# *Plasmodium knowlesi* Ligand-receptor Process in Baboon (*Papio anubis*) Placenta

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**Abstract**

Pregnancy associated malaria poses many risks to both women and their infants. It is characterized by the accumulation of infected erythrocytes in the intervillous spaces of the placenta leading to adverse reactions. Studies using the *P. knowlesi*-Olive baboon model of pregnancy malaria have demonstrated this phenomenon though the mechanisms and molecules involved are not known. This study sought to identify the ligands and receptor molecules that permit accumulation of infected erythrocytes in the placenta of *P. knowlesi* infected Olive baboons and to further test placental isolates for adhesion to purified receptors. Sequences of known *Plasmodium* erythrocyte binding antigens and human placental receptors were BLASTed against the genome of *P. knowlesi* and *P. anubis* respectively. Hits generated were analysed and characterized to determine the prospective ligands and receptors in *P. knowlesi* and *P. anubis* respectively. Also, four adult female baboons (*P. anubis*) were infected with *P. knowlesi* parasites and their placentas sampled. Infected erythrocytes isolated from these placentas were tested for binding against purified receptors. We identified Predicted CSPG 4 partial and Predicted HAPLN 1 as the putative receptor molecules in the Olive baboon. Further, the *P. knowlesi* erythrocyte binding proteins (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) matched closely to the placental *P. falciparum* ligand *Var2csa*. However, static binding assays with *P. knowlesi* infected erythrocytes did not show any binding to purified receptors. This study has identified and proposed receptors and ligands involved in the adherence process in *P. knowlesi* infected Olive baboons during pregnancy.

**Keywords:** *Plasmodium knowlesi*, Olive baboon, receptor, ligand, malaria, pregnancy

## 1. Introduction

Malaria is a public health problem that mainly affects pregnant women and children under 5 years old. During pregnancy, *Plasmodium falciparum* infected erythrocytes adhere to the intervillous spaces of the placenta, a phenomenon called sequestration (Costa *et al.,* 2006; Sherman *et al.,* 2003). Mature forms of the parasite which are commonly absent in peripheral circulation tend to dominate the placenta (Maubert *et al.,* 2000). Sequestration is mediated by receptor-ligand interactions between parasite-induced ligands on the erythrocyte membrane and cellular adhesion molecules (CAM) on the surface of vascular endothelial cells. Several studies have characterized these CAMs involved in adhesion of infected erythrocytes (IEs) in the placenta: chondroitin sulphate A (CSA) (Reeder *et al.,* 1999), hyaluronic acid (HA) (Beeson *et al.,* 2000), CD36 (Febbraio *et al.,* 2001). The best characterized ligand in *P. falciparum* is *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP 1) encoded by the highly variable *var* gene family (Smith *et al.,* 2001). The P*f*EMP 1 proteins are distantly related to the schizont-infected cell agglutination antigens (SICA), that are encoded by the SICAvar genes in *P. knowlesi,* and share binding signature motifs (Korir and Galinski, 2006).

Recently, the monkey malaria parasite *Plasmodium knowlesi* has become an important public health concern as it can infect humans (Cox-Singh *et al.,* 2010, 2008; Sabbatani *et al.*, 2010; White, 2008). Studies have demonstrated that its infection of non-human primates parallels that of *P. falciparum* thereby making it a suitable model for study of human malaria (Anderios *et al.,* 2010; Dutta *et al.,* 1982, 1981, 1978; Langhorne and Cohen, 1979; Ozwara *et al.,* 2003). The *P. knowlesi*-Olive baboon model has been shown to display clinical and pathological symptoms during pregnancy similar to *P. falciparum* in humans (Mustafa *et al.,* 2010). However, despite evidence showing the accumulation of parasites in the intervillous spaces of the placenta, the mechanism used by *P. knowlesi* IEs to sequester is not known and the molecules involved have not been determined.

In this study, we used bioinformatics analyses and *in vitro* static based adhesion assays to identify and characterize the ligand and receptor molecules involved in the accumulation of *P. knowlesi* infected erythrocytes in the placenta of Olive baboons.

## 2. Materials and Methods

### 2.1 Ethics statement

The proposal for this study was reviewed and passed as ethically acceptable by the Institutional Animal Care and Use Committee (IACUC) and the Institution's Scientific Ethical Review Committee (ISERC) of the Institute of Primate Research-Kenya where this work was done.

### 2.2 Study design

The study involved: a descriptive *in silico* study to identify the putative ligands and receptor molecules used by *P. knowlesi* and the Olive baboon to adhere to the placenta, *in vivo* study to investigate the effects of *P. knowlesi* malaria in Olive baboons during pregnancy and an *in vitro* study to determine the binding phenotype of *P. knowlesi* infected erythrocytes isolated from baboons during pregnancy (Figure 1).
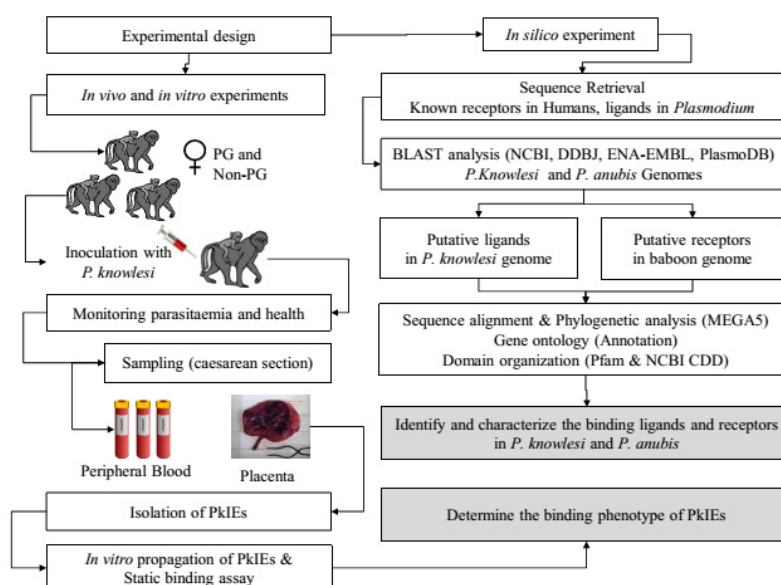


Figure 1. Schematic diagram of the study's experimental design

Four adult female baboons were screened and maintained in the company of an adult male for mating to occur. Pregnancy status and gestation periods of the baboons were confirmed by ultrasound. The baboons were infected together with four non-pregnant baboons (control) on gestation day 150 with $2 \times 10^5$ *P. knowlesi* blood stage parasites cultured overnight (Ozwara *et al.,* 2003). Following infection, level of parasitaemia was determined daily from day 3 post infection. When patent parasitaemia was established in baboons (day 7-9 post infection), caesarean sections (CS) were performed by an attending veterinarian to obtain intact sterile placental tissue (Mustafa *et al.,* 2010). Peripheral blood was also collected and processed together with the placentae to acquire infected erythrocytes. These isolated infected erythrocytes were then tested to determine their binding phenotypes in a static binding assay.

### 2.3 In vivo and in vitro experiments

2.3.1 Parasites

Baboons were infected using *P. knowlesi* H strain parasites originally from Pk1(A+) clone (Barnwell *et al.,* 1983) and whose genome has been fully sequenced (Pain *et al.,* 2008). *P. falciparum* lines CS2 and 3D7 were used to monitor the performance of static binding assay (obtained through MR4 as part of the BEI Resources Repository, NIAID, NIH, *P. falciparum* CS2, MRA-96, and *P. falciparum* 3D7 KAHRP knockout, MRA-554, deposited by SJ Rogerson and AF Cowman respectively) (Cowman, 1995; Rogerson *et al.*, 1995).

