Expression analysis of EBA-175 paralogues in *P. falciparum* field isolates
2.0 Introduction:

Invasion of erythrocytes by *Plasmodium sp.* merozoites is a complex multistep process. The initial interaction with an erythrocyte can take place on any part of the merozoite surface, which is followed by reorientation of merozoite in such a way that its apical end is in contact with the erythrocyte surface. After apical reorientation, an irreversible junction develops between the apical surface of the merozoite and the erythrocyte. Apical organelles such as rhoptries and micronemes discharge their contents to create an invagination on the erythrocyte surface. The merozoite then enters the invagination and the junction moves around the merozoite as a circumferential ring. Finally the junction fuses at the posterior end of merozoite, bringing the parasite within a vacuolar membrane, known as parasitophorous vacuole, thus completing the invasion process. Reorientation of merozoites at the surface of erythrocytes and subsequent host-parasite interactions involved in the process of invasion has been observed by videomicroscopy (Dvorak et al., 1975) and ultrastructural studies (Bannister et al., 1975; Aikawa et al., 1978; Miller et al., 1979). These observations have suggested that parasite molecules at the apical pole and at the surface of merozoite perform specific and possibly, adhesive functions that are essential for invasion into red cells.

The human malaria parasite, *P. vivax* and the simian malaria parasite, *P. knowlesi*, are completely dependent upon interaction with Duffy blood group antigen for invasion of human erythrocytes (Miller et al., 1975; Miller et al., 1976). Adams et al., cloned genes for three different ligands $\alpha$, $\beta$ and $\gamma$ from *P. knowlesi*, which exhibited different specificities for receptors on human and rhesus erythrocytes (Adams et al., 1992). *P. knowlesi* $\alpha$ binds to Duffy antigen on human and rhesus erythrocytes whereas *P. knowlesi* $\beta$ uses sialic acid residues on rhesus erythrocytes. Receptor for *P. knowlesi* $\gamma$ on rhesus erythrocytes still remains unidentified.
Duffy binding proteins (DBPs) of *P. vivax* and *P. knowlesi* share similar exon-intron structure and the nucleotide sequences at the exon-intron boundaries are well conserved. The extracellular region of DBP contains two conserved cysteine rich domains, regions II and VI separated by a non-homologous hydrophilic region. In order to study function of DBP in erythrocyte invasion, *P. knowlesi* DBP was disrupted using homologous recombination. Deletion of PkαDBP resulted in complete loss of invasion of human erythrocytes by *P. knowlesi*, demonstrating the critical role of PkDBP-Duffy antigen interaction in red cell invasion. Electron microscopic studies with *P. knowlesi* knock out (PkaKO) parasites showed that PkaKO merozoites interact with Duffy positive human erythrocytes. Furthermore, initial attachment and apical reorientation take place normally, however, junction formation was not observed, suggesting that PkDBP plays an essential role in junction formation during invasion of human erythrocytes by *P. knowlesi* merozoites (Singh et al., 2005).

Unlike *P. vivax* and *P. knowlesi*, *P. falciparum* does not use the Duffy antigen as a receptor for invasion. A 175-kDa *P. falciparum* protein referred as Erythrocyte Binding Antigen-175 (EBA-175) interacts with sialic acid residues on glycophorin A for invasion into erythrocytes (Sim et al., 1994). *P. falciparum* laboratory strains as well as field isolates are able to invade erythrocytes treated with neuraminidase or trypsin, which cleaves sialic acid residues and glycophorin A respectively (Okayeh et al., 1999; Baum et al., 2003). This suggests that sialic acid/glycophorin A independent receptors can be used for erythrocyte invasion by *P. falciparum*. However, EBA-175 interacts only with sialic acid residues on glycophorin A, suggesting that molecules other than EBA-175 might be involved in mediating alternate invasion pathways.
EBA-175 gene structure is similar to the DBP family of *P. vivax* and *P. knowlesi* except that it contains tandem duplication (F1 and F2) of the N-terminal cysteine rich region. Analysis of genome database of *P. falciparum* laboratory strain 3D7 has identified four EBA-175 paralogues. EBA-175 paralogues (EBA-181, EBA-165 and EBA-140) share similar exon-intron structure as that of EBA-175 (Fig.2.1). The homology between EBA-181/165/140 and EBA-175 is higher in F1 and F2 domains when compared to other domains. Alignment of the binding domain (F2) shows that all cysteines are conserved between EBA-175 and EBA-181/165/140 (Fig.2.2).

The role of EBA-175 paralogues in mediating erythrocyte invasion has been studied in detail. EBA-181 binds to an unknown receptor on erythrocyte surface, which is neuraminidase and chymotrypsin sensitive and trypsin resistant. EBA-140 has been shown to use sialic acid residues on glycophorin C for invasion into erythrocytes. EBA-165 is a pseudogene that is transcribed but not translated. The differential binding specificities of EBA-181 and EBA-140 might allow the parasites to invade enzyme treated erythrocytes. Thus, if EBA-175 paralogues define invasion phenotype of the parasite then EBA-175 paralogues might be differentially expressed in *P. falciparum* field isolates with distinct invasion phenotypes. In order to assess the role of EBA-175 paralogues in mediating alternate invasion pathways, we have analyzed the expression pattern of EBA-175 paralogues both at transcriptional and translational level in field isolates with distinct invasion phenotypes.
Figure 2.1. EBA-175 paralogues found in *P. falciparum*. The gene structure of *P. falciparum* sialic acid binding protein (EBA-175) is similar to DBP of *P. vivax* and *P. knowlesi* except that EBA-175 contains a tandem duplication (F1 and F2) of the N-terminal cysteine rich region. EBA-181, EBA-165 and EBA-140 also share the similar gene structure, however, the homology is higher in the F2 domain when compared with other domains.
Expression Analysis: Introduction

Figure 2.2. Alignment of F2 domain of EBP paralogues. EBA-181, EBA-165 and EBA-140 show homology up to 30% with EBA-175. Cysteines (highlighted in blue) are conserved between EBA-175 and EBA-181, EBA-165 and EBA-140. Conserved aminoacids are shown in red while semiconserved amino acids are shown in blue.
2.1 Materials and Methods:

2.1.1 Collection of blood:
O+ve blood was collected in 10% Citrate-phosphate-dextrose buffer and centrifuged at 800xg for 5min. Plasma with buffy coat was removed to obtain packed erythrocytes. These erythrocytes were stored at 4°C for 3-4 weeks.

