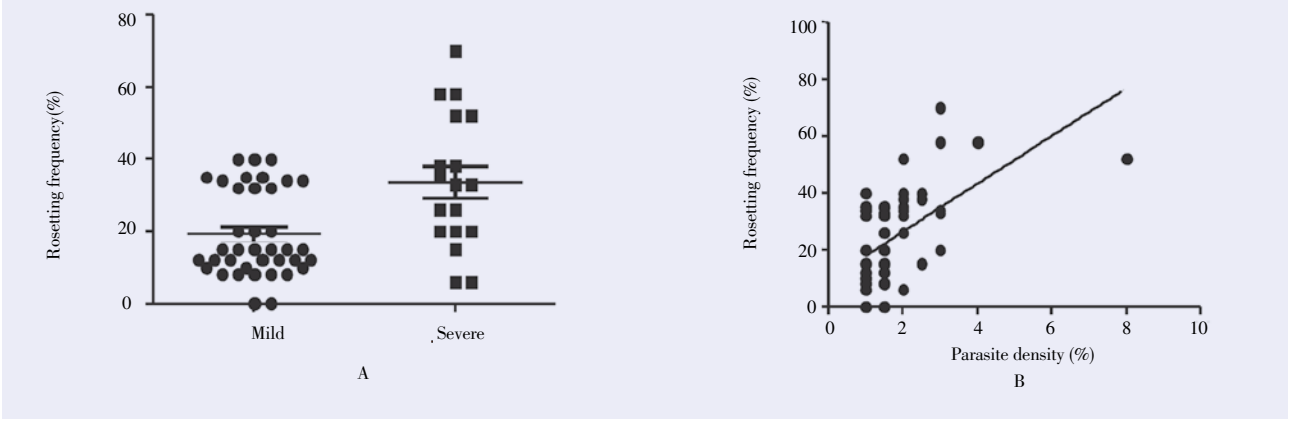

Figure 2. Significant association of rosetting frequency with severe malaria and its positive correlation with parasite density.

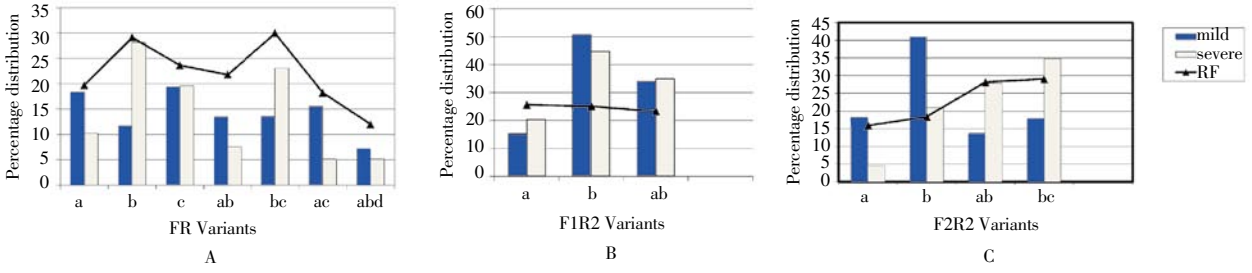

Figure 3. Mean rosetting frequency distribution amongst genetic variants of DBL 1– α and percentage distribution of these variants.
A: DBL α –FR variants; B: DBL α –F1R2 variants; C: DBL α –F2R2 variants in mild and severe malaria cases.

4. Discussion

The pathogenesis of severe malaria is multifaceted and several factors are involved in determining whether an individual with *P. falciparum* infection develops severe malaria. *P. falciparum* isolates that form rosettes with uninfected RBCs have been associated with severe malaria in many African studies but not in South East Asia or PNG[5]. In the present study, we have observed a significantly high rosetting frequency in severe malaria group of patients compared to that of mild malaria. The reasons for such discrepancies in different endemic regions are not known clearly and can be attributed to the regional differences of parasite population or host genetic make-up and/or their interactions. The observation of high frequency of complement receptor 1 (CR1) deficiency mutant in PNG[16], and high incidence of α-thalasemia along with HbE variant in South East Asian populations, which impairs rosette formations[17] supports the endemic differences of host genetic factors. Although, the mechanisms by which rosetting becomes virulence are still unknown, the possible mechanisms include (Ⅰ) greater microvascular obstruction to blood flow[18]; (Ⅱ) elevated parasite densities in favoring invasion of the merozoites directly to the uninfected RBCs in the rosette, without passing through the host plasma[19]; and (Ⅲ) immune evasion by shielding the infected red blood cell (iRBC) from phagocytic cells or antibodies[20].

