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Erythrocyte invasions and receptor heterogeneity in field isolates of Nanay river basin Iquitos

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ABSTRACT

Objective: To determine whether the requirements for sialic acid varies and whether several types of silaic acid independent receptors utilized for invasion mechanisms of fresh filed isolates collected around Nanay river basin, Iquitos. **Methods:** The field isolates were cultured as described previously by Jensen and Trager and MR4 protocol with little modifications. The erythrocytes preparation and subsequent enzyme treatment was done as described previously by Sharma. with little modification. Invasion assay was performed as described previously by Sharma *et al* with little modification. **Results:** The Nanay river basin isolates showed five types of invasion mechanisms or types of receptors-ligand interactions. Here we observed that an equal numbers of neuraminidase sensitive and resistant invasion receptor-ligand interaction profiles as the most common receptor-ligand invasion profiles. Neuraminidase resistance trypsin sensitive chymotrypsin sensitive ($NM_RT_SCT_S$) invasion of receptor-ligand interaction profile was found in seven isolates, Five field isolates and one reference strain showed neuraminidase sensitive, trypsin sensitive and chymotrypsin resistant ($NM_ST_SCT_R$) invasion of receptor-ligand interactions, six isolates including one reference strains dd2 showed neuraminidase sensitive, trypsin and chymotrypsin resistance ($NM_ST_RCT_R$) indicating its dependence on sialic acids and independence of trypsin and chymotrypsin sensitive proteins. Four isolates showed neuraminidase sensitive, trypsin sensitive and chymotrypsin sensitive ($NM_ST_SCT_S$) invasion of receptor-ligand interactions, seven isolates were neuraminidase resistant, trypsin sensitive and chymotrypsin resistance ($NM_RT_SCT_R$) invasion of receptor-ligand interactions, indicating its dependence on trypsin sensitive proteins. **Conclusions:** The Nanay river basin isolates showed five types of invasion mechanisms or types of receptors-ligand interactions. A full understanding of theses invasion mechanisms may allow the development of novel prophylactic and therapeutic strategies that block erythrocyte receptor-ligand invasion mechanisms.

1. Introduction

Plasmodium falciparum (P. falciparum) is the causative agent of the most virulent form of human malaria and up to 2.7 million deaths annually have been attributed to this pathogen. Entry of malaria parasites into its host erythrocyte initiates the intra-erythrocyte asexual cycle that is central to the pathogenesis of this devastating pathogen. The process by which a merozoite invades RBC is crucial to the survival of the parasite and ensures maintenance of the blood stage infection. Interference with this process would prevent disease as merozoites are short-lived outside the host cell. Merozoite function is to gain entry to a new red cell. Once entered inside a new red cell where it changes markedly, then grows, and divides. After 48 h, the products of this division are released as sixteen or so new merozoites[1,2]. RBC invasion by merozoite comprises of several sequential steps: initial attachment of any part of the merozoites to RBC membrane, reorientation to allow the apical end of the parasite to contact with RBC membrane, release of the contents of the apical organelles, junction formation, membrane invagination, and finally parasite entry[2]. However, the molecular mechanisms by which these processes occur remain poorly understood.

The process of invasion is a crucial, rapid stage in the life cycle of human malarial parasite *P. falciparum*. The existence of several types of molecular receptor interactions is considered to be a survival mechanism of the malarial parasite. So far, five important invasion ligand-receptor mechanisms of *P. falciparum* have been