2.3.2 Animals

Female baboons (*P. anubis,* weighing between 12–23kg) were used. They were screened to ensure they were not

infected with *P. knowlesi*, hemoprotozoans, gastrointestinal parasites and Simian Immunodeficiency Virus (SIV) before inclusion in the study. Each baboon was housed in an individual squeeze back cage of dimensions $0.6 \times 0.6 \times 0.8$ meters at the bio-containment facility (according to Institute of Primate Research Standard Operating Procedures and guidelines for holding of animals) and fed on commercial non-human primate diet (Olobo and Black, 1990).

2.3.3 Parasite preparation and culture

To obtain infected erythrocytes from placenta and peripheral blood a method by Beeson and co-workers was used (Beeson *et al.,* 2000).

Laboratory *P. falciparum* lines CS2 and 3D7 were used as controls for testing binding to CSA, HA and CD36 (Cowman, 1995; Rogerson *et al.,* 1995). *In vitro* cultures were initiated with cryopreserved parasites using standard culturing techniques (Trager and Jensen, 1976). The parasite pellet was mixed with 5ml of complete RPMI 1640 medium [supplemented with 37.5mM HEPES, 7mM D-glucose, 6mM NaOH, 25µg/ml of gentamicin sulphate, 2mM L-glutamine, and 10% human serum] at pH7.2 and transferred to a T-25cm$^3$ culture flask (Corning, UK). The parasitaemia was adjusted to 2% using human PCV (O+ve erythrocytes), gassed with a mixture of 90% nitrogen, 5% carbon dioxide, and 5% oxygen and incubated at 37°C (Trager and Jensen, 1976).

*In vitro* culture of *P. knowlesi* parasites was initiated with cryopreserved parasites previously isolated from infected baboon placenta and peripheral blood. The pellet obtained was transferred into culture to a starting erythrocyte PCV of 2.5% in complete culture medium [RPMI 1640 (Sigma) supplemented with 2.5% baboon PCV, 20% baboon heat inactivated serum and 15µg/ml gentamycin solution (Sigma)]. Cultures were transferred into sterile labelled T-25cm$^3$ culture flasks, gently mixed, gassed with 5% $CO_2$, 5% $O_2$, and 90% $N_2$ for 30 seconds, and incubated at 36.5°C (Ozwara Suba and LUMC, 2005). Synchronization of *in vitro* cultures was done using 5% sorbitol to obtain homogenous staged parasites (Lambros and Vanderberg, 1979) and in cases of excess yields from propagation, they were cryopreserved using standard techniques (Moll *et al.,* 2008).

2.3.4 Static adherence assay

Samples obtained were cultured as described for up to 24 hours until mature trophozoite forms predominated (Beeson *et al.,* 2000). Laboratory parasite clones (*P. falciparum* 3D7 and CS2) were cultured using standard culturing techniques (Trager and Jensen, 1976). The parasitaemia was adjusted to 3% and 1% haematocrit for all adhesion assays.

Purified receptors used in this study were: Recombinant Human CD36/SR-B3 Fc Chimera (R & D Systems, UK), Chondroitin 4 sulphate sodium salt from bovine trachea, CSA (Sigma) and Hyaluronic acid from bovine vitreous humour, HA (Sigma).

A method by Fatih and others was used for binding experiments with modifications (Fatih *et al.,* 2012). Briefly, three identical areas of each Petri dish (60×15mm diameter, Falcon 1007, Becton Dickenson, Oxford, UK) were treated with 2µl aliquots of purified CSA, HA each at 100µg/ml and 50µg/ml and CD36 at 50µg/ml and 25µg/ml. Control areas were treated with phosphate buffered saline (PBS) and three marked areas were left untreated. The dishes were incubated in a humid chamber at 37°C for two hours before aspirating excess protein and blocking all areas with 1% w/v bovine serum albumin in PBS for 2 hours at 37°C. The blocking solution was removed and *in vitro* matured infected erythrocyte cultures were added to 3 ml warmed binding buffer (RPMI 1640 media supplemented with 2% D-glucose) to a final haematocrit of 3%. Each protein and the PBS control were represented in triplicate per dish and duplicate dishes were seeded per isolate. Dishes were seeded with 1.5ml of the prepared *in vitro* cultured cell suspension per isolate and additional assay dishes with *P. falciparum* clone CS2 and 3D7 as assay performance controls. The dishes were incubated at 37°C for 1 hour, with gentle mixing at 10 minutes intervals. Unbound cells were removed by gentle washing seven times with binding buffer. Bound cells were fixed with 1% v/v glutaraldehyde (Sigma) for 1 hour and stained with 10% Giemsa (BDH, VWR international Ltd, England) for 20 minutes. Using an inverted light microscope at × 300 magnification the number of bound infected erythrocytes was enumerated from non-overlapping fields for each protein and PBS treated area. The area of the field of view was equivalent to 0.19635mm$^2$. The results were expressed as the number of infected cells (IE) bound/ mm$^2$ to each of the proteins or control [IE/mm$^2$ = (1/0.19635) × mean number of bound IE per field] (Fatih *et al.,* 2012).

*2.4 Bioinformatics*

2.4.1 Sequence retrieval and homology search of *P. anubis* genome

Coding sequences (CDS) of the human receptor molecules chondroitin sulphate A (CSA) hyaluronic acid (HA) and CD36 (thrombospondin receptor) [GenBank: 126091140, 194018435 and 188536058 respectively] were

retrieved from NCBI (NCBI, http://www.ncbi.nlm.nih.gov ). These were aligned to NCBI's non-redundant protein and nucleotide databases, the DNA Database of Japan and the European Molecular Biology Laboratory's European Bioinformatics Institute (DDBJ, http://www.ddbj.nig.ac.jp/; ENA-EMBL, http://www.ebi.ac.uk/ena/; NCBI, http://www.ncbi.nlm.nih.gov) databases to identify their homologues in non-human primates. The BLASTn or BLASTx algorithms were used while maintaining the default parameters (Altschul *et al.*, 1990). The non-human primate (NHP) homologues identified were retrieved from NCBI's GenBank and individually aligned to the entire genome of *P. anubis* at NCBI (Zinner *et al.*, 2013) using BLASTn and BLASTx. The hits were manually inspected and those with highest percentage identities (PID) and lowest expectation values (E-values) were designated as the putative receptor molecules in *P. anubis*.

2.4.2 Characterization of putative receptor molecules in *P. anubis* genome

Putative protein sequences from *P. anubis* were aligned with sequences of known human receptors using MUSCLE and evolutionary history inferred using the Neighbor-Joining method in MEGA5 with bootstrap consensus tree inferred from 1000 replicates (Tamura *et al.*, 2011). To determine the probable function and location of the putative molecules, they were aligned to NCBI's non-redundant database to determine closest matching homologues. They were further characterized by comparing their domain organization to those of the known human placental receptor molecules in Pfam and NCBI CDD domain databases (Finn *et al.*, 2010; Marchler-Bauer *et al.*, 2011).

2.4.3 Sequence similarity search of *P. knowlesi* genome

Protein sequences of known erythrocyte binding ligands in *Plasmodium* were retrieved from PlasmoDB (Aurrecoechea *et al.*, 2009) and NCBI [PlasmoDB: PF3D7_1200600, PF3D7_0731500, PF3D7_0102500, PF3D7_0424300, PF3D7_1301600, PKH_000490, PKH_134580, PKH_062300, PVX_110810; GenBank: 23507807, 34525754, 6165411, 74766456, 29293851, 226438086]. These sequences were used in a BLAST search against the entire *P. knowlesi* genome hosted at PlasmoDB (Pain *et al.*, 2008). Further, the SICAvar antigen (SICAvar HB205, *P. knowlesi* from Malaysia 205kDa Pk1 (B+) 1+) [GenBank: AF078128.2] was also used as a query for BLAST against *P. falciparum* and *P. knowlesi* genomes hosted at PlasmoDB to determine its homologues. The hits generated were inspected, those with highest percentage identities and an expectation value (E-value) of < 1e-04 were designated as the putative erythrocyte binding ligands in the *P. knowlesi* genome.