2.1.2 Parasite culture:
*P. falciparum* laboratory strain and field isolates were cultured using methods described by Trager and Jensen (Trager and Jensen, 1976). Parasite cultures were maintained under mixed gas (5%CO₂, 5%O₂ and 90% N₂) at 37°C in O+ve erythrocytes and RPMI-1640 supplemented with 10% O+ve human sera, 0.2% sodium bicarbonate, 0.2% glucose and gentamicin (10μg/ml). The cultures were maintained at 4% hematocrit and 5-10% parasitemia. Parasite stage and parasitemia was checked by making a thin smear on glass slide, fixing with methanol and staining with 5-10% Giemsa stain (Sigma).

2.1.3 Sorbitol synchronization of the parasite:
*P. falciparum* cultures were synchronized using sorbitol (Lambros and Vanderberg, 1979). Parasite cultures with majority at the early ring stage were centrifuged at 500xg for 5min. After washing the culture once with incomplete RPMI-1640, it was incubated with 7-10 pellet volumes of 5% sorbitol at room temperature for 5-7min with intermittent mixing. Sorbitol lyses the mature trophozoites and schizonts, leaving rings unaffected. After incubation with 5% sorbitol, it was centrifuged at 500xg for 5min. The culture was then washed once with incomplete RPMI and the pellet was resuspended in complete RPMI-1640 at a hematocrit of 4%. This was further allowed to grow at 37°C under mixed gas.
2.1.4 **Enrichment of the parasite at the trophozoite or schizont stage by Percoll method:**

*P. falciparum* cultures with majority of infected erythrocytes at schizont stage were washed with incomplete RPMI. Parasite pellet was resuspended in incomplete RPMI and layered onto equal volume of 65% Percoll. This was centrifuged at 800xg for 20min. Schizonts obtained at the interface were collected and washed twice with incomplete RPMI. The culture was again resuspended in complete RPMI and allowed to grow further.

2.1.5 **Enzyme treatments:**

2.1.5.1 Neuraminidase treatment:

100μl of erythrocytes were washed twice with incomplete RPMI. Erythrocytes were then incubated with 300mU of neuraminidase (Roche) in 14ml of incomplete RPMI (pH 6.8) at 37°C for 2hrs. Erythrocytes were washed twice with incomplete RPMI and further used for erythrocyte invasion assays.

2.1.5.2 Trypsin treatment:

275μl of washed erythrocytes were resuspended in 5.5ml of incomplete RPMI and treated with 1mg/ml of TPCK treated Trypsin (Sigma) for 2hrs at 37°C. Erythrocytes were washed with incomplete RPMI and used for invasion assays.

2.1.6 **Erythrocyte Invasion assays:**

Schizonts were purified by floatation over 65% Percoll as described above. Percent purity of schizont preparation was determined using Giemsa stain while hematocrit of purified schizonts was determined using hemocytometer. Purified schizonts were diluted with complete RPMI and uninfected erythrocytes (normal, neuraminidase, and trypsin treated erythrocytes) to a final hematocrit of 2% (2X10⁸ erythrocytes/ml) and a
final parasitemia of 2% (4X10^6 erythrocytes/ml). 200µl of this mix was added to 96well flat bottom microtitre plate. Parasites were further incubated for 24-26hrs at 37°C under mixed gas. Smears were made and rings were scored. Percent invasion in enzyme treated erythrocytes was expressed with respect to invasion into normal erythrocytes. Invasion rates above 20% of normal were considered as positive for invasion into enzyme treated erythrocytes.

2.1.7 Preparation of genomic DNA from *P. falciparum*:
Parasite culture was centrifuged at 500xg for 5min and resuspended in incomplete RPMI. Saponin (Sigma) was added to a final concentration of 0.05% to lyse RBC membrane. Following centrifugation at 4000xg for 5min, the parasite was resuspended in lysis buffer (40mMTris-Cl pH-8.0, 80mM EDTA pH-8.0, 2% SDS, 0.1mg/ml Proteinase K (Roche)) and incubated at 37°C for 3hrs with intermittent shaking. After lysis of the parasite, equal volume of phenol (Sigma) was added, mixed well and centrifuged at 8000xg for 10min. Upper aqueous layer was transferred to a fresh tube. Extraction was performed similarly with chloroform (Sigma). RNase (Sigma) was added at a final concentration of 10µg/ml and incubated for 1hr at 37°C. Extraction was repeated again in a similar manner with phenol: chloroform. Genomic DNA was precipitated from upper aqueous layer by addition of 1/10 volume of sodium acetate (pH-5.2) and 2.5 volumes of absolute ethanol. After overnight incubation at -20°C, genomic DNA was pelleted by centrifugation at 8000xg for 30min at 4°C. Genomic DNA was washed once with 70% ethanol and air-dried. The pellet was dissolved in 25-100µl of DNase-RNase free water. Concentration of the genomic DNA was measured spectrophotometrically by taking O.D. at 260nm. Genomic DNA was analyzed by separation on a 0.8% agarose gel.
2.1.8 Preparation of total RNA from \textit{P. falciparum}:

\textit{P. falciparum} was grown till late schizont stage to a parasitemia of \(-10\%\) in a 10ml culture dish at 4\% hematocrit. Infected erythrocytes were washed with diethyl pyrocarbonate (DEPC) treated phosphate buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄) and further lysed using 0.5\% saponin as described above. After lysis of infected erythrocytes, parasites were collected by centrifugation at 4000xg for 5 min at 4°C and resuspended in 1ml of TRIzol reagent (Invitrogen). The parasite pellet was passed through pipette several times in order to break parasite clumps and further incubated for 5min to permit complete dissociation of nucleoprotein complex. Thereafter, 200\µl of chloroform was added per ml of TRIzol reagent, the tubes were shaken vigorously for 15sec and further incubated for 2-3min. The tubes were centrifuged at 12000xg for 10min at 4°C. RNA in the upper aqueous layer was collected and transferred to a fresh tube. In order to precipitate RNA, 500\µl of isopropanol per ml of TRIzol was added and the tubes were incubated for 15min at room temperature. RNA was pelleted by centrifugation at 12000xg for 10min at 4°C. RNA pellet obtained was further washed with 75\% ethanol prepared in DEPC treated water. This was followed by centrifugation at 8000xg for 10min. After carefully aspirating out ethanol from the tubes, the pellet was briefly air-dried and dissolved in 200\µl DNase-RNase free water. RNA obtained was DNase treated to remove DNA contamination from RNA sample and purified using High Pure RNA isolation kit (Roche) as per procedure described by manufacturer. Briefly, 400\µl of lysis/binding buffer (4.5M guanidine hydrochloride, 50mM Tris-HCl, 30\% TritonX-100 (w/v) pH-6.6) was added to the RNA and mixed well. This was added onto the column of glass fiber fleece and centrifuged for 1min at 8000xg.10\µl of DNasel in 90\µl of DNase incubation buffer was added on top of the column. The column was incubated for 15min at room temp. 500\µl of wash buffer I (5M guanidine hydrochloride, 20mM Tris-HCl pH-6.6) was added and the
column was centrifuged at 8000xg for 1min. The same procedure was repeated for washing with wash buffer II (20mM NaCl, 2mM Tris-Cl pH7.5). Finally 200μl of wash buffer II was added to the column and centrifuged at 8000xg for 2min to remove residual wash buffer. RNA was eluted in 50μl DNase-RNase free water. 1μl of RNase inhibitor (RNasin) was added to the RNA sample. Yield of RNA was measured by measuring absorbance of RNA at 260nm. RNA obtained was stored at -70°C till further use.

2.1.9 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):
RT-PCR was performed in two steps: first strand synthesis where total RNA was reverse transcribed to single stranded DNA, followed by PCR using specific primers designed to amplify EBA-175 and its paralogues.

2.1.9.1 First strand cDNA synthesis
Total RNA was reverse transcribed using either random hexamers or a gene specific primer RT6. RT6 is a degenerate primer based on conserved sequences from region VI of EBPs. RT6 is designed to specifically reverse transcribe EBA-175 and its paralogues. SuperscriptRTII (Invitrogen) first strand synthesis system was used for RT-PCR as per manufacturer's protocol. RNA/primer mixtures were prepared in 0.2ml DEPC treated PCR tubes as mentioned below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample</th>
<th>No RT control</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>2μg</td>
<td>2μg</td>
</tr>
<tr>
<td>10mM dNTP mix</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Gene specific primer (RT6)</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random Hexamers</td>
<td>1μl</td>
<td>1μl</td>
</tr>
</tbody>
</table>
| DEPC treated water             | to 10μl | to 10μl
Each sample was incubated at 65°C for 5 min and then immediately chilled on ice for at least 1min. The following mixture was prepared in the indicated order:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT buffer</td>
<td>2µl</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>4µl</td>
</tr>
<tr>
<td>0.1mM DTT</td>
<td>2µl</td>
</tr>
<tr>
<td>RNaseout</td>
<td>1µl</td>
</tr>
</tbody>
</table>

9µl of reaction mixture was added to each RNA/primer mixture, mixed gently and incubated for 42°C for 2 min. 1µl of SuperscriptRTII was added to each tube except the no RT control and further incubated for 50min at 42°C. The reaction was terminated by heating at 70°C for 15min, followed by chilling on ice. The tubes were centrifuged briefly and incubated with 1µl of RNaseH for 20min at 37°C before proceeding with amplification by PCR using specific primers.

### 2.1.9.2 PCR amplification of cDNA:

cDNA obtained was used for PCR using primers specific for EBA-175, EBA-181, EBA-165 and EBA-140. Primers used for PCR amplification are as follows:

- **EBA175**: 5’ gtatcatctggtattcaagg3’ and 5’atttttataatctgaatgtaattcttc 3’
- **EBA181**: 5’tgtaataatgagtgttatacc 3’ and 5’ctacaccttaactgc 3’
- **EBA165**: 5’ ataagatgagataaatgc3’ and 5’ctctatctagtaataattgta 3’
- **EBA140**: 5’tccgaaaatgaagataattctg 3’ and 5’ctctcttaacaaaaatttatatttt 3’

The following mixture was added to 0.2ml PCR tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>5µl</td>
</tr>
<tr>
<td>10mM dNTP mix (Roche)</td>
<td>1µl</td>
</tr>
<tr>
<td>10µM sense primer</td>
<td>1µl</td>
</tr>
<tr>
<td>10µM antisense primer</td>
<td>1µl</td>
</tr>
</tbody>
</table>
| Taq DNA polymerase(5U/µl)  | 0.5µl }
cDNA 1μl
Water 40.5μl

Target DNA was denatured for 5min initially and then 30 cycles of PCR were performed with conditions optimized for each gene. PCR conditions for amplification of EBA-175 and its paralogues are as follows:

**EBA-175**
- 94°C - 5min
- 94°C - 30sec
- 57°C - 1min $\times$ 30
- 72°C - 1min
- 72°C - 10min

**EBA-181**
- 94°C - 5min
- 94°C - 30sec
- 52°C - 1min $\times$ 30
- 72°C - 1min
- 72°C - 10min

**EBA-165**
- 94°C - 5min
- 94°C - 30sec
- 47°C - 1min $\times$ 30
- 72°C - 1min
- 72°C - 10min

**EBA-140**
- 94°C - 5min
- 94°C - 30sec
- 55°C - 1min $\times$ 30
- 72°C - 1min
- 72°C - 10min

10μl of the PCR amplified product was analyzed on 1% agarose gel.

**2.1.10 Real time PCR:**

Real time RT-PCR was performed according to the protocol described by Blair et al. (Blair et al., 2002). Total RNA was isolated at late schizont stage of the parasite following TRizol method. After DNase treatment, 500ng of total RNA was reverse transcribed using oligodT. For real time transcript quantification, cDNA was used in a 5' fluorogenic assay using the chemistry of Taqman system (Fig.2.3). Probes (Qiagen) were labeled with a fluorophore (FAM- 6-carboxy-fluorescein and HEX-hexachloro-6-carboxy-fluorescein) at the 5' end and a quencher (TAMRA-6-carboxy-tetramethyl-rhodamine) at 3' end. Quencher in proximity with fluorophore does not allow fluorophore to
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give emission spectra. When PCR reaction goes to annealing phase, both primer and probe anneals to the target DNA. During the extension phase, while amplifying target DNA, Taq polymerase due to its 5’-3’ exonuclease activity, cleaves the labeled probe. This releases fluorophore from quencher and hence gives emission spectra at a particular wavelength. Primers and probes specific for EBA-175, EBA-181, EBA-165 and EBA-140 were used. Primers used to amplify EBA-175 and its paralogues are:

