Molecular characterization of *Plasmodium falciparum* PHISTb proteins as potential targets of naturally-acquired immunity against malaria [version 2; peer review: 3 approved with reservations]

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Abstract

**Background:** *Plasmodium falciparum* causes the deadliest form of malaria in humans. Upon infection, the host's infected red blood cells (iRBCs) are remodelled by exported parasite proteins to provide a niche for parasite development and maturation.

**Methods:** Here we analysed the role of three PHISTb proteins Pf3D7_0532400, Pf3D7_1401600, and Pf3D7_1102500 by expressing recombinant proteins and evaluated antibody responses against these proteins using immune sera from malaria-exposed individuals from Kenya and The Gambia in Africa.

**Results:** Children and adults from malaria-endemic regions recognized the three PHISTb proteins. Responses against PHISTb proteins varied with malaria transmission intensity in three different geographical sites in Kenya (Siaya and Takaungu) and The Gambia (Sukuta). Antibody responses against PHISTb antigens Pf3D7_1102500 and Pf3D7_1401600 were higher in Sukuta, a low transmission region in Gambia, compared to Siaya, a high transmission region in western Kenya, unlike Pf3D7_0532400. Anti-PHIST responses indicate negative correlation between antibody levels and malaria transmission intensity for Pf3D7_1102500 and Pf3D7_1401600. We report a correlation in antibody responses between schizont and gametocyte extract, but this is not statistically significant (cor=0.102, p=0.2851, CI=95%) and, Pf3D7_0532400 (cor=0.11, p=0.249, CI=95%) and Pf3D7_1401600 (cor=0.02, p=0.7968, CI=95%). We report a negative

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correlation in antibody responses between schizont and PF3D7_1102500 (cor=-0.008, p=0.9348, CI=95%). There is a correlation between gametocyte extract and PF3D7_1401600 (cor=-0.0402, p=0.6735, CI=95%), PF3D7_1102500 (cor=0.0758, p=0.4271, CI=95%) and PF3D7_0532400 (cor=0.155, p=0.1028, CI=95%). Acquisition of anti-PHIST antibodies correlates with exposure to malaria for PF3D7_0532400 (p=0.009) but not PF3D7_1102500 and PF3D7_1401600 (p=0.507 and p=0.15, respectively, CI=95%). Children aged below 2 years had the lowest antibody levels which do not correlate with age differences.

**Conclusions:** Collectively, these findings provide evidence of natural immunity against PHISTb antigens that varies with level of malaria exposure and underscore their potential as possible serological markers to *P. falciparum* infection aimed at contributing to malaria control through vaccine development.

**Keywords**
P. falciparum, Immunity, PHISTb, naturally acquired immunity, antibody-antigen response
Amendments from Version 1

In the introduction, we made changes to provide a detailed description of PHIST family of genes, in consideration to previously published data. This highlights fundamental concepts regarding these genes, as we narrow down to the three PHISTb genes which we focused on for the functional assays. Additionally, we justify the rationale for settling on the three PHISTb antigens (Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500) for assessment of antibody responses from three different geographical locations with varying malaria intensities. Moreover, we screened our serum samples with gametocyte extracts to gain a broader understanding of immune responses. Interestingly, we find a similar pattern of antibody responses to gametocyte antigens as is the case with schizont extract. We have provided additional figures in light to this. We believe this addition is valuable the field. We have provided additional information on key definitions to enhance understanding of terms and concepts used in the paper.

Any further responses from the reviewers can be found at the end of the article.

Introduction

Malaria is a global health problem, with over 228 million cases reported worldwide in 2018, and the majority of disease burden occurs in sub-Saharan Africa, with *P. falciparum* infections accounting for most malaria deaths. Efforts to prevent and control malaria have met several challenges in terms of drug and insecticide resistance exhibited by the parasites and vectors. The need to expand the current malaria control toolbox is ever urgent. An effective malaria vaccine would be a vital component for improving disease prevention and curtailing transmission. However, the most advanced malaria vaccine, ‘RTS,S’, only offers partial protection in children and is yet to be fully implemented in disease endemic countries. A key component in designing new intervention strategies will rely on our understanding of the parasite biology to identify metabolic process or molecules amenable to disruption and disease control. In our previous work, we used DNA microarrays to investigate parasite adaptation to malaria transmission intensity at the transcript level. Among the key changes we observed was consistent upregulation of *Plasmodium* helical interspersed subtelomeric (PHIST) genes in low malaria transmission areas. The expression of the PHIST genes was also linked to that of the master regulator of gametocyte commitment the *P. falciparum* Ap2g transcription factor.