2.4.4 Characterization of putative binding ligands in *P. knowlesi* genome

To predict the molecular function and cellular location of the putative erythrocyte binding molecules identified, they were aligned against the NCBI non-redundant database to determine closest matching homologues. Their protein parameters and inferred gene ontology (GO) terms were obtained from PlasmoDB and SIB's ProtParam tool (ExPASy - ProtParam tool, http://web.expasy.org/protparam/; PlasmoDB, http://www.plasmodb.org). Furthermore, their domain organization was obtained in a domain search against the Pfam and NCBI CDD domain databases and compared to the known *P. falciparum* erythrocyte binding ligand (Var2csa) (Finn *et al.*, 2010; Marchler-Bauer *et al.*, 2011).

*2.5 Statistical analysis*

Statistical analyses were performed using R 2.15.2 (R Core Team, 2012). The Mann-Whitney U-test and the Kruskal-Wallis test were used to evaluate statistical significance of the data since distributions were skewed from normal (Fatih *et al.*, 2012; Heddini *et al.*, 2001). Probability values of *P* < 0.05 were considered significant.

**3. Results**

*3.1 Receptor molecules (CSPG 4 and HAPLN 1) discovered in P. anubis are similar to human receptors CSA and HA*

To determine the putative receptors in *P. anubis*, sequences of known human placental receptors were BLASTed against the genome of *P. anubis* from NCBI server and the hits generated analysed. The human receptor molecules CSA (CSPG 4), HA (HAPLN 1) and CD36 all aligned to their homologues in non-human primates with high identities and low expectation values (PID 80–99%, E-value <0.0) (Table 1). Human CSA aligned to predicted chondroitin sulphate proteoglycan (CSPG 4 and CSPG 4-like) nucleotides and proteins, human HA aligned to predicted hyaluronan and protein link protein 1(HAPLN 1 and variants 2 and 3), while CD36 molecule aligned to CD36 molecules (platelet glycoprotein isoforms and transcript variants 2, 3 and 4).

The non-human primate homologues were used as queries to BLAST the *P. anubis* genome. The BLAST hits showed that the individual sequences aligned in similar manner to the non-redundant nucleotide and protein

databases of *P. anubis*. The top BLAST hits had identities ranging from 83 to 99% with low expectation values (E-value=0.0). The predicted CSPG 4 homologues aligned to CSPG 4 and CSPG 4-like partial sequences in *P. anubis* genome (PID=>90%, E=0.0), while the predicted HAPLN 1 homologues aligned to HAPLN 1 (PID=>93%, E=0.0). The BLAST also identified other CSPGs namely neurocan, versican, aggrecan and brevican. This suggests that the baboon has the receptor molecules CSPG 4 and HAPLN 1 that are similar to the human receptors CSA and HA.

Table 1. BLAST hits of known human receptors to non-human primates

| Query | Predicted Sequences producing HSPs (proteins) | Organism | % ID |
|---|---|---|---|
| *H. sapiens* CSPG4 (8305bp) | CSPG 4 | *Gorilla gorilla gorilla* | 99 |
| | LQP: CSPG 4 | *Pan paniscus* | 99 |
| | CSPG 4 | *Pan troglodytes* | 99 |
| | CSPG 4, partial | *Papio anubis* | 97 |
| | CSPG 4 | *Saimiri boliviensis boliviensis* | 95 |
| | CSPG 4-like, partial | *Macaca mulatta* | 94 |
| | CSPG 4 | *Otolemur garnettii* | 89 |
| | CSPG 4 | *Nomascus leucogenys* | 92 |
| | LQP: CSPG 4 | *Callithrix jacchus* | 95 |
| | CSPG 4-like, partial | *Pan troglodytes* | 94 |
| *Homo sapiens* HAPLN 1 (4678bp) | HAPLN 1 isoform 3 | *Pan troglodytes* | 99 |
| | HAPLN 1 | *Gorilla gorilla gorilla* | 99 |
| | HAPLN 1 | *Saimiri boliviensis boliviensis* | 97 |
| | LQP: HAPLN 1 | *Papio anubis* | 98 |
| | Proteoglycan link protein | *Macaca fascicularis* | 98 |
| | HAPLN 1 | *Nomascus leucogenys* | 99 |
| | HAPLN 1 isoform 2 | *Pongo abelii* | 98 |
| | HAPLN isoform 1 | *Macaca mulatta* | 98 |
| | HAPLN 1 | *Otolemur garnettii* | 97 |
| | unnamed protein product | *Macaca fascicularis* | 98 |
| | HAPLN 1 isoform 2 | *Callithrix jacchus* | 97 |
| *Homo sapiens* CD36 molecule (thrombospondin receptor) (CD36), transcript variant 1, (4727bp) | PG 4 isoform 4 | *Pan troglodytes* | 99 |
| | PG 4 | *Pongo abelii* | 98 |
| | PG 4 | *Gorilla gorilla gorilla* | 98 |
| | PG 4 isoform 1 | *Nomascus leucogenys* | 96 |
| | PG 4 | *Macaca mulatta* | 95 |
| | PG 4 isoform 1 | *Saimiri boliviensis boliviensis* | 92 |
| | PG 4 | *Callithrix jacchus* | 92 |
| | PG 4-like | *Otolemur garnettii* | 88 |
| | PG 4 isoform 6 | *Nomascus leucogenys* | 97 |
| | PG 4 isoform 4 | *Saimiri boliviensis boliviensis* | 91 |
| | PG 4-like | *Otolemur garnettii* | 75 |

**Key:** *Macaca mulata* (Rhesus macaque), *Pongo abelii* (Sumatran orangutan), *Gorilla gorilla* (Gorilla), *Nomascus leucogenys* (White-cheeked gibbon), *Pan troglodytes* (Chimpanzee), *Papio anubis* (Olive baboon),

*Callithrix jacchus* (White-tufted ear marmoset), *Macaca fascularis* (Crab-eating macaque), *Homo sapiens* (man), *Pan paniscus* (Bonobo – Pigmy chimpanzee), *Saimir boliviensis boliviensis* (Black-capped squirrel monkey), *Otolemur garnetii* (Bushbaby).

CSPG (chondroitin sulphate proteoglycan), HAPLN (hyaluronan and protein link protein), LQP (low quality protein), PG (platelet glycoprotein)

*3.2 Putative P. anubis receptor sequences cluster with Macaque monkeys' sequences*

Phylogenetic relationship was inferred using the putative *P. anubis* receptor sequences and sequences from human and non-human primate receptors. Putative sequences from *P. anubis* clustered to homologous sequences in macaque monkeys *M. mulatta* and *M. fascularis*, the natural hosts of *P. knowlesi* (Figure 2 A, B and C). In general, the human receptor sequences (CSPG, HAPLN 1 and CD36 antigen) clustered with homologous sequences from higher non-human primates like gorilla and chimpanzees. However, it was noted that all the homologous sequences share a common ancestral origin as demonstrated by branching of the phylogenetic tree and bootstrap values. The CSPG core protein sequences (neurocan, aggregan, versican and brevican) from *P. anubis* all clustered together forming a clade that diverged from other CSPG homologues in *P. anubis* and other non-human primates (Figure 2A).