EBA175RTF: 5’ aatttctgtaaaatattgtgcaccatag 3’ and
EBA175RTR: 5’gatactgcacaacacagattttcttg 3’
EBA181RTF: 5’gcgggtagtacaatattagatgattc3’ and
EBA181RTR: 5’ tgttgttgtgtcaaaaattagtgttcttg 3’
EBA165RTF: 5’attaatctgtacatcacatacgca3’ and
EBA165RTR: 5’ acgcccatcatgcacatt 3’
EBA140RTF: 5’gcaaaataaatgcaacaatgaata3’ and
EBA140RTR: 5’ aacaaggacccggtgaacta 3’

18srRNA was used as internal control. The sequence of the primer used is as follows:

18srRNARTF: 5’gctgactacgtccctgc3’ and
18srRNARTR: 5’ acaattcatcatatctttcaatcggta 3’

Probes for EBA-175 and its paralogues were labeled with 5’FAM and 3’TAMRA. Probe for 18srRNA was labeled with 5’HEX and 3’ TAMRA. The sequence of probes is as follows:

EBA175PR: atgaagaaatcccattaaaaacatgcactaaag
EBA181 PR: aaatgacagaaggtagcgaaagtgatgttggag
EBA165 PR: atagaaacaacagctgagaacaatataggtggttaagt
EBA140 PR: ccatggaatatgtacctattctgcagacaaagg
18SrRNA PR: ttgtacacaaacgccgctgctg

Various dilutions of genomic DNA from 0.6ng to 600ng were used as standard. Liver stage antigen-1, which is expressed during liver stage of the parasite, was taken as negative control to check for any relaxed transcription during blood stage of the parasite.
Figure 2.3. Real-time RT-PCR using Taqman system. A. Total RNA was reverse transcribed using oligodT, followed by PCR amplification using specific primers and probe for EBA-175 and its paralogues. The Taqman probe bears a fluorophore at each end. The fluorescence signal generated by the reporter dye (R) is quenched by the second fluorophore (Q). Probe cleavage by the 5' exonuclease activity of the Taq DNA polymerase (POL) during PCR extension separates the reporter and quencher, fluorescence is recorded (B). Unbound probes are not hydrolyzed and consequently, fluorescence is proportional to the quantity of the target product present.
Following reaction was set up in 96 well PCR plates:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Supermix (Biorad)</td>
<td>12.5µl</td>
</tr>
<tr>
<td>10µM sense primer</td>
<td>2µl</td>
</tr>
<tr>
<td>10µM antisense primer</td>
<td>2µl</td>
</tr>
<tr>
<td>10µM 18srRNA sense primer</td>
<td>0.75µl</td>
</tr>
<tr>
<td>10µM 18srRNA antisense primer</td>
<td>0.75µl</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>2µl</td>
</tr>
<tr>
<td>cDNA</td>
<td>1µl</td>
</tr>
<tr>
<td>Water</td>
<td>4µl</td>
</tr>
</tbody>
</table>

PCR reaction was set up in real time PCR instrument that has an optical system to collect emission spectra at different wavelengths (iCycler- Biorad). The real time PCR consisted of one cycle of 95°C for 10min, followed by 50 cycles of 95°C for 15sec, 60°C for 1min and 72°C for 1min. Serial dilutions of genomic DNA were used as reference control. Each PCR was performed in duplicate. Well factor solution (Biorad), which is a mixture of all fluorescent dyes, was used as a blank for real time PCR. Initially, 25µl of well factor solution was aliquoted into 96 wells of PCR plate and kept for two cycles of 95°C for 10sec and 60°C for 45sec in the iCycler. Well factor plate was then removed from the PCR block and the experimental plate was kept for further cycles. Data analysis was performed using software provided by iCycler, which determines threshold values (Cₜ) and reduced fluorescence values for amplified product. Cₜ values obtained for EBA-175/165/140/181 were normalized with Cₜ values of 18srRNA. These values were further converted to genomic equivalents using Growth algorithm in Microsoft Excel software. Using genomic equivalent of DNA as standard for each gene, expression levels of EBA-175/181/165/140 were estimated. Expression levels of EBA-181/165/140 were estimated with respect to EBA-175 in 3D7 and field isolates.
2.1.11 Detection of EBA-175 paralogues at translational level:
Regions III-V of EBA-181, EBA-165 and EBA-140 were expressed as GST fusion proteins as described below. The fusion proteins were purified using glutathione sepharose columns. 25μg of purified protein was injected into mice for raising antibodies. Sera was collected 10-14 days after every boost and the titre of antibodies was determined by ELISA.

2.1.11.1 Cloning region III-V of EBA-181/165/140 in a GST fusion vector:
Regions III-V of EBA-181/165/140 were PCR amplified from genomic DNA of 3D7 using primers with BamH1 and Not I sites. The sequences of primers used for amplification of EBA-181/165/140 are as follows:

- EBA181R3D: 5’caggagatccaaaaatatcaagtactgaaccaaatg3’
- EBA181R3U: 5’agccgacgagccgacattctctgtgtgtatcattatg3’
- EBA165R3D: 5’ caggagatccaaaaatatcaagtactgaaccaaatg3’
- EBA165R3U: 5’ tgaatctcgcccgctccttttctatattctatag3’
- EBA140R3D: 5’ acggtaggatcttttggtgtgtaaagcctaatag3’
- EBA140R3U: 5’ agccgacgagccgacattctctgtgtgtatcattatg3’

PCR condition for amplification of EBA-175 and its paralogues is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>200ng</td>
</tr>
<tr>
<td>10X Pfu buffer</td>
<td>5μl</td>
</tr>
<tr>
<td>10μM Sense primer</td>
<td>3μl</td>
</tr>
<tr>
<td>10μM Antisense primer</td>
<td>3μl</td>
</tr>
<tr>
<td>5mM dNTP mix</td>
<td>5μl</td>
</tr>
<tr>
<td>Pfu DNA polymerase (Stratagene)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Water</td>
<td>up to 50μl</td>
</tr>
</tbody>
</table>