The PHIST family forms part of the parasite exportome and are exclusively found in parasites that infect primates and are greatly expanded in *P. falciparum* and *P. reichnovii*. The exact number of proteins in different species of plasmodium parasites is not clear. There are 39 PHIST proteins in *P. vivax*, but studies on the gene family *Pv_fam_e* shows presence of 44 *rad* genes and 21 *phist* genes. For *P. knowlesi*, Sargeant *et al.* report the presence of 27 PHIST proteins, while Pain *et al.* confirm 38 proteins and PlasmoDB lists 39 proteins. The PHIST family of proteins is defined by a conserved domain of approximately 150 amino acids that have been predicted to form four consecutive alpha helices. Depending on presence and position of conserved tryptophan residues within the PHIST domain, the family has been divided further into three subgroups including PHISTA, PHISTb and PHISTc. PHISTA proteins amount to 26 different proteins, and possess two conserved tryptophan residues. PHISTb proteins comprise of 24 members and have 300-600 amino acid residues. They have a unique C-terminal amino acid stretch following the PHIST domain. Some PHISTb proteins localize and interact with the human host cytoskeleton. PHIST proteins localize within the iRBCs and have been implicated in molecular and cellular function of the infected cells. These include surface display of PIEMP1, change in cell rigidity, gametocytogenesis and cerebral and pregnancy-associated malaria. The PHISTc subgroup has 18 members and exhibits the most diversity in length of amino acids varying from 200-1200. The PHIST domain is located next to the C terminus of the protein, similar to PHISTA.

PHIST genes have been associated with parasite adaptation to malaria transmission. Among them were PHISTb genes Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500. Pf3D7_0532400 is a lysine-rich membrane-associated protein that directly associates to infected red blood cell (iRBC) cytoskeletons and enhances cytoadherence to Cd36. Pf3D7_1102500 is a gametocyte export protein and interacts with the cytoskeleton, while Pf3D7_1401600 contains an MEC domain, its function is largely unknown apart from an association with placental malaria affects iRBC membrane rigidity and is upregulated in sexually committed parasites.

Recent reports by Rono *et al.*, identified some PHISTb genes to linked to parasite adaptation to local malaria transmission forces. The expression of PHIST genes Pf3D7_1401600 and Pf3D7_1102500 were strongly correlated with malaria parasites from low transmission regions. Pf3D7_0532400 has been implicated in PIEMP1 surface display on infected erythrocytes and involves the interactions between the PHIST protein and ATS domain of PIEMP1. The role of PHISTb proteins in cyto-adherence to iRBCs and the differential expression of the proteins in high versus low malaria transmission areas establishes the need to study them further, and assess them as potential targets of naturally acquired immunity against malaria.

In this study we investigated the role of PHISTb antigens Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500 as targets of naturally acquired immunity. Recombinant PHISTb antigens were expressed and evaluated for antibody responses in a cohort of African children who have shown clinical protection against malaria during infancy.

Methods

Ethical statement

Written informed consent was obtained from parents/guardians of children taking part in the study and adults whose samples were used. Ethical approval was received from the Kenya Medical Research Institute Scientific and Ethics Review Committee under protocol number 3149 for this study.
Amplification and cloning of PHISTb genes

Total RNA was extracted from 100µL of pelleted iRBCs from culture-adapted P. falciparum 3D7 parasites from the KEMRI-Wellcome laboratories, at the trophozoite stage using Trizol reagent (Life Technologies, Thermo Fisher Scientific, USA, Catalog number 15596026) kit according to the manufacturer’s instructions. Prior to cDNA synthesis, 2µL of the sample (RNA) was treated with DNase I for 20 minutes at 37°C. Reverse transcription of the DNase treated RNA was performed using a cDNA synthesis SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA, Catalog number 18080085). 2µL of the cDNA was subsequently used as template to amplify PHISTb genes by PCR employing gene-specific primers in a 20µL final reaction volume that had 1mM GoTaq master mix (Promega Corporation, USA, Catalog number M7122) (10µL), 0.5mM Forward primer, 0.5mM Reverse primer (Supplementary Table 1, Extended data[^a]), supplemented with 2.5mM MgCl$_2$ under the following thermocycler conditions; initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds, extension at 68°C for 2 minutes, and a final elongation step of 68°C for 7 minutes. The PCR products were resolved by 1% ethidium bromide-stained agarose gel electrophoresis for 1 hour at 110V (Bio-Rad Model Powerpac Basic Power Supply, Bio-Rad Laboratories, Inc., USA) (Supplementary Figure 1, Extended data[^a]) and PHISTb DNA bands of interest purified using the QIAquick PCR Purification Kit (Qiagen, Germany, Catalog 28104) and cloned into pEXP5-CT TOPO vector (Invitrogen) as per manufacturer’s instructions.