The variants HAPLN 2, 3 and 4 from *P. anubis* all clustered together forming a clade divergent from the HAPLN 1 sequence in *P. anubis* and other non-human primates homologues (Figure 2B). It was noted that the *P. anubis* sequences were closer to the lower monkeys (marmoset, bushbaby) than higher monkeys (gorilla and chimpanzee) that clustered to human HAPLN 1 sequence forming a clade. Likewise, the scavenger receptor class B member (SRCBM) proteins 1 and 2 diverged from similar ancestry to platelet glycoprotein 4 (PG 4) from *P. anubis* and other non-human primate homologues (Figure 2C).
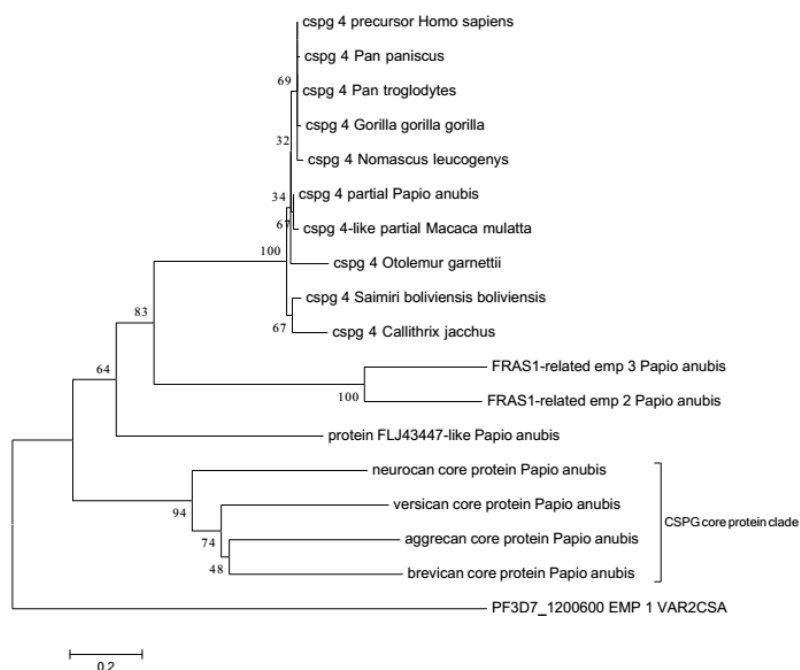


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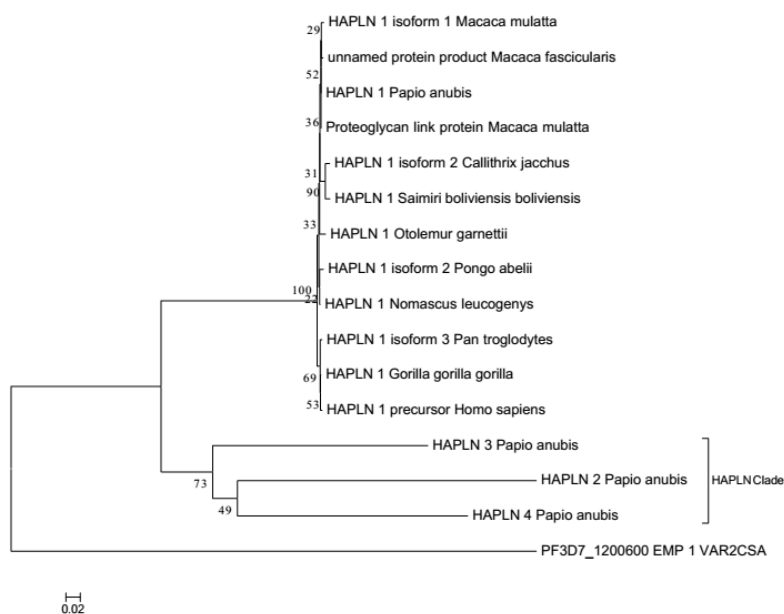
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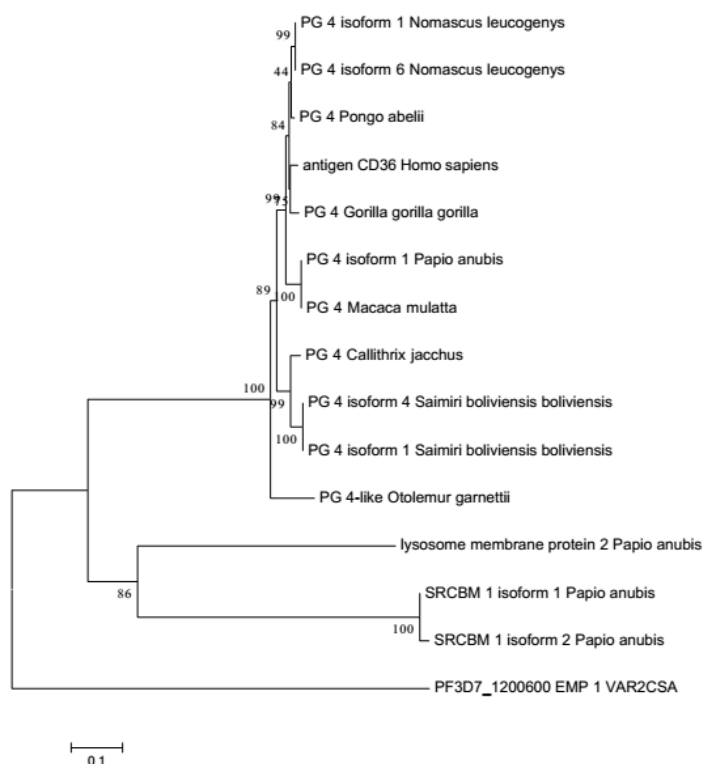


C

Figure 2. Phylogenetic relationships of sequences from *P. anubis* and other non-human primates.

The Neighbor-Joining trees were constructed using the Poisson correction method for 18 CSPG sequences (A), 16 HAPLN sequences (B) and 15 CD36 sequences (C). The numbers at the juncture of two branches are the percent of 1000 bootstrap trees with the same branch length. *P. falciparum* sequence Pf3D7_1220600 EMP 1 VAR2CSA was included as an out-group.

The *P. anubis* sequences with the highest identities and least E-values from BLAST analysis were designated as the likely molecules that mediate placental erythrocyte binding (Table 2). The top hits for the CSPG receptor

were CSPG 4 and CSPG 4-like partial sequences. However, other hits were discovered which are a group of aggregate proteins associated with CSPGs (neurocan, aggregan, versican and brevican core proteins). Equally, the top hit for the HAPLN 1 receptor was HAPLN 1 in the *P. anubis* genome. Other hits that were closely associated were; HAPLN 2, 3 and 4 which are variants of HAPLN 1. The human CD36 molecule receptor identified similar CD36 molecule transcript variants 1 and 2 along with other scavenger receptor class B member proteins in the *P. anubis* genome.