PCR cycling parameters used for amplification of each paralogue are as follows:
Amplified DNA fragments were purified using PCR purification kit (Qiagen). The GST fusion vector pGEX4T1 (Amersham Biosciences) and PCR amplified DNA fragments were digested with *BamH* I and *Not* I at 37°C for 2 hrs. Digested vector and inserts were resolved by agarose gel electrophoresis (1%) and purified using gel extraction kit (Qiagen). Concentrations of gel-purified digested fragments were estimated by measuring absorbance at 260nm. EBA-181/165/140 fragments were ligated with pGEX4T1 vector at 16°C for 16-18hrs at a molar ratio of 1:3 (vector: insert). The ligation mix was then transformed in *E.coli* DH10β chemical competent cells, prepared by calcium chloride method (Sambrook et al., 1989). For transformation, ligation mix was added to 200μl of DH10β and incubated for 30min on ice. Cells were given a heat shock at 42°C for 90sec and immediately chilled on ice for at least 1min. 1ml of LB medium was added to competent cells and cells were further grown for 1hr at 37°C. Cells were pelleted down at 2000xg for 5min and plated on LB agar containing ampicillin (100μg/ml) (LBAmpl100). LBAmpl100 plates were incubated for 16-18hrs at 37°C. Colonies obtained were replica plated on fresh LBAmpl100 plate and incubated for 8-10hrs. Colonies were grown in LBAmpl100 medium at 37°C for 16-18hrs. Cells were collected by centrifugation. Plasmids were isolated using plasmid isolation kit (Qiagen) and analyzed for presence of inserts after digestion with *BamH*I and *Not*I.

<table>
<thead>
<tr>
<th></th>
<th><strong>EBA-181</strong></th>
<th><strong>EBA-140</strong></th>
<th><strong>EBA-165</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>5min</td>
<td>5min</td>
<td>94°C - 5min</td>
</tr>
<tr>
<td>94°C</td>
<td>30sec</td>
<td>94°C - 30sec</td>
<td>94°C-30sec</td>
</tr>
<tr>
<td>59°C</td>
<td>1min X 30</td>
<td>61°C - 1min X 30</td>
<td>58°C-1min X30</td>
</tr>
<tr>
<td>72°C</td>
<td>2.5min</td>
<td>72°C - 2.5min</td>
<td>72°C - 2.5min</td>
</tr>
<tr>
<td>72°C</td>
<td>10min</td>
<td>72°C -10min</td>
<td>72°C - 10min</td>
</tr>
</tbody>
</table>
Positive clones of pGEX4T181, pGEX4T165, and pGEX4T140 were transformed into BL21(DE3) cells for protein expression.

2.1.11.2 Expression and purification of region III-V of EBA-181/165/140:

*E. coli* strain BL21(DE3) with plasmids pGEX4T181, pGEX4T165, and pGEX4T140 were grown overnight at 37°C in 10ml LBAmpl00. 10ml of overnight culture was inoculated into 1L LBAmpl00 media supplemented with 0.2% glucose. After reaching an O.D. between 0.6-0.8 culture at 600nm, culture was induced with 0.2mM IPTG and further incubated for 3hrs at 37°C. Cells were harvested at 3000xg for 10min at 4°C and washed once with Tris buffered saline (TBST: 20mM Tris pH-7.5, 150mM NaCl, TritonX-100). *E. coli* cells were lysed in TBST by sonication. The soluble fraction containing EBA-181RIII-V, EBA-165RIII-V, and EBA-140RIII-V was separated by centrifugation at 20000xg for 20min at 4°C. Supernatant obtained was loaded on to GSTbind resin (Novagen) pre equilibrated with TBST. Column was washed with 20 column volumes of TBST and further with 10 column volumes of 20mMTris pH-7.5 with 400mM NaCl. Column was again equilibrated with TBS. Protein bound to GST on the column was eluted with 50mM Tris (pH-8.0) containing 20mM glutathione (reduced). Fractions were analyzed on SDS-PAGE (10%). Proteins were stained with Comassie Brilliant Blue R-250 (Sigma).

2.1.11.3 Raising antibodies against the EBA-181RIII-V, EBA-165RIII-V, and EBA-140RIII-V in mice:

Polyclonal antibodies were raised against EBA-181RIII-V, EBA-165RIII-V, and EBA-140RIII-V in BALB/c mice. 25μg of purified recombinant protein was formulated in complete Freund's adjuvant and injected in mice intraperitoneally. Priming was followed by two booster immunizations with recombinant proteins formulated in Freund's incomplete adjuvant on days 28 and 69. Bleeds were collected on days 0, 14, 42, 84 and are referred to
as prebleed, first bleed, second bleed and third bleed respectively. For collection of sera, blood was allowed to coagulate by incubation at 37°C for 1hr and overnight at 4°C. After centrifugation at 3000xg for 5min, sera was transferred to a fresh tube and stored at -70°C till further use.

Endpoint titres of antibodies against each protein were determined using ELISA. 500ng of recombinant proteins EBA-181RIII-V, EBA-165RIII-V, and EBA-140RIII-V was coated on to ELISA plate (Nunc) and incubated overnight at 4°C. ELISA plates were washed once with PBS and blocked with 300μl of 2% BSA in PBS at 37°C for 2hrs. Serial dilutions of sera were prepared in 1% BSA in PBS. The wells were washed once with PBST and further incubated with 100μl of various dilutions of sera for 1 hr at 37°C. The wells were washed thrice with PBST and incubated for 1hr at 37°C with anti-mouse IgG goat antibodies conjugated with horseradish peroxidase (1:2000). The wells were washed thrice with PBST and developed using O-phenylene diamine (OPD-1mg/ml) as substrate. O.D. was recorded at 492nm using ELISA reader (Molecular Devices).

2.1.11.4 Immunoprecipitation:
Parasites were purified at trophozoite stage following Percoll method. Purified trophozoites were washed with methionine-cysteine deficient RPMI medium and cultured in Cys-Met-RPMI medium (Sigma) supplemented with 1% human serum, 2mM glutamine (Invitrogen) and containing 200μCi/ml of 35S methionine-cysteine mix for 16h. The supernatant was harvested by centrifugation and used for immunoprecipitation.

Radiolabeled supernatant was first precleared with protein A agarose beads (equilibrated with NETT buffer: 50mM Tris pH-7.5, 150mM-NaCl, 1mM EDTA and 0.5% Triton X-100) for 30min at 4°C. Supernatant was collected after centrifugation at 800xg for 2min. Antibodies against EBA-175, EBA-181, EBA-165 and EBA-140 were added to the supernatant and incubated
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for 1 hr on ice with intermittent shaking. Protein A agarose beads were then added to the supernatant and incubated for 45min-1hr under rocking conditions. Beads were washed four times with NETT buffer. Protein A beads were boiled in 2X SDS reducing loading buffer and the proteins were analyzed by SDS-PAGE and fluorography.