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documented. Of these, four from EBA family as EBA-175, a 175 kD erythrocyte binding antigen with glycophorin A(GPA)[3,4], EBL-1 with glycophorin B (GPB)[5], EBA-140, a 140 kD erythrocyte binding antigen with glycophorin C (GPC) and EBA-181 a 181 kD erythrocyte binding antigen with the erythrocyte receptors B and 4.1[2,6], and one from multigene family. *Pf*RH4 binds with complement receptor-1. At least three more unidentified receptors on the erythrocyte surface as *Pf*RH1 with receptor 'Y'. *Pf*RH2 with the receptor 'Z'[2] and *Pf*RH5 with the receptor 'W'[7] have been considered to play a very important role in invasion mechanisms. The binding properties of *Pf*RH proteins are considered to determine the invasion phenotypes of *P. falciparum* isolates. The receptors 'Y' is a neuraminidase sensitive and binds with *Pf*RH1 of parasite ligand and the experiments in neuraminidase treated erythrocytes resulted in reduced invasion, showed sialic acid dependent invasion mechanisms[3]. Receptor 'Z' binds with *Pf*RH2 including *Pf*RH A and B, both are identical and different only at C-terminal region and its genetic deficient erythrocytes experiments showed that 'Z' receptors are involved in sialic acid independent mechanisms of invasion. The receptors 'W' binding with *Pf*RH5 in a manner of sialic acid independent invasion mechanisms. *Pf*RH5 are also shown to be involved in invasion in laboratory experiments because of *Pf*RH5's polymorphic nature which is believed to be involved in its receptor binding specificity[3], however the erythrocyte receptors *Pf*RH1, *Pf*RH2 including *Pf*RHa & *Pf*RHb and *Pf*RH5 remain unknown, hence the studies on invasion mechanisms and its ligand-receptors interactions attracted a large number of researchers around the world. The invasion mechanisms and related receptors-ligand interactions are identified by using genetically deficient erythrocytes having no receptors or mutant receptors on the surface and by removing the surface receptors believed to be involved in invasion mechanisms by treating the erythrocytes with specific enzymes[8]. The most commonly used enzyme are neuraminidase which removes sialic acid molecules from the erythrocyte surface which enables to determine whether the isolate is sialic acid dependent or independent. Trypsin the enzyme which removes glycophorins A and C; and chymotrypsin remove surface glycophorin B and band 4.1[8]. Most of the invasion studies have reported about the laboratory maintained isolates of *P. falciparum* not much report is available about the invasion mechanisms used by the field isolates. A study from fresh field isolates of India had shown that most of the fresh isolates 12 out of 15 utilized sialic acid independent invasion mechanisms and only 3 isolates were shown as dependents on sialic acid and glycophorin A. however, a study of field isolates from Gambia had shown that most of the isolates used the sialic acid dependent invasion mechanisms and are neuraminidase and trypsin sensitive[2]. These contrasting inferences from two geographic locations highlighted the additional studies on invasion mechanisms and receptors utilized by field isolates to design a vaccine based on erythrocyte stage antigens targeting specific invasion pathways[2]. Therefore, we investigated the erythrocyte invasion strategies and receptor heterogeneity to determine whether the requirements for sialic acid varies and whether several types of silaic acid independent receptors utilized for invasion mechanisms of fresh filed isolates collected around Nanay river basin, Iquitos.

2. Materials and methods

2.1. Study area and parasites

In Iquitos, peoples living in close proximity, and often refers to a group that shares some common values is called community. One of such community is called Zungarococha, in the San Juan District south of Iquitos, Peru. The San Juan district is an epicenter of *P. falciparum* and *Plasmodium vivax* (*P. vivax*) transmission. The community of Zungarococha is a network of four villages: Zungarococha town, Puerto Almendra, Ninarumi and Llanchama. The villages are situated on a road which curves along the Nanay River, in the order listed above, with a distance of approximately 1 km between ZG, PA, and NA (well within An. darling migration range) and approximately 5 km between ZG and LL (although possible, not likely to have mosquito migration between ZG and LL). Zungarococha was selected based upon existing reports of locally acquired *P. falciparum* malaria infections, a high *P. falciparum* incidence rate compared with communities in and surrounding Iquitos. The fact that the community is composed of four villages each separated by approximately two kilometers. Adults and children diagnosed with severe/no severe *P. falciparum* malaria based on clinical symptoms and detection of parasites in blood smears were enrolled in the study. Blood samples were collected immediately prior to treatment after obtaining informed consent from the children's parents or guardians, and protocols were approved by ethical review boards in the Peru. The isolates were collected into heparinized tubes and processed within 12 h of collection for parasite culture.

2.2. Parasite culture

The field isolates were cultured as described previously by Jensen and Trager[9] and MR4 protocol with little modifications. The collected heparinized blood sample was washed 3 times in 37 ℃ prewarmed washing medium by centrifuging 8 min at 600 *g* and buffy coat was removed after each centrifugation. The parasites were cultured in A^+ human erythrocytes at a hematocrit of approximately 5% in 25 cm^2 or 75 cm^2 flasks containing 5 or 20 mL, respectively, of RPMI1640 medium supplemented with 10% human serum type A^+, 20 μg/mL of gentamycin and 100 μm incubated in an atmosphere of 5% CO_2, 3% O_2, 92% N_2 at 37 ℃. The parasitemia was determined at 18 or 36 h, by Giemsa-stained blood smears. The two reference strains Dd2 and 3d7 were cultured by adopting the above methods used as controls in invasion assays.