Table 2. Putative sequences in the Olive baboon (*P. anubis*) genome used as receptor molecules

| Nucleotide | Accession no | Protein | Accession no |
|---|---|---|---|
| **CSPG** | | | |
| CSPG4, partial | XM_003901231.1 | CSPG 4, partial | XP_003901280.1 |
| CSPG 4-like, partial | XR_163612.1 | neurocan core protein | XP_003915287.1 |
| PUP (FLJ43447) | XM_003901225.1 | FRAS1-related EMP 3 | XP_003899274.1 |
| neurocan (NCAN) | XM_003915238.1 | PUP (FLJ43447) | XP_003901274.1 |
| | | aggrecan core protein | XP_003901403.1 |
| | | versican core protein | XP_003899948.1 |
| | | brevican core protein | XP_003892883.1 |
| | | FRAS1-related EMP 2 | XP_003913840.1 |
| **HAPLN 1** | | | |
| HAPLN 1 | XM_003899897.1 | HAPLN 1 | XP_003899946.1 |
| | | HAPLN 3 | XP_003901408.1 |
| | | HAPLN 4 | XP_003915288.1 |
| | | HAPLN 2 | XP_003892880.1 |
| **CD36 molecule** | | | |
| CD36 molecule, transcript variant 1 | XM_003896397.1 | PG 4 isoform 1 | XP_003896446.1 |
| CD36 molecule, transcript variant 2 | XM_003896398.1 | LMP 2 | XP_003898782.1 |
| | | SRCBM 1 isoform 2 | XP_003907409.1 |
| | | SRCBM 1 isoform 1 | XP_003907408.1 |

**Key**: HAPLN 1 (hyaluronan and proteoglycan link protein 1), CSPG (chondroitin sulphate proteoglycan 4), CD36 (CD36 molecule, thrombospondin receptor), EMP (extracellular matrix protein), PG (platelet glycoprotein), PUP (putative uncharacterized protein), LMP (lysosome membrane protein), SRCBM (scavenger receptor class B member)

*3.3 P. knowlesi EBP-alpha, beta and gamma as putative ligands for placental adhesion*

To determine prospective ligands in *P. knowlesi*, sequences of known *Plasmodium* erythrocyte binding antigens were BLASTed against the genome of *P. knowlesi* and hits generated were analysed. The *P. knowlesi* SICAvar antigen (SICAvar-HB205) sequence aligned to various molecules in *P. falciparum* genome but the hits were of high expectation values (E-value > 0.062, cut off <1e-04). However, hits against *P. knowlesi* were of low expectation values (E-value 0.0, cut off <1e-04) with high identity to its variants (SICAvar antigen and SICAvar antigen (fragment)).

Fifty seven (57) protein sequences were selected following BLAST analysis as the putative ligands in *P. knowlesi* genome (E-value cut off <1e-04) (Table 3). The top hits are the erythrocyte binding proteins – *alpha*, *beta* and *gamma*. They aligned to *P. falciparum* Var2csa, a known binding ligand, with low expectation values (4.3e-09 to 5.3e-13, cut off <1e-04). Thirty four of the sequences (60%) are conserved *Plasmodium* proteins of unknown function, some are merozoite surface proteins and others are annotated for different functions. Since the *P. knowlesi* erythrocyte binding ligands (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) demonstrated high identities and low expectation values against the known erythrocyte binding ligands, we designated them as the putative ligands used for adhesion.

Table 3. Putative sequences in the *P. knowlesi* genome used as ligand molecules

| Sequences producing HSP (proteins) | Accession number |
|---|---|
| Erythrocyte binding protein (beta) | PKH_000490 |
| Erythrocyte binding protein (gamma) | PKH_134580 |
| Erythrocyte binding protein (alpha) | PKH_062300 |
| Reticulocyte binding protein, putative | PKH_070003 |
| Chromosome associated protein, putative | PKH_051770 |
| Conserved *Plasmodium* protein, unknown function | PKH_060900, PKH_061100, PKH_103220, PKH_143120, PKH_121410, PKH_094540, PKH_092240, PKH_041690, PKH_031040, PKH_126570, PKH_131300, PKH_052170, PKH_101030, PKH_081490, PKH_062110, PKH_100260, PKH_125560, PKH_126220, PKH_133810, PKH_030800, PKH_050220, PKH_081890, PKH_145780, PKH_090490, PKH_112280, PKH_133180, PKH_062110, PKH_083470, PKH_146600, PKH_081330 |
| RNA binding protein, putative | PKH_142610 |
| Ada2-like protein, putative | PKH_081460 |
| Atypical protein kinase, ABC-1 family, putative | PKH_133270 |
| CCAAT-box DNA binding protein subunit B | PKH_094400 |
| Dynein heavy chain, putative | PKH_130100, PKH_092040 |
| Eukaryotic translation initiation factor 4 gamma | PKH_141200 |
| Hypothetical protein, conserved in Apicomplexans | PKH_130600 |
| Hypothetical protein, conserved in *Plasmodium* | PKH_130670, PKH_140190 |
| Merozoite adhesive erythrocytic binding protein | PKH_094500 |
| Merozoite surface protein | PKH_145630 |
| *Plasmodium* exported protein, unknown function | PKH_120020 |
| Pre-mRNA splicing factor, putative | PKH_060650 |
| Protein phosphatase, putative | PKH_093290 |
| Rhomboid protease, putative | PKH_133640 |
| Rhoptry associated membrane antigen, putative | PKH_010540 |
| Ribonuclease, putative | PKH_113360 |
| Splicing factor, putative | PKH_123140 |
| Tryptophan-rich antigen | PKH_146990 |
| Ubiquitin transferase, putative | PKH_112120 |
| Zinc finger protein, putative | PKH_100710 |

(Source PlasmoDB ver 9: March 2013)

*3.4 Analogous domain organization of putative ligands in P. knowlesi and receptors in P. anubis to P. falciparum and humans respectively*

To characterize the putative receptor molecules in *P. anubis* their domain organization was compared to known human receptors using Pfam and NCBI CDD databases. The putative baboon sequences showed similar domain organization to the human receptor sequences (Table 4). The predicted CSPG 4, partial sequence of *P. anubis* and CSA (CSPG) of human origin had the domain Laminin-G. Likewise, the predicted HAPLN 1 sequence of *P.*

*anubis* and HA (HAPLN 1) of human origin had the domains immunoglobulin V-set and extracellular link protein. The human receptor CD36 molecule (thrombospondin receptor) also had similar domain organization as the predicted *P. anubis* receptors. Similar domains suggest that they are conserved in the Olive baboon and could have evolved to perform a similar function like in humans.

Table 4. Domain organization of putative baboon and known human receptor molecules' sequences

| Sequence | Organism | Pfam and NCBI CDD Domains |
|---|---|---|
| **CSPG** | | |
| CSA (CSPG) | *H. sapiens* | Laminin Domain (G1 and G2) |
| Predicted: CSPG 4, partial | *P. anubis* | Laminin Domain (G1 and G2) |
| PREDICTED: neurocan core protein; PREDICTED: aggrecan core protein; PREDICTED: LQP versican core protein; PREDICTED: brevican core protein | *P. anubis* | Immunoglobin V-set domain, Xlink (Extracellular link domain), Xlink (Extracellular link domain), EGF-like domain, Lectin C (Lectin C-type domain), Sushi (Sushi domain – SCR repeat) |
| PREDICTED: FRAS1-related EMP 3; PREDICTED: LQP FRAS1-related EMP 2 | *P. anubis* | Calx-beta domain |
| PREDICTED: LQP: PUP FLJ43447-like | *P. anubis* | None |
| **HAPLN 1** | | |
| HA (HAPLN 1) | *H. sapiens* | Immunoglobin V-set domain ; Xlink (Extracellular link domain), Xlink (Extracellular link domain) |
| Predicted: HAPLN 1; PREDICTED: LQP: HAPLN 3; PREDICTED: HAPLN 4; PREDICTED: HAPLN 2 | *P. anubis* | Immunoglobin V-set domain ; Xlink (Extracellular link domain), Xlink (Extracellular link domain) |
| **CD36 molecule** | | |
| CD36 (Thrombospondin receptor) | *H. sapiens* | CD36 (CD36 family) |
| Predicted: PG 4 isoform 1; PREDICTED: LMP 2; PREDICTED: SRCBM 1 isoform 2; PREDICTED: SRCBM 1 isoform 1 | *P. anubis* | CD36 (CD36 family) |

(Source Pfam and NCBI CDD website)

**Key**: PG-4 (platelet glycoprotein 4), HAPLN 1 (hyaluronan and proteoglycan link protein 1), PUP (putative uncharacterized protein), LQP (low quality protein), LMP (lysosome membrane protein), SRCBM (scavenger receptor class B member), CSPG (chondroitin sulphate proteoglycan), SCR (short consensus repeats)