2µL of the ligation product was transformed into TOP10 chemically-competent *E. coli* cells and grown overnight in LB agar plates with ampicillin. A colony PCR to identify target colonies containing the correct recombinant plasmids with PHISTb gene inserts was performed (Supplementary Figure 1, Extended data[^a]). 2µL of plasmid DNA with PHISTb gene inserts was used to amplify genes by PCR using gene-specific primers in a 20µL reaction volume that had 1mM GoTaq master mix (Promega Corporation, USA, Catalog number M7122) (10µL), 0.5mM Forward primer, 0.5mM Reverse primer (Supplementary Table 1, Extended data[^a]), supplemented with 2.5mM MgCl$_2$ under the following thermocycler conditions; initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds, extension at 68°C for 2 minutes, and a final elongation step of 68°C for 7 minutes. The PCR products were resolved through 1% ethidium bromide-stained agarose gel electrophoresis for 1 hour at 110V (Bio-Rad Model Powerpac Basic Power Supply, Bio-Rad Laboratories, Inc., USA) (Supplementary Figure 1, Extended data[^a]) and PHISTb DNA bands of interest purified using the QIAquick PCR Purification Kit (Qiagen, Germany, Catalog 28104) and cloned into pEXP5-CT TOPO vector (Invitrogen) as per manufacturer’s instructions.

Sample preparation, liquid chromatography with tandem mass spectrometry analysis, and antigen validation

We confirmed the identity of the expressed PHISTb antigens using mass spectrometry. Briefly, 10 µg of the purified PHISTb proteins were separately denatured in 50mM Tris-HCl (Sigma-Aldrich, United States) containing 8M urea (Sigma-Aldrich, United States), pH 8. Subsequently, the denatured proteins were reduced with 40mM dithiothreitol (Sigma-Aldrich, United States) at room temperature with shaking for 1 hour and alkylated in the dark for 1 hour with 80mM iodoacetamide (Sigma-Aldrich, United States). Proteins were precipitated with four times the sample volume of cold acetone for 1 hour at -20°C and the protein pellet obtained after discarding the supernatant following centrifugation for 10 minutes at 14,000 xg at room temperature. Proteins were resuspended in 15 µL of 6M urea in 50mM Tris-HCl (pH 8) buffer and digested with trypsin/Lys-C mix (Promega) according to the manufacturer’s instructions using the two step in-solution digestion. Peptides obtained were desalted using C18 spin columns according to manufacturer’s instructions (Thermo Scientific), dried in a Speedvac concentrator and re-suspended in 15µL of resuspension solvent (99% H$_2$O, 1% acetonitrile, 0.1% formic acid).

The validation of PHISTb proteins was conducted following established protocol[^17]. As previously described, a minimum of two unique peptides for a protein was considered a positive identification for each expressed PHISTb antigen.

**ELISA Analysis**

Antibody responses to recombinant PHISTb antigens and schizont extract were measured using a published ELISA protocol[^18]. Briefly, Dynex 4HBX Immunolon plates (Dynex Technologies Inc.) were coated with 100 µL of 0.05 µg/mL recombinant PHISTb antigen diluted in coating buffer (15mM Na$_2$CO$_3$, pH 9.4) and 100µL of crude schizont extract diluted 1:6000 as described previously[^19]. An overnight incubation was done at 4°C followed blocking for 5 hours at room temperature with 1% skimmed milk diluted in 1× PBS with 0.05% Tween-20. Subsequently, 100µL of sera diluted 1:1000 in blocking buffer was added in each well and incubated overnight at 4°C. At each of these steps the plates were washed four times in 1× PBS with 0.05% Tween-20. They were incubated for
4 hours at room temperature with 100µL of horse radish peroxidase-conjugated polyclonal rabbit anti-human IgG (Dako, Catalog number EC 3.2.1.17), diluted 1:5000 in blocking buffer. The plates were washed four times and incubated with 100µL of developing buffer (0.1 M citric acid, 0.2 M Na₃HPO₄, 4mg O-phenylenediamine dihydrochloride tablets (Sigma-Aldrich, United States), 8µL hydrogen peroxide and 20mL distilled water. After 20 minutes, the reaction was stopped with 25µL 2M H₂SO₄ and absorbance read at an optical density (OD) of 492nm. A serial dilution of a purified immunoglobulin reagent (malaria immune globulin, MIG) obtained from a pool of semi immune malaria adults was included for each PHISTb antigen to derive a standard dilution curve that allowed conversion of OD reading to antibody concentration relative to those in MIG as previously described. Pooled hyper-immune sera obtained from Kilifi adults was added to each well as a positive control, while sera from malaria naïve European adults was included as a negative control for all the antigens tested. Samples were assayed in duplicate for quality control and results having a coefficient variation greater than 20% was repeated.