2.1.11.5 Immunofluorescence assay:
A thin smear of late stage schizonts was fixed with chilled mixture of acetone (90%) and methanol (10%) at -20°C for 1 hr. Fixed slides were dried and blocked with 5% BSA in PBS at 37°C for 1hr. The slides were incubated for 1hr with a mixture of rabbit sera raised against EBA-175 (1:100) and mouse sera raised against EBA-181/165/140 (1:100). After washing slides thrice with PBST, fluorescein-isothiocyanate (FITC) anti-mouse labeled antibody and Cy3-labeled anti-rabbit antibody was added at a dilution of 1:100 and further incubated for 1hr. Slides were washed again with PBST and mounted using Antifade reagent (Bio-rad). Dual-color fluorescence images were visualized using fluorescence microscope (Nikon).
2.2 Results:

2.2.1 *P. falciparum* field isolates exhibit diverse invasion phenotype

Erythrocyte invasion studies performed with *P. falciparum* shows that the parasite could use multiple receptors on erythrocyte surface for invasion. Out of fifteen field isolates studied by Okoyeh et al., four field isolates were chosen which exhibited diverse invasion phenotypes. RAJ116 could not invade neuraminidase and trypsin treated erythrocytes while JDP8 could invade both neuraminidase and trypsin treated erythrocytes. RAJ68 could invade neuraminidase treated erythrocytes but not trypsin treated erythrocytes while RAJ104 could invade trypsin treated erythrocytes but not neuraminidase treated erythrocytes.

Erythrocyte invasion studies were repeated to show that these parasites have retained their invasion phenotypes. Invasion rates above 20% of normal were considered as positive for invasion into enzyme treated erythrocytes. Erythrocytes invasion assays with 3D7 and *P. falciparum* field isolates showed that parasites have not changed their ability to invade neuraminidase and trypsin treated erythrocytes (Fig.2.4). In order to assess role of EBA-175 paralogues in mediating alternate invasion pathways, we tested whether EBA-175 paralogues are differentially expressed in *P. falciparum* field isolates. Expression of EBA-175 and its paralogues (EBA-181, EBA-165, EBA-140) was examined in these field isolates both at transcriptional and at translational level.

2.2.2 *EBA-175* paralogues are transcribed in all *P. falciparum* field isolates.

Total RNA isolated at late schizont stage of the parasite, was reverse transcribed using either random hexamers or degenerate primer (RT6) based on conserved sequences of region VI which would specifically reverse
Expression Analysis: Results

<table>
<thead>
<tr>
<th>Parasite Isolate</th>
<th>Invasion into erythrocytes (% of normal)</th>
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<tr>
<td></td>
<td>Neuraminidase</td>
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<tr>
<td>3D7</td>
<td>72 ± 0.2</td>
</tr>
<tr>
<td>RAJ116</td>
<td>6 ± 0.1</td>
</tr>
<tr>
<td>RAJ104</td>
<td>2 ± 0.1</td>
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<tr>
<td>RAJ68</td>
<td>40 ± 0.4</td>
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<tr>
<td>JDP8</td>
<td>29 ± 0.7</td>
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Figure 2.4. Invasion phenotypes of *P. falciparum* field isolates used for studying the expression pattern of EBA-175 and its paralogues. Purified *P. falciparum* schizonts were incubated in triplicate wells with normal and enzyme-treated erythrocytes. Invasion rates above 20% of normal erythrocytes were considered as positive for invasion into enzyme treated erythrocytes.
transcribe EBA-175 and its paralogues. cDNA obtained was then amplified using region II specific primers for EBA-175, EBA-181, EBA-165, EBA-140. Expression at transcriptional level was tested in 3D7 and *P. falciparum* field isolates with distinct invasion phenotypes. RAJ116, which does not possess alternate invasion pathways, as well as RAJ68, RAJ104 and JDP8, which use alternate invasion pathways, transcribe EBA-175, EBA-181, EBA-165 and EBA-140. Fig.2.5 shows the expression of EBA-175, EBA-181, EBA-165 and EBA-140 in one of the field isolates JDP8. Similar pattern was observed for 3D7 and other *P. falciparum* field isolates. However, the method followed here is not quantitative and differences in invasion phenotype might be related to differential levels of expression of EBA-175 paralogues. Thus, Real time RT-PCR was performed to study the expression of EBA-175, EBA-181, EBA-165 and EBA-140 quantitatively in 3D7 and four field isolates. Total RNA was isolated at late schizont stage and cDNA synthesis was performed using oligodT. Real time PCR was performed using fluorogenic 5’ nuclease assay (Taqman) as described by Blair et al. Genomic equivalents of EBA-175/181/165/140 were normalized with 18srRNA. Normalization of genomic equivalents of EBA-165 with 18srRNA showed that EBA-165 is consistently expressed in low amounts in all field isolates (Fig.2.6A). Also, the variation in transcript levels of EBA-181 and EBA-140 with respect to EBA-175 was observed to be in between 1.5 to 2 fold (Fig.2.6B). Although EBA-181 and EBA-140 are expressed at significant levels in these field isolates but no correlation was observed between expression of EBA-175 paralogues and invasion phenotypes of these parasites.
Expression Analysis: Results

Figure 2.5. EBA-175/181/165/140 is transcribed at late schizont stage of the parasite JDP8. Total RNA was isolated and reverse transcribed using random hexamers and region VI (R6) specific primer and then PCR was performed using region II specific primer of EBA-175 (A), EBA-181 (B), EBA-140 and EBA-165 (C). RT negative (no reverse transcriptase added) was used as control to check for genomic DNA contamination in the RNA preparation.
Figure 2.6. Real time RT-PCR analysis of EBA-165 (A) and EBA-175/181/140 (B) in 3D7 and field isolates with distinct invasion phenotypes. Total RNA was isolated and reverse transcribed using oligodT and then PCR was performed using specific primer of EBA-175 and its paralogues. Genomic equivalents of EBA-175/181/165/140 were normalized with 18srRNA. Genomic equivalents of EBA-181/140 were calculated with respect to EBA-175.
2.2.3 **EBA-175 paralogues are translated at late schizont stage of the parasite.**

The divergent regions, III-V of EBPs were expressed as a GST fusion protein in *E. coli* BL21(DE3) cells, in order to raise specific antibodies against each EBA-175 parologue. Regions III-V of EBA-181, EBA-165 and EBA-140 expressed as fusions with GST tags were affinity purified using a glutathione sepharose column. The GST fusion proteins were eluted with 20mM glutathione (reduced) (Fig.2.7) and used to immunize mice to raise specific antibodies against each protein (Fig.2.8A). Immunization of mice with EBA-181, EBA-165 and EBA-140 showed ELISA titres of 1:352000, 1:576000 and 1:464000 respectively after second boost (Fig.2.8B). These antibodies were used to detect EBA-175 paralogues in 3D7 and field isolates using immunoprecipitation and immunofluorescence assays.