To characterize the putative ligand molecules in *P. knowlesi* their protein parameters and gene ontology (GO) terms were determined from PlasmoDB and ProtParam. The GO terms denote the inferred biological process involved in, cellular component and molecular function of the proteins. The putative ligand molecules are located on different chromosomes in the *P. knowlesi* genome. Majority of them are found along chromosomes 12 to 14 (44%), few along chromosomes 1 to 7 (24%) and the rest (29%) along chromosomes 8 to 11 (Table 5). Most of the molecules (71%) are not annotated for any function in their GO terms. The rest are annotated for various functions not related to adhesion. Notably, some are annotated for the biological process of pathogenesis, are integral to the membrane in location and function in either binding or receptor activity (PKH_062300, PKH_134580, PKH_000490, PKH_094500, PKH_140190 and PKH_083470). The molecules have an average molecular weight of 182,449Da (range 53,905-792,939Da) and average isoelectric point of 6.67 (range 3.84-10.4). The molecules (PKH_062300, PKH_134580, PKH_000490, PKH_094500, PKH_140190 and PKH_083470) could be involved in binding in *P. knowlesi* since they show similar gene ontology to the *P.*

*falciparum* binding ligand var2csa.

Table 5. Protein parameters and gene ontology of the putative ligand molecules in *P. knowlesi*

| Accession No. | Location | GO Term Name | Mol.Wt (Da) | pI |
|---|---|---|---|---|
| PKH_121410 | Chr12: 630,081 to 634,832 | Carbohydrate metabolic process (BP), extracellular region (CC), carbohydrate binding (MF) | 176959 | 5.08 |
| PKH_120020 | Chr12: 3,896 to 6,013 | Chromosome telomeric region (CC), telomeric DNA binding (BP) | 57166 | 9.57 |
| PKH_031040 | Chr3: 523,717 to 527,129 | Intracellular transport (BP) | 68156 | 4.06 |
| PKH_060900 | Chr06: 426,927 to 430,622 | None | 141313 | 4.29 |
| PKH_061100 | Chr06: 502,113 to 507,287 | None | 192739 | 10.02 |
| PKH_092240 | Chr09: 986,223 to 991,010 | None | 181982 | 4.37 |
| PKH_041690 | Chr04: 734,902 to 740,466 | None | 217077 | 5 |
| PKH_131300 | Chr13: 615,512 to 619,429 | None | 149710 | 4.13 |
| PKH_052170 | Chr05: 965,222 to 968,315 | None | 97857 | 4.29 |
| PKH_101030 | Chr10: 514,632 to 519,997 | None | 173506 | 8.82 |
| PKH_081490 | Chr08: 680,039 to 686,932 | None | 255988 | 7.67 |
| PKH_062110 | Chr06: 940,976 to 946,909 | None | 205736 | 9.79 |
| PKH_130670 | Chr13: 304,702 to 307,452 | None | 102905 | 3.84 |
| PKH_126570 | Chr12: 2,845,961 to 2,850,019 | None | 57705 | 9.6 |
| PKH_130600 | Chr13: 275,798 to 283,680 | None | 292616 | 8.25 |
| PKH_100260 | Chr10: 170,011 to 175,479 | None | 209315 | 6.59 |
| PKH_125560 | Chr12: 2,399,748 to 2,402,267 | None | 97360 | 10.4 |
| PKH_126220 | Chr12: 2,675,025 to 2,697,654 | None | 792939 | 8.57 |
| PKH_133810 | Chr13: 1,830,784 to 1,835,121 | None | 167741 | 4.74 |
| PKH_030800 | Chr03: 399,430 to 403,702 | None | 100445 | 8.61 |
| PKH_050220 | Chr05: 148,645 to 151,737 | None | 121772 | 6.65 |
| PKH_081890 | Chr08: 866,815 to 872,536 | None | 195316 | 5.08 |
| PKH_090490 | Chr09: 210,532 to 213,774 | None | 127731 | 9.59 |
| PKH_112280 | Chr11: 1,140,808 to 1,143,552 | None | 106513 | 9.19 |
| PKH_133180 | Chr13: 1,534,695 to 1,539,509 | None | 182749 | 9.14 |
| PKH_103220 | Chr10: 1,455,731 to 1,466,680 | None | 159469 | 5.15 |
| PKH_126570 | Chr12: 2,845,961 to 2,850,019 | None | 57705 | 9.6 |
| PKH_146600 | Chr14: 2,950,491 to 2,956,205 | None | 208322 | 9.01 |
| PKH_081330 | Chr8: 602,756 to 604,958 | None | 53905 | 6.17 |
| PKH_130600 | Chr13: 275,798 to 283,680 | None | 292616 | 8.25 |
| PKH_070003 | Chr7: 8,828 to 18,024 | None | 344108 | 6.69 |
| PKH_145630 | Chr14: 2,529,932 to 2,532,694 | None | 101555 | 4.43 |
| PKH_145780 | Chr14: 2,586,597 to 2,602,757 | Nucleotide binding (BP) | 639217 | 5.28 |
| PKH_062300 | Chr6: 1,032,863 to 1,036,679 | Pathogenesis (BP), integral to membrane (CC), receptor activity | 120703 | 5.29 |

| PKH_134580 | Chr13: 2,177,465 to 2,181,275 | Pathogenesis (BP), integral to membrane (CC), receptor activity | 121212 | 4.9 |
|---|---|---|---|---|
| PKH_000490 | 1,863 to 5,920 | Pathogenesis (BP), integral to membrane (CC), receptor activity | 130481 | 4.79 |
| PKH_094500 | Chr9: 2,060,514 to 2,067,075 | Pathogenesis (BP), membrane (CC), apical complex (CC), binding (MF) | 226916 | 7.74 |
| PKH_140190 | Chr14: 74,144 to 77,074 | Protein binding | 108375 | 5.78 |
| PKH_083470 | Chr8: 1,606,360 to 1,609,299 | Protein binding (MF) | 112853 | 4.48 |
| PKH_094540 | Chr09: 2,106,607 to 2,111,130 | RNA binding (BP) | 135372 | 4.27 |
| PKH_143120 | Chr14: 1,429,011 to 1,438,213 | RNA binding (BP), binding (MF), transferase activity (MF) | 194311 | 4.17 |

(Source PlasmoDB ver 9: March 2013)

**Key**: Biological process (BP), cellular component (CC), molecular function (MF)

Following Pfam analysis less than half of the sequences (30%) were annotated for various domain organizations while 22 sequences (67%) failed to be annotated. In comparison only 11 sequences (33%) failed to be annotated with the rest having various domain organizations in the NCBI CDD analysis (Table 6). Among the annotated sequences, the erythrocyte binding proteins (PKH_000490, PKH_062300, and PKH_134580) had the domains; duffy binding protein N, duffy binding domain, and EBA-175 VI which are similar to the reference *P. falciparum* ligand (Var2csa) which has 7 duffy binding domains. Seven sequences (21%) had the domain Smc (Chromosome aggregation ATPases), and the remaining sequences (36%) had different domains. The *P. knowlesi* erythrocyte binding proteins (PKH_000490, PKH_062300, and PKH_134580) share some domains with the *P. falciparum* binding ligand var2csa indicating that they may be performing similar functions.