2.3. PCR typing of P. falciparum isolates

The PCR typing of patient isolates were carried out to determine if they were genotypically distinct and contain single or multiple genotypes as described elsewhere[10,11]. The two polymorphic blood stage antigens, merizoite surface protein-1 (MSP-1) block 2 were subtyped by nested PCR using oligonucleotide primers specific for MSP-1. PCR products encoding blocks 2 and 3 of MSP-2 were subtyped using oligonucleotide primers specific for FC27 and 3D7 subtypes as previously described[11].

2.4. Preparation of erythrocytes

The erythrocytes preparation and subsequent enzyme

treatment was done as described previously by Sharma *et al*[8] with little modification. Briefly the erythrocytes were collected from a female Iquitos donor, age of 34 y O^+ 10 mL of venous blood was collected in two separate 5 mL dextrose vacutainer and blood was transferred under sterile conditions to a sterile 10 mL centrifuge tube and centrifuged at 2 000 *g* × 5 min and serum and buffy coat is removed and again centrifuged with additionally 10 mL wash medium. The washed erythrocytes were suspended in wash medium and stored at 4 ℃. The washed erythrocytes were treated with enzymes pre diluted in incomplete RPMI 1640 medium as trypsin 1 mg/mL (Sigma, New York); soybean trypsin inhibitor 0.5 mg/mL (Sigma, New York) and chymotrypsin 1 mg/mL (Sigma, New York). The enzyme neuraminidase (Vibrio cholerae neuraminidase; Calbiochem, Lima Peru) were diluted in double distilled water and stored at 4 ℃ in a 2.5 mL microtube 50 mU of neuraminidase in 1 mL of incomplete RPMI1640 were added and another tube treated to be control 1 mL of incomplete medium alone added. A volume of 100 μL of prepared donor erythrocytes were added into each tube having enzyme (neuraminidase, trypsin and chymotrypsin) or incomplete RPMI1640 medium only as control. The samples were placed on a rotating plate for 1 h at 37 ℃ then washed with 1ml of incomplete medium. Trypsin and chymotrypsin treated erythrocytes were added with soybean trypsin inhibitor to inactivate the remaining trypsin. Then all samples were incubated on a rotating plate for 5 min at 37 ℃ and washed twice then, re suspended in incomplete RPMI1640 and stored at 4 ℃.

2.5. Agglutination assay on enzyme treated erythrocytes.

The purpose of this assay was to ensure enzyme treatment performed successfully and removed surface epitopes successfully. After pretreatment with enzymes, 40 μL of 5% hematocrit sample was added to the equal volume of monoclonal anti-M antibody, anti-N antibody or peanut lectin (sigma) and incubated for up to 5 min with anti-S antibody or 25 min with anti-M antibody and peanut lectin at 37 ℃. The agglutination was assesses after centrifugation for 1 min at 150 *g*. The agglutination showed that complete loss of anti-M mediated agglutination evidence of successful trypsin digestion, complete loss of anti-S mediated agglutination is the evidence of chymotrypsin activity and gaining peanut lectin mediated agglutination by neuraminidase activity.

2.6. Invasion assay

Invasion assay was performed as described previously by Sharma *et al*[12] with little modification. The schizont enrichment was done by 65% percoll gradient, washed twice. The parasites were mixed with human erythrocytes untreated and enzyme treated at a final parasitemia of 1%-2%. The cultures were plated in 96 well plates at a volume of 50 μL with duplicate wells for each sample. The sample is then incubated in parasite culture chamber at 37 ℃ for 24-48 h and blood smears were prepared in duplicate and numbers of ring stage parasites present were determined by light microscope examination. Fields (50) or approximately 5 000 cells were counted. The invasion is determined by the percentage rings normalized to 100% of invasion into human erythrocytes. When no new-ring infected erythrocytes were seen and the invasion is considered as 100% inhibited, similarly when invasion of enzyme treated erythrocytes was higher than the control and is considered negative invasion due to increased invasion. Similar procedures were adopted for the two reference strains DD2 and 3D7 and all the experiments were performed in triplicate. Percent inhibition is called as follows;

$$\text{Percent inhibition: } \frac{\text{No. of rings in control} - \text{No. of rings in tested} \times 100}{\text{No .of rings in control}}$$

2.7. Statistical analysis

Invasion of isolates were tested by Krwskal-wallis *H* test; these treat the values of percent inhibition of invasion in a non-parametric manner and compares the different groups to each other. Correlations were determined with spearman's rank order correlation.