The experimental evidences to support or refute these hypotheses are very few and culture adapted *P. falciparum* clone showed no increased invasion efficiency in rosetting compared to non rosetting parasites[21]. However, a positive correlation between rosetting and parasitemia have been observed in natural isolates of Kenya[19], Thailand[22], PNG[5] and by us in the present study (Correlation coefficient $\rho = 0.613$, $P<0.001$). Owing to the fact that rosetting is an inherent property of a given *P. falciparum* isolate and in the culture shown to be not directly influenced by the parasitemia[19], association of increased rosetting frequency with high parasite density *in vivo* irrespective of whether it is associated or not with severe malaria could be due to enhancement of invasion and/or immune evasion. Furthermore, the significant association of high parasite density with severe malaria suggests that rosetting mediated higher parasitemia might have contributed to the development of severity of the disease.

Since DBL 1-α, the rosetting domain of PfEMP1 is highly variable in length and amino acid sequences[23], to explore the hypothesis that differences in parasite strains with respect to DBL 1-α polymorphisms could have certain role in rosetting leading to severe malaria, we tried to correlate genetic polymorphisms in DBL 1-α and its association with clinical outcome of the disease. The results showed that parasite strains having the single fragment of F2R2 region were associated with mild malaria whereas multiple fragments were associated with severe malaria ($P<0.0001$). Although, no such relations were obtained for FR region, the percentage distribution of 'a' fragment appeared to be more in mild malaria cases, whether it was alone or with other fragments. It is not possible at this point to explain how such variations are associated with clinical outcome.

However, the fact that the iRBCs express only one PfEMP-1 molecule irrespective of whether the iRBCs contain single or multiple parasite clones[24], PfEMP-1 fragment of the same size can differ significantly in sequence[10], and sequencing of PfEMP-1 RNA could have helped in identifying the region potentially involved in rosetting are the drawback of present study. Furthermore, in order to find out whether the multiple fragments were the consequence of multiplicity of infections, when we analyzed a subset of mono infected samples (revealed from MSP1 and MSP2 typing, data not shown) for DBL 1-α diversity, all these multiple fragments were observed which indicates that differences in parasite strains with respect to DBL 1-α polymorphisms might have contributed to different clinical manifestations. Moreover, the observation of high rosette frequency for 'b' allele of FR region and multiple fragments of F2R2 region supports the strain specific association of disease severity. However, the role of multiplicity of infections, which may also produce multiple fragments in multiply infected clinical isolates, cannot be ruled out. As the rosetting domain of PfEMP1, the DBL 1-α binds to multiple host receptors[9] the significant association of multiple fragments of F2R2 region with severe malaria suggests several receptor-ligand interactions as the possible mechanisms of pathogenesis. Alternatively, the high percentage distribution of smaller fragments ('a' of FR and 'a' and 'b' of F2R2) with mild malaria suggests the lack of adequate rosetting epitopes in these fragments that might have contributed to low rosetting frequency in mild malaria cases which in turns reflected the low parasite density in these group of patients.

Moreover, the absence of significant association of rosetting frequency with severe malaria in certain geographical areas[5] and the observation of very low or no rosetting (frequency<5%) in some parasite isolates derived from severe malaria in the present study suggests the involvement of some other unidentified factors in malaria pathogenesis and it remains to be ascertained whether rosetting itself is important in pathogenesis or is simply a marker for some other factor, which mediates the disease process.

In conclusion, our study reveals that rosetting mediated higher parasitemia might have contributed to the development of severity of the disease and may be dependent on parasite strains or host genetic make-up and/or their interactions. Further works need to be carried out to explore the DBL 1-α diversity in relation to rosette formations in different endemic regions and its association with clinical outcome.

Conflict of interest statement

The authors have no conflicts of interest connecting to the work reported in this paper

Acknowledgements

The authors thankfully acknowledge Indian Council of Medical Research, New Delhi for financial support and to the Director RMRC, Bhubaneswar for providing necessary laboratory facilities for the study. We also acknowledge University Grant Commission (UGC) and Council of Scientific and Industrial Research (CSIR), New Delhi for providing fellowships to Mrs. Ronnaly Rout and Mr G Dhangadamajhi respectively to carry out the research work. The authors are grateful to the patients who participated in the study.

References

[1]Mackintosh CL, Beeson JG, Marsh K. Clinical features and pathogenesis of severe malaria. *Trends Parasitol* 2004; **20**: 597-603.

[2]Rowe JA, Claessens A, Corrigan RA, Arman M. Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. *Expert Rev Mol Med* 2009; **11**: e16.

[3]Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. *Nature* 2002; **415**: 673-9.

[4]Dondorp AM, Ince C, Charunwatthana P, Hanson J, van Kuijen A, Faiz MA, et al. Direct *in vivo* assessment of microcirculatory dysfunction in severe falciparum malaria. *J Infect Dis* 2008; **197**: 79-84.