Immunoprecipitation using antibodies against F2 domain of EBA-175, raised in rabbits detected the expression of EBA-175 in 3D7 and field isolates with distinct invasion phenotypes (Fig.2.9). Immunoprecipitation of EBA-175 paralogues was performed in 3D7 and field isolates. In 3D7, antibodies against EBA-181 immunoprecipitated an 181kDa band while antibodies against EBA-140 precipitated distinct bands around 140kDa. EBA-165, reported as a pseudogene, which is transcribed but not translated, and normal mouse serum served as negative controls (Fig.2.10A). Antibodies against EBA-181, EBA-165 and EBA-140 were then used for performing immunoprecipitation from field isolates. Similar pattern of immunoprecipitation was observed in field isolates as in 3D7. EBA-181 and EBA-140 are translated in field isolates with distinct invasion phenotypes (Fig.2.10B, C, D, E).
Figure 2.7. Purification of EBA-181 (A), EBA-165 (B), EBA-140 (C) using GST column. Lane1: Molecular weight Marker (Amersham biosciences). Lane2-9: elutes of the recombinant protein collected using 20mM glutathione.
A. **Days of sera collection**

| Days of injection | Days |  
|-------------------|------|---
| Priming          | 0    | 14 | 42 | 84  |

B. **Expression Analysis: Results**

**Figure 2.8.** Antibodies were raised against EBA-181RIII-V, EBA-165RIII-V, and EBA-140RIII-V for detection of protein in the parasites.

**A. Immunization schedule**—Prebleed was collected on day 0. Mice were primed on day 1 with 25μg of recombinant proteins formulated in complete Freund's adjuvant, followed by booster doses formulated in incomplete Freund's adjuvant on day 28 and 69. Bleeds were collected on day 14, 42 and 84 days and are referred as first bleed, second bleed and third bleed.

**B.** ELISA titres of antibodies raised in mice against EBA-181RIII-V, EBA-165RIII-V, and EBA-140RIII-V after second boost.

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Figure 2.9. Immunoprecipitation of EBA-175 from *P. falciparum* field isolates. Rabbit sera raised against F2 domain of EBA-175 was used for immunoprecipitation of EBA-175 from metabolically labeled culture supernatants of 3D7 and field isolates. Preimmune rabbit sera was added to metabolically labeled culture supernatant of 3D7, which served as negative control.
Figure 2.10. Immunoprecipitation of EBA-181/165/140 from *P. falciparum* field isolates and 3D7. Antibodies against region III-V were used to immunoprecipitate EBA-181/165/140 from metabolically labeled culture supernatants of parasites. Preimmune sera from mice was used as a control which is represented by lane 1 of A, B, C, D, E. Antibodies against EBA-181 immunoprecipitated a band of 181kDa in 3D7 and all field isolates as seen in lane 2 of A, B, C, D, E. EBA-140 antibodies detected two-three bands depending upon parasite strain as observed in lane 4 of A, B, C, D, E. EBA-165 was not expressed at late schizont stage as antibodies against EBA-165 could not immunoprecipitate any specific protein as seen in lane 3 of A, B, C, D, E.
To study the localization of EBA-175 paralogues, immunofluorescence assays were performed. Colocalization of EBA-175 with EBA-181/165/140 was observed using antibodies, which were used to perform immunoprecipitations. Immunofluorescence experiments with *P. falciparum* field isolates and 3D7 showed the same subcellular localization of EBA-175 with EBA-181/140 in late stage schizonts, suggesting that EBA-181 and EBA-140 are also present in micronemes. However, EBA-165 did not show any staining, as EBA-165 is not expressed at late schizont stage of the parasite. Fig.2.11 shows immunofluorescence assay performed with one of the parasite RAJ116.
Figure 2.11. Colocalization of EBA-175 with EBA-181, EBA-165 and EBA-140 proteins in *P. falciparum* field isolate RAJ116. Acetone: methanol fixed smears of parasites were incubated with rabbit sera raised against F2 domain of EBA-175 and mice sera raised against region III-V of EBA-181, EBA-165 and EBA-140, followed by incubation with anti-rabbit cy3 and anti-mouse FITC. Dual-color fluorescence images were visualized using fluorescence microscope (Nikon) and images were merged using Adobe Photoshop software.
2.3 Discussion:

*Plasmodium sp.* is an obligate intracellular parasite, belonging to phylum Apicomplexa. The survival of obligate intracellular parasites depends upon the successful invasion of an appropriate host cell. The success of *Plasmodium* infection in vertebrate hosts initially depends upon the invasion of hepatocytes by sporozoites. Thereafter, an infected hepatocyte releases thousands of merozoites into the bloodstream for invasion into erythrocytes. The process of invasion starts with the reversible attachment of merozoite on the red cell surface of host. The parasite then establishes an intimate association, involving reorientation to put the apical structures in contact with the host membrane. Organelles present at the apical end, rhoptries and micronemes release their contents to form a junction, where the host and parasite membrane are in contact with each other. Proteins located in the apical organelles bind to receptors on host cell membrane and are involved in recognition and selection of erythrocytes. The binding of merozoites from *Plasmodium sp.* to erythrocytes is dependent on at least two families of parasite ligands, Duffy binding like (DBL) and Reticulocyte-binding like (RBL) family. RBL protein family localizes to rhoptries of merozoite while DBL protein family is expressed in micronemes of merozoite.