3. Results

We investigated on the 30 *P. falciparum* field isolates collected around Nanay river basin, Iquitos for invasion receptors-ligand interaction profiles and results are presented in Table 1. To facilitate the analysis of field isolates, we also studied the two reference strains DD2 and 3D7. We found that the percentage of inhibition of invasion for neuraminidase enzyme treated erythrocytes ranging from 14.9% to 98.5%; inhibition of trypsin treated erythrocytes ranging from 15.3% to 96.5% and chymotrypsin treated erythrocytes inhibition ranging from 23.8% to 93.8%.

The Nanay river basin isolates showed five types of invasion mechanisms or types of receptors-ligand interactions. Here we observed that an equal numbers of neuraminidase sensitive and resistant invasion receptor-ligand interaction profiles as the most common receptor-ligand invasion profiles. Neuraminidase resistance trypsin sensitive chymotrypsin sensitive ($NM_RT_SCT_S$) invasion of receptor-ligand interaction profile was found in seven isolates, indicating that they were sialic acid independent and dependence on trypsin and chymotrypsin sensitive proteins. Five field isolates and one reference strain showed neuraminidase sensitive , trypsin sensitive and chymotrypsin resistant ($NM_ST_SCT_R$) invasion of receptor-ligand interactions, indication its dependence on sialic acid and trypsin sensitive proteins for their invasion of receptor-ligand interactions, six isolates including one reference strains dd2 showed neuraminidase sensitive, trypsin and chymotrypsin resistance ($NM_ST_RCT_R$) indicating its dependence on sialic acids and independence of trypsin and chymotrypsin sensitive proteins. Four isolates showed neuraminidase sensitive, trypsin sensitive and chymotrypsin sensitive ($NM_ST_SCT_S$) invasion of receptor-ligand interactions, indicating dependence of sialic acid as well as trypsin and chymotrypsin sensitive proteins with unknown receptors-ligand interactions and seven isolates were neuraminidase resistant, trypsin sensitive and chymotrypsin resistance ($NM_RT_SCT_R$) invasion of receptor-ligand interactions, indicating its dependence on trypsin sensitive proteins. Each blood sample was subjected to PCR amplification of two different polymorphic loci: MSP-1(block2) and MSP-2 (block-3) to determine whether the field isolates in this study were clonal or a composite of multiple parasite lines. PCR typing based on the polymorphic markers MSP-1 and

Table 1

Alternative pathways of Peruvian Amazon field isolates collected from severe and uncomplicated *P. falciparum* malaria.