[5]Mercereau-Puijalon O, Guilotte M, Vigan-Womas I. Rosetting in *Plasmodium falciparum*: A cytoadherence phenotype with multiple actors. *Trans Clin Biol* 2008; **15**: 62-71.

[6]Kraemer SM, Smith JD. A family affair: *var* genes, PfEMP1 binding, and malaria disease. *Curr Opin Microbiol* 2006; **l9**: 374-80.

[7]Ralph SA, Scheidig- Benatar C, Scherf A. Antigenic variation in *Plasmodium falciparum* is associated with movement of *var* loci between subnuclear locations. *Proc Natl Acad Sci* 2005; **102**: 5414-9.

[8]Mayor A, Bir N, Sawhney R, Singh S, Pattnaik P, Singh SK, et al. Receptor-binding residues lie in central regions of Duffy-binding-like domains involved in red cell invasion and cytoadherence by malaria parasites. *Blood* 2005; **105**: 2557-63

[9]Normark J, Nilsson D, Ribacke U, Winter G, Moll K, Wheelock CE, et al. PfEMP1-DBL-1 α amino acid motifs in severe disease states of *Plasmodium falciparum* malaria. *Proc Natl Acad Sci USA* 2007; **104**: 15835-40.

[10]Horata N, Kalambaheti T, Craig A, Khusmith S. Sequence variation of PfEMP1-DBLalpha in association with rosette formation in *Plasmodium falciparum* isolates causing severe and uncomplicated malaria. *Malar J* 2009; **8**: 184-95.

[11]Ranjit MR. The epidemiology of malaria in Orissa. *ICMR Bull* 2006; **36**: 29-38.

[12]World Health Organization . *WHO expert committee on malaria: WHO technical report series 892,I-V*. Geneva: World Health Organization; 2000.

[13]Sambrook J, Russel DW. *Molecular cloning- A laboratory manual*. The 3rd ed. New York: Col Spring Harbor Laboratory Press; 2001, p. 6.4-6.11.

[14]Ozarkar AD, Prakash D, Deobagkar DN, Deobagkar DD. Distinct polymorphism of DBL α domain of the *var* gene in laboratory-cultured and clinical field isolates of *Plasmodium falciparum* malaria parasites. *Curr Sci* 2007; **93**: 219-23.

[15]Rowe JA, Handel IG, Thera MA, Deans AM, Lyke KE, Koné A, et al. Blood group O protects against severe *Plasmodium falciparum* malaria through the mechanism of reduced rosetting. *Proc Natl Acad Sci* 2007; **104**: 17471-6.

[16]Cockburn IA, Mackinnon MJ, O'Donnell A, Allen SJ, Moulds JM, Baisor M, et al. A human complement receptor 1 polymorphism that reduces *Plasmodium falciparum* rosetting confers protection against severe malaria. *Proc Natl Acad Sci* 2004; **101**: 272-7.

[17]Fucharoen S, Sanchaisuriya K, Fucharoen G, Panyasai S, Devenish R, Luy L. Interaction of hemoglobin E and several forms of alpha-thalassemia in Cambodian families. *Haematologica* 2003; **88**: 1092-8.

[18]Nash GB, Cooke B.M, Cralson J, Wahlgren M. Rheological properties of rosettes formed by red blood cells parasitized by *Plasmodium falciparum*. *Br J Haematol* 1992; **82**:757-63.

[19]Rowe JA, Obeiro J, Marsh K, Raza A. Short report: Positive correlation between rosetting and parasitaemia in *P. falciparum* clinical isolates. *Am J Trop Med Hyg* 2002; **66**: 458-60.

[20]Deans AM, Rowe JA. *Plasmodium falciparum*: Rosettes do not protect merozoites from invasion-inhibitory antibodies. *Exp Parasitol* 2006; **112**: 269-73.

[21]Clough B, Atilola FA, Pasvoi G. The role of rosetting in the multiplication of *Plasmodium falciparum*: rosette formation neither enhances nor targets parasite invasion into uninfected red cells. *Br J Haematol* 1998; **100**: 99-104.

[22]Chotivanich K, Udomsangpetch R, Simpson JA, Newton P, Pukrittayakamee S, Looareesuwan S, et al. Parasite multiplication potential and the severity of falciparum malaria. *J Infect Dis* 2000; **181**: 1206-9.

[23]Fowler EV, Peters JM, Gatton ML, Chen N, Cheng Q. Genetic diversity of the DBL alpha region in *Plasmodium falciparum var* genes among Asia-Pacific isolates. *Mol Biochem Parasitol* 2002; **120**: 117-26.

[24]Wang CW, Hermsen CC, Sauerwein RW, Arnot DE, Theander TG, Lavstsen T. The *Plasmodium falciparum var* gene transcription strategy at the onset of blood stage infection in a human volunteer. *Parasitol Int* 2009; **58**:478-80.