*P. vivax* and *P. knowlesi* DBP interact with Duffy blood group antigen while *P. falciparum* protein of 175kDa (EBA-175) binds to sialic acid residues on glycophorin A. Analysis of genome sequence of 3D7 has identified genes, which share homology with EBA-175 and are referred to as EBA-181, EBA-165 and EBA-140. The receptor binding specificities of EBA-181 and EBA-140 differ from EBA-175. EBA-181 binds to the surface of erythrocytes in a sialic acid dependent manner to a trypsin-resistant/chymotrypsin-sensitive receptor. Although the receptor for EBA-140 is neuraminidase and trypsin sensitive, which is similar to EBA-175, its specificity, however, differs from EBA-175 as EBA-140 could bind to erythrocytes that lack glycophorin A.
Moreover, EBA-140 showed reduced binding to Gerbich erythrocytes (altered glycophorin C and missing glycophorin D), suggesting that EBA-140 may bind to sialic acid residues on glycophorin C. Furthermore, it has been observed that *P. falciparum* laboratory strains and field isolates could mediate invasion pathways independent of sialic acid residues/glycophorin A interaction. The identification of EBA-175 paralogues from genome sequence of *P. falciparum* provides candidate ligands that might function in parasites with alternate invasion pathways. Thus if EBA-175 paralogues define invasion phenotype of the parasite, then EBA-175 paralogues might be differentially expressed in *P. falciparum* field isolates with distinct invasion phenotypes. We further analyzed the role of EBA-175 paralogues in alternate invasion pathways by studying their expression in *P. falciparum* field isolates with distinct invasion pathways.

In order to assess the role of EBA-175 paralogues in multiple invasion pathways, field isolates (RAJ116, JDP8, RAJ68 and RAJ104), which have distinct invasion pathways, were selected for analysis. RAJ116 cannot invade neuraminidase and trypsin treated erythrocytes and thus is completely dependent on sialic acid residues on glycophorin A for erythrocyte invasion. RAJ68 and RAJ104 invades either neuraminidase or trypsin treated erythrocytes respectively whereas JDP8 was able to invade both neuraminidase and trypsin treated erythrocytes at similar invasion rates compared to invasion into normal erythrocytes. Thus RAJ68, RAJ104, JDP8 invade through sialic acid/glycophorin A independent pathways.

Expression of EBA-181, EBA-165 and EBA-140 was analyzed in *P. falciparum* field isolates both at transcriptional and translational level. RT-PCR studies showed that EBA-175, EBA-181, EBA-165 and EBA-140 are transcribed in all the four *P. falciparum* field isolates tested. Quantitative RT-PCR analysis of EBA-175 paralogues in 3D7 and *P. falciparum* field isolates showed that EBA-181 and EBA-140 are transcribed at levels comparable to that of EBA-175. The transcriptional levels of EBA-165 were
observed to be consistently low in all *P. falciparum* field isolates. However, there was no correlation of expression pattern with invasion phenotype of the parasite.

In order to analyze expression at protein level, antibodies raised against regions III-V of EBA-181, EBA-165 and EBA-140 were used to perform immunoprecipitation from metabolically labeled culture supernatants. Initially, expression of EBA-175 was tested in 3D7 and field isolates. EBA-175 which mediates invasion through sialic acid residues on glycophorin A is translated in 3D7 and all field isolates. Furthermore, the expression of EBA-181 and EBA-140 was detected in 3D7 and *P. falciparum* field isolates, irrespective of invasion phenotype. The expression of EBA-165 was not detected in any of the parasites, confirming it as a pseudogene. Also, antibodies raised against EBA-175/181/165/140 were also used for immunofluorescence assay at late schizont stage of the parasite. EBA-181 and EBA-140 colocalized with EBA-175, suggesting that they are expressed in the same subcellular compartments. These studies suggest that EBA-175 paralogues are alone not sufficient for mediating alternate invasion pathways.

Although, RAJ116 expresses EBA-181 at levels similar to JDP8, RAJ104 and RAJ68, it still does not invade trypsin treated erythrocytes, suggesting that expression of EBA-181 is not sufficient for invasion into trypsin treated erythrocytes. In *P. vivax*, reticulocyte-binding proteins PvRBP-1 and PvRBP-2 help parasite to specifically invade reticulocytes. Members of Py235 protein family of *P. yoelii* are homologous to PvRBP-1 and PvRBP-2 and have been thought to be involved in defining specificity of red cells for invasion by merozoites. *P. falciparum* homologs of PvRBP-1 and PvRBP-2, namely PfRh1, PfRh2a, and PfRh2b have been shown to bind red cells and play a role in erythrocyte invasion. Furthermore, there exists an apparent redundancy in PfRh family of proteins at the level of gene number and sequence. Although, members of this family are expressed at late schizont
stage but considerable variations occur in their transcription pattern (Taylor et al., 2002). Besides changes in their transcription pattern, variations were also found at protein level. For example PfRh1, which is correctly targeted to the apical end of FCB1 merozoites, does not localize properly in 3D7 and T996 parasites. This diversity in the PfRh protein family allows the parasite to invade erythrocytes with diverse receptors. Recently, Stubbs et al., showed that expression of PfRh4 correlates with sialic acid independent invasion. Also, silencing of PfRh4 occurs over time when parasites were cultured again in normal erythrocytes, showing that the switch in invasion pathways could occur in both directions. Thus, PfRh4 is essential for switching invasion pathways where activation and deactivation of sialic acid independent invasion is regulated by differential gene expression and silencing of PfRh4 (Stubbs et al., 2005). Thus, it could be possible that in addition to EBA-175 paralogues, PfRh family of proteins might be necessary for mediating alternate invasion pathways in *P. falciparum*. It has been observed in case of *P. vivax*, PvRBP-1 and PvRBP-2 helps in selection of reticulocytes, which is followed by binding of DBP to Duffy blood group antigen for invasion into reticulocytes. Similarly, PfRh proteins with the help of DBPs might help *P. falciparum* to mediate multiple invasion pathways. This allows the parasite to counter the selection pressure from human host. It has been reported that glycophorins A and B are rapidly evolving genes in human lineage (Wang et al., 2003). Thus, binding of EBA-181 and EBA-140 to different receptors on erythrocyte surface might allow the parasite to evolve better in malaria endemic areas. In contrast to *P. vivax*, which has disappeared from West Africa because of prevalence of Duffy negative blood group, *P. falciparum* has been successful in endemic areas because it has generated multiple DBL ligands to create multiple pathways for invasion, thus making invasion of erythrocytes with different genetic backgrounds possible.