Invasion pathways	Isolates	Community	Percentage of mean inhibition		
			Neuraminidase(50 mU/mL)	Trypsin(1 mg/mL)	Chymotrypsin(1 mg/mL)
$Nm_RT_SCT_S$	7033E	Zungarococha	24.2±5.2	75.9±3.9	57.8±2.7
	1372E	Almendra	22.1±6.8	78.3±6.1	67.2±3.8
	4141E	Shiriara	32.1±4.8	73.7±5.2	70.2±7.2
	4372R	Ninarumi	14.9±5.0	90.3±5.0	75.1±1.3
	4731P	Zungarococha	21.1±3.0	82.5±3.5	73.3±4.2
	5561E	Ninarumi	23.7±3.0	88.2±7.0	63.1±1.0
	7182E	Almendra	17.3±4.2	90.8±3.8	89.1±4.7
$Nm_RT_SCT_R$	6018E	Zungarococha	14.3±3.7	80.2±6.8	45.2±3.8
	6015E	Rio Napo	13.8±2.8	83.7±5.3	42.0±3.7
	6010E	Zungarococha	10.2±1.9	87.3±7.1	55.7±2.8
	7013E	Rio Napo	12.3±3.3	91.5±5.0	50.5±7.0
	6011E	Rio Napo	13.7±4.0	96.5±7.8	53.8±6.1
	7033E	Ninarumi	13.8±3.8	92.9±6.3	45.9±4.2
	3769E	Rio Napo	14.3±5.2	85.7±5.2	44.9±3.8
$Nm_ST_SCT_S$	6510E	Ninarumi	81.7±7.2	89.0±5.1	93.8±7.1
	8761E	Zungarococha	91.2±5.1	85.3±4.3	88.7±8.8
	1811E	Rio Napo	90.1±5.2	95.2±5.3	91.3±4.7
	1903E	San Juan	90.0±6.1	93.2±7.0	87.9±4.0
$Nm_ST_SCT_R$	1720S	Rio Napo	89.3±5.8	91.5±3.3	44.0±3.7
	1375S	Almendra	92.9±3.9	88.5±4.5	47.5±4.0
	1375S	Zungarococha	97.7±7.2	90.2±6.1	46.2±5.2
	1377S	Ninarumi	96.3±5.3	90.8±7.0	41.7±7.0
	1378S	Llanchama	98.5±6.1	89.8±3.8	44.9±4.3
	3D7	Reference	72.1±5.0	76.2±3.8	23.8±4.7
$Nm_ST_RCT_R$	Dd2	Reference	80.8±6.2	26.2±4.1	31.2±3.7
	1221S	Ninarumi	91.7±4.2	15.3±3.2	11.8±4.8
	1439S	Almendra	88.6±5.8	29.8±4.3	17.6±3.0
	1459S	Zungarococha	90.2±6.1	22.5±6.0	10.0±2.9
	1988S	Ninarumi	90.8±3.3	19.8±5.0	18.2±4.8
	1989S	Almendra	87.9±4.3	23.0±6.1	13.7±3.1

$Nm_ST_RCT_R$: Neuraminidase sensitive trypsin resistant chymotrypsin resistant; $Nm_ST_SCT_R$: neuraminidase sensitive trypsin sensitive chymotrypsin resistant; $Nm_ST_SCT_S$: Neuraminidase sensitive trypsin sensitive chymotrypsin sensitive; $Nm_RT_SCT_R$: Neuraminidase resistant trypsin sensitive chymotrypsin resistant; $Nm_RT_SCT_S$: Neuraminidase resistant trypsin chymotrypsin sensitive.

MSP-2 indicates that each *P. falciparum* isolate used in the assays was unique and contains a single *P. falciparum* genotype (Figure 1, 2).

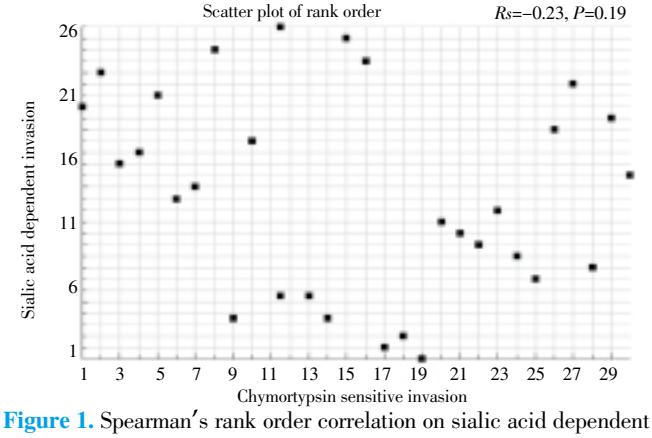

Figure 1. Spearman's rank order correlation on sialic acid dependent and chymotrypsin invasion.

There is a lack of significant correlation on invasion of enzyme treated erythrocytes between sialic acid dependent invasion and chymotrypsin. *Rs* and *P* values are derived by spearman's rank order correlation (supported by free on line spearman rank order correlation software programme version 1.1.23-r7 (Wessa 2011).

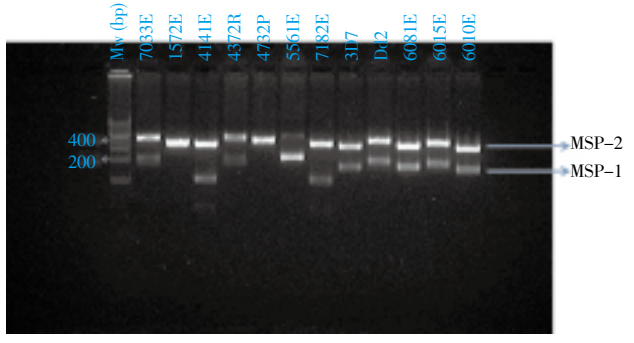

Figure 2. PCR typing of *P. falciparum* field isolates based on msp-1 and msp-2.