Table 6. Domain organization of the putative ligand molecules in *P. knowlesi* genome

| Accession No. | | Pfam Domains |
|---|---|---|
| PF3D7_1200600 | | Duffy binding domains (7) |
| PKH_094500 | | Apical membrane antigen 1 (AMA-1), EBA-175 VI |
| PKH_060900 | | CDC45 |
| PKH_125560 | | cwf21 domain |
| PKH_070003 | | DUF2937 (unknown function) |
| PKH_000490, | PKH_062300, PKH_134580 | Duffy BP N, Duffy binding domain, DBP, EBA-175 VI |
| PKH_030800 | | KLRAQ (predicted coiled-coil domain) |
| PKH_140190 | | LisH |
| PKH_130670, PKH_092240, PKH_126570, PKH_052170, PKH_081490, PKH_130600, PKH_133810, PKH_081890, PKH_112280, PKH_083470, PKH_081330, | PKH_061100, PKH_041690, PKH_131300, PKH_101030, PKH_062110, PKH_100260, PKH_050220, PKH_090490, PKH_133180, PKH_146600, PKH_145630 | None |
| PKH_126220 | | PapD-like |

| PKH_103220 | | Tryptophan-Threonine-rich Plasmodium antigen C terminal (TryThrA_C) |
|---|---|---|
| | | **NCBI CDD Domains** |
| PKH_094500 | | Apical membrane antigen 1 (AMA-1), EBA-175 VI, PTZ0012 (MAEBL provisional) |
| PKH_060900, PKH_092240, PKH_081490, PKH_133810, PKH_081330, PKH_126220, | PKH_130670, PKH_101030, PKH_062110, PKH_146600, PKH_145630, PKH_133180 | None |
| PKH_125560 | | cwf21 domain |
| PKH_070003 | | PTZ00440 |
| PKH_000490, PKH_134580 | PKH_062300, | Duffy BP N, Duffy binding domain, DBP, EBA-175 VI |
| PKH_030800 | | SH3_and_anchor |
| PKH_140190 | | LisH |
| PKH_061100 | | 2A1904 K+-dependent Na+/Ca+ exchanger |
| PKH_126570, | PKH_081890 | COG2433Uncharacterized conserved protein |
| PKH_131300 | | DNA_pol-phi super family |
| PKH_100260 | | Inter-Src homology 2 (iSH2) helical domain of Class IA Phosphoinositide 3-kinase; COG1340Uncharacterized archaeal coiled-coil protein |
| PKH_050220, PKH_112280, PKH_041690, PKH_130600 | PKH_090490, PKH_083470, PKH_052170, | Smc, SMC_prok_B |
| PKH_103220 | | Tryptophan-Threonine-rich Plasmodium antigen C terminal (TryThrA_C) |

(Source Pfam and NCBI CDD)

**Key**: Smc (Chromosome segregation ATPases), SMC_prok (structural maintenance of chromosomes, prokaryotes)

*3.5 Binding of P. knowlesi infected erythrocytes to purified receptors*

*P. knowlesi* infected erythrocytes isolated from pregnant Olive baboons were tested for binding against immobilized purified receptors. None of the *P. knowlesi* infected erythrocytes isolated from either baboon placenta (Pan3443) or peripheral blood (Pan3614 and Pan3443) bound to the purified receptors CD36, CSA or HA (Figure 3B, C and D). The two parasite clones used to optimize the static binding assay bound variably to the purified receptors being tested (Figure 3A). In general, *P. falciparum* CS2 bound at high levels to the CSA and HA receptors (Median, 3820 and 1681 IEs/mm$^2$ respectively) while *P. falciparum* 3D7 bound at high levels to CD36 (Median, 785 IEs/mm$^2$). Binding to the receptors CSA and HA was significantly different in PfCS2 ($p$=0.00) unlike in Pf3D7 where this was not significant ($p$=0.80). The level of binding of the receptors CSA and HA when compared between the two parasite clones was significantly different ($p$=0.00 and $p$=0.00) respectively/
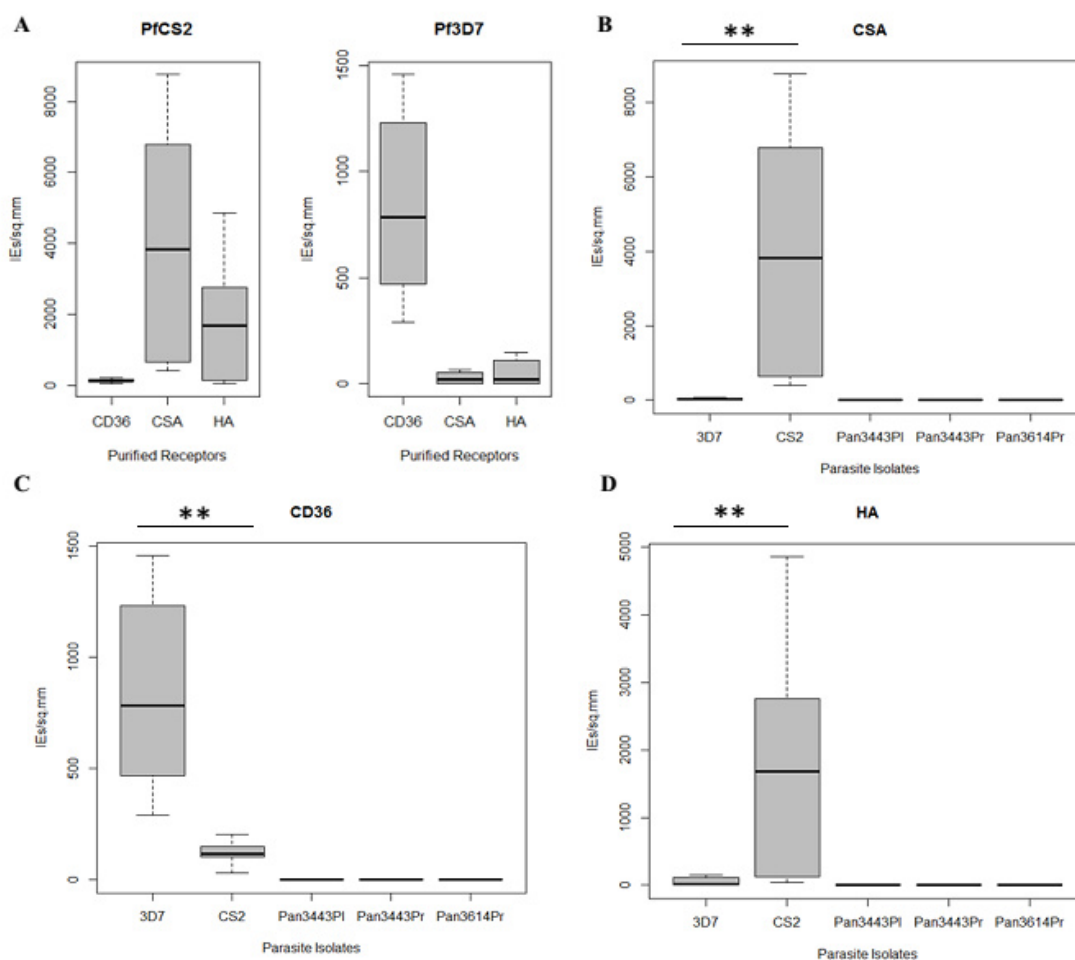
Figure 3. Box and whisker plots of infected erythrocytes binding to purified receptors CSA, HA and CD36

(A) Binding of *P. falciparum* CS2 and 3D7 IEs used as control. (B, C and D) Binding of *P. knowlesi* infected erythrocytes from placenta (PAN3443Pl) and peripheral blood (PAN3614Pr, PAN3443Pr) to purified receptors. Data represents the median and interquartile ranges of adherent infected erythrocytes per $mm^2$. PfCS2 and Pf3D7 were used as assay performance controls. **$p < 0.05$

## 4. Discussion

In the current study, we elucidated the factors that mediate binding in *P. knowlesi* and the Olive baboon using two approaches: (1) comparing putative receptor and ligand sequences in baboon with human and *P. knowlesi* with *P. falciparum* respectively and (2) adhesion assays.

In *P. anubis*, we identified the putative receptors as: predicted CSPG 4, predicted HAPLN 1 and CD36 molecule transcript variants 1, 2 for the corresponding human receptors CSA (CSPG), HA (HAPLN) and CD36 respectively. Previous studies have shown that non-human primates are phylogenetically closer to human beings (Galinski and Barnwell, 2012) and this would explain why the data obtained showed high identities and low e-values for the top BLAST hits. Phylogenetic analyses showed that the putative receptors identified in *P. anubis* all cluster together with sequences from macaques particularly *M. mulatta* and *M. fascularis* which are known natural hosts of *P. knowlesi* (Cox-Singh *et al.,* 2010, 2008). Since the Olive baboon is susceptible to *P. knowlesi* with similar disease outcomes to humans (Mustafa *et al.*, 2010; Ozwara *et al.,* 2003) these data suggest that the putative molecules identified could be used for adhesion. On the other hand, receptor sequences from human receptors clustered with sequences from the great apes; gorilla, bonobo and chimpanzee which are phylogenetically closer to humans (Galinski and Barnwell, 2012; Sibal and Samson, 2001). Despite this clustering, the putative receptors have common ancestry to the human receptors since they diverged from base and their evolutionary distance is close.

The putative receptors identified in *P. anubis* had similar domain organization to the known human receptors.

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The Laminin-G (LamG) domains common in human and *P. anubis* CSPG sequences are $Ca^{2+}$ mediated receptors with binding sites for steroids, beta1 integrins, heparin, sulfatides, fibulin-1, and alpha-dystroglycans. They play a role in signal transduction via cell-surface steroid receptors, adhesion, migration and differentiation through mediation of cell adhesion molecules (Marchler-Bauer *et al.,* 2011). Likewise, HAPLN 1 sequences had the domains immunoglobulin V-set and extracellular link protein. The link proteins are involved in interaction with HA and contribute to the structural integrity of different tissues (Marchler-Bauer *et al.,* 2011). Lastly, the CD36 family domain identified in the CD36 molecule of both *P. anubis* and human genome is thought to be a novel class of scavenger receptors with a role in signal transduction and cell adhesion (Marchler-Bauer *et al.,* 2011). CD36 is ubiquitous in system, mostly found in vasculature, not in placental tissue (Febbraio *et al.,* 2001; McCormick *et al.,* 1997). These data suggest that the identified molecules in *P. anubis* are likely used for adhesion due to their similar domain structure to the human receptors.

We identified 57 protein sequences from BLAST results as the putative molecules in the *P. knowlesi* homologous to *Plasmodium* binding ligands. Although there was no clear homology observed due to high expectation values, an E-value of <1e-04 was used as cut off to select the best aligning sequences. Majority of these protein sequences were conserved *Plasmodium* proteins of unknown function while some have been annotated for functions other than adhesion like Ada2-like protein, Dynein heavy chain or Ribonuclease. The putative ligand molecules aligned to sequences that are conserved in either *Plasmodium vivax* Sal-1 or *Plasmodium cynomolgi* strain B organisms but which have not been annotated for any function. This suggests close homology between these three species and is consistent with previous studies that have shown *P. knowlesi* and *P. vivax* to be phylogenetically related (Carlton *et al.,* 2008).

Notably, the top hits identified were the *P. knowlesi* erythrocyte binding proteins; EBP-*alpha* (PKH_062300), EBP-*beta* (PKH_000490), and EBP-*gamma* (PKH_134580). These were initially used as queries in this study on the premise that they could be homologous to molecules used in binding of *P. knowlesi* IEs to the placenta of Olive baboons. They are known erythrocyte invasion ligands (Chitnis and Miller, 1994). They have the domains: duffy binding protein N, duffy binding domain, and EBA-175 VI that are similar to the duffy binding domains of the principal *P. falciparum* ligand (Var2csa). Similarly, they are annotated for the biological process of pathogenesis, are integral to the membrane in location and function in either binding or receptor activity (GO terms). This suggests that they could be performing similar functions to the DBL domains of var2csa and are likely the *P. knowesi* ligands responsible for binding to the placenta of Olive baboons.

Sequence similarity searches showed that SICAVar antigen molecule in *P. knowlesi* does not align to *P. falciparum* binding ligands but aligns to its variants in *P. knowlesi*. The SICAvar and KIR genes are the largest variant gene families in the published genome of *P. knowlesi* (Pain *et al.,* 2008). These antigens found on the surface of infected erythrocytes are associated with parasite virulence and have been shown to be essential in antigenic variation (Korir and Galinski, 2006). They have shown that despite the *P. knowlesi* SICAvar and *P. falciparum* var antigen families encoding proteins that enable antigenic variation in the respective organisms, they do not share significant level of sequence identity when aligned. Although distantly related, some *P. knowlesi* SICA peptides show identity with a particular *P. falciparum* EMP1, mapping throughout all characterized domains, including the externally exposed cysteine-rich domains that are characteristic of both proteins (Korir and Galinski, 2006).

We tested *P. knowlesi* infected erythrocytes (*Pk*IEs) isolated from the placenta and the peripheral blood of Olive baboons for their ability to bind to purified receptors in a static binding assay. We found that none of the *Pk*IEs adhered to either CSA, HA or CD36, the receptors responsible for *P. falciparum* sequestration in the placenta and microvasculature in humans (Beeson *et al.,* 2000; Fried and Duffy, 1996). A cerebral malaria study by Fatih and co-workers (2012), found that *Pk*IEs from human subjects bound in a variable manner to the endothelial receptors ICAM-1 and VCAM but not to CD36. In retrospect, *P. knowlesi* malaria in the Olive baboon during pregnancy showed that the infection led to the accumulation of parasites in the intervillous spaces of the placenta leading to pathology (Mustafa *et al.,* 2010). In our study, we used frozen samples instead of fresh isolates in binding assays which might have interfered with the binding capacity of the infected erythrocytes. A study by Ochola and others (2011) reported that they had obtained different patterns of association between binding and clinical phenotypes by using freshly grown parasite isolates than frozen ones (Newbold *et al.,* 1997). Placental isolated parasites are usually in the trophozoite stage and can be used for binding assays directly unlike peripheral parasites that need to be cultured to the trophozoite stage before being used in assays (Beeson *et al.,* 2002). *P. knowlesi* isolates may have failed to bind to purified receptors because these receptors could be unique to *P. falciparum* isolates. Since the data presented here suggests that there are receptor molecules in the baboon that mediate adhesion, they may have a modified structure making them different from human receptors.

Our study has investigated the ligands and receptors that mediate *P. knowlesi* infected erythrocytes' (*Pk*IEs) binding in the Olive baboon. We attest that the molecules discovered are likely the ones mediating adhesion of parasites in the placenta. We propose further work on molecular biology to corroborate our findings. The findings in this study further affirm the close likeness of the *P. knowlesi* infection in baboons to the *P. falciparum* infection in humans. They show that the baboon can be a good model for *in vivo* placental malaria studies at the pre-clinical level. This will be useful in evaluating placental malaria vaccines and other therapeutic agents that can prevent malaria infection during pregnancy leading to better outcomes for mothers and their unborn.

## 5. Conclusion

The present study has identified and proposed: CSPG 4, CSPG 4-like and HAPLN 1 sequences as the putative receptors molecules in *P. anubis* and shown they share similar domains to the human receptors. We have also shown that the *P. knowlesi* erythrocyte binding proteins (EBP-*alpha*, EBP-*beta* and EBP-*gamma*) could be playing a role in infected erythrocyte adhesion apart from their traditional role of invasion. Static binding using *Pk*IEs however did not show any binding to purified receptors. The findings presented further affirm that the *P. knowlesi*-Olive baboon model can provide a useful pre-clinical model for evaluating placental malaria vaccines and other therapeutic agents.

## Acknowledgements

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