PCR products MW-molecular weight (bp).

4. Discussion

The results from our experiments showed that the isolates of Nanay river basin having five types of invasion receptor-ligand interaction profiles. The parasites had the invasion on neuraminidase resistance ($NM_RT_SCT_S$) but dependence on

trypsin and chymotrypsin sensitive proteins; involved the cells through complement receptor-1 on erythrocyte surface with *Pf*RH4 ligand[1]. Isolates invaded the erythrocytes through sialic acid dependence and trypsin sensitive proteins ($NM_ST_SCT_R$) utilized the GPA receptors on the erythrocyte surface interacted with EBA-175 ligand[3,4]. The isolates which showed its dependence only on sialic acid molecular ($NM_ST_RCT_R$) invaded through PfRH1 ligand interacted with unknown receptors called 'Y' on erythrocyte surfaces. The isolates invaded the cells through dependency on sialic acid. Trypsin and chymotrypsin sensitive proteins ($NM_ST_SCT_S$) on unknown receptor ligand interactions. Similarly isolates invaded the cells through trypsin sensitive proteins alone ($NM_RT_SCT_R$) through unknown receptor-ligand interactions. The results are favorably supported by the findings of Triglia *et al*[8] reported that *P. falciparum* parasites have varying capabilities to invade sialic acid deficient erythrocytes; and in addition to sialic acid another erythrocyte receptor/ligand is required for the invasion process. Thus, there is existence of diversity in the utilization of receptors on the erythrocyte surfaces by the malarial parasite. Crosnier *et al*[3] reported that erythrocytes treated with all these enzymes and related surface molecules removed are invaded by the parasites through *Pf*RH5 ligand with the Ok blood group antigen, basigin, is a receptor for *Pf*Rh5, a parasite ligand that is essential for blood stage growth across all laboratory-adapted and field strains as $Nm_RT_RCT_R$ and the polymorphisms in ligand *Pf*RH5 influences its receptor binding specificity. The parasites also invading the cells which are sensitive to all the enzymes $NM_ST_SCT_S$ through unknown receptor-ligand interactions. Similarly we noted that erythrocytes which were sensitive only to trypsin treatment invaded successfully by the parasites through unknown mechanisms. In this study, we found the invasion of sialic acid and chymotrypsin sensitive receptors while the parasite isolates were largely dependent on sialic acid independent and trypsin sensitive receptors called complement receptor 1. No correlation was observed between the utilization of sialic acid dependent and chymotrypsin-sensitive invasion receptor-ligand infection profiles, Figure 2 (Rs =-0.23; P-0.19) represent distinct invasion pathways. The result is favorably supported by the findings of Lobo *et al*[7] reported by using 14 distinct field isolates from the legal Amazon areas of brazil and confirmed the existence of flow invasion profiles and significant diversity of invasion pathways in nature Jennings *et al*[13] reported that *P. falciparum* utilizes multiple ligand-receptor interactions for the invasion of human erythrocytes through the reticulocyte binding protein homolog (*Pf*RH) family have been shown to be critical for directing parasites to alternative erythrocyte receptors that define invasion pathways. Similarly Gaur *et al*[14] reported that some lines of *P. falciparum* invaded both sialic acid as well as sialic acid independent erythrocytes with an ability to change its invasion requirements for erythrocyte sialic acid suggests a switching mechanism permitting invasion by alternative pathways. Our results confirmed the presence of two main receptor types that binds to a sialic acid dependent ligand and another one binds to sialic acid independent ligand. The sialic acid independent ligands bind with different types of sialic acid independent receptors having different affinities for binding. The EBA and *Pf*RH proteins play critical roles in receptor binding and invasion of host erythrocytes. The precise roles of these parasite ligands in invasion process remain to be fully understood. A number of other as yet unidentified parasite ligands localized in the apical organelles may also be crucial for invasion[3]. A full understanding of theses invasion mechanisms may allow the development of novel prophylactic and therapeutic strategies that block erythrocyte receptor-ligand invasion mechanisms.

Conflict of interest statement

We declare that we have no conflict of interest.

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