Data analysis
Statistical analysis was performed in R Studio (version 1.2.1335). Antibody sero-positivity was defined as three standard deviations above the mean of responses from 3 malaria naïve individuals done in duplicate. Individuals were categorized as being high or low responders if the OD₄₉₂nm value was above or below the median antibody response. We compared antibody responses between the three recombinant PHISTb antigens using Kruskal-Wallis test. Spearman’s correlation was used to evaluate association between antibody responses for purified PHISTb antigens with age. Wilcoxon test was used to compare the differences in antibody responses in the three geographical locations (Siaya, Takaungu and Sukuta) and among different age groups.

Study population
The study used human sera from children (n = 544) previously collected samples from individuals residing in malaria endemic regions in Africa namely Sukuta (Latitude: 13.4070° Longitude: 13.41033 -16.70815) in the Gambia, Kilifi County (Latitude: -3.6667 Longitude: 39.7500), and Siaya County (Latitude: -0.0833 Longitude: 34.2500) in Kenya. Sera obtained from three blood samples of malaria naïve adults (volunteers from United Kingdom and Sweden) were used as negative controls. Pooled hyper-immune sera from Malawian adults was included as positive controls. Details of samples used for the study are described elsewhere. Briefly, the samples are from a cross-sectional survey conducted among children aged below the age of nine years during periods of low and high malaria transmission in Sukuta (The Gambia) and Siaya (Kenya). This period was characterized by a rise in prevalence of malaria between 1985 and 2004 across Africa. The rate of parasite prevalence during this period was 37% for Sukuta, 83% for Siaya, and 49% and 74% for Kilifi North and Kilifi South (Takaungu), respectively.

Definitions
A clinical episode is defined as the body temperature above 37.5°C with >2500 parasites per microlitre of blood. Clinical malaria reflects a history of fever that is accompanied by peripheral parasitemia ≥100/µL. Clinical protection can be defined as a very low incidence of clinical malaria.

Preparation of recombinant PHISTb antigens
PHISTb antigens were produced using bacterial expression system. The expressed parasite antigens were confirmed by western blot and proteomic analysis (Supplementary Figure 1 and Supplementary Table 2, Extended data). Next we established whether PHIST antigens were recognized by pooled malaria hyper immune sera. Indeed, we observed a dose-dependent response curve against the PHIST antigens by PHIS and MIG but not malaria naïve adults from Europe (Figure 1).

Antibody levels against crude schizont extracts have been used as a marker of malaria exposure and transmission intensity. Previous work by Snow et al. established that antibody responses to malaria parasite antigens in children increased with disease endemicity. Using the same cohort of African infants previously described, we evaluated responses to schizont extracts in infants from Sukuta in Gambia, and Siaya and Takaungu in Kenya by schizont ELISA. As observed before, antibody responses to schizont extracts were highest in Siaya and lowest in Sukuta, which mirrors disease endemicity (Figure 2). Next, we evaluated for anti-PHIST responses. Unlike responses to schizont extracts, anti-PHIST responses for Pf3D7_1102500 and Pf3D7_1401600 were significantly high in Sukuta (low transmission region) compared to Siaya (high transmission); however, we did not see a similar pattern for Pf3D7_0532400 (Figure 2). Therefore, these data suggest a negative association between anti-PHIST antibody levels and malaria transmission for two PHIST proteins Pf3D7_1102500 and Pf3D7_1401600.

To investigate this further, we evaluated correlations between antibody responses for the different PHIST antigens, schizont and gametocyte extracts. We observed a positive correlation in antibody response between the crude schizont extract and Pf3D7_0532400 (p-value = 0.00582), but not the other two PHIST antigens (Pf3D7_1401600 and Pf3D7_1102500), which we previously found to elicit high responses in low transmission areas. However, significant correlation was observed between responses to Pf3D7_1401600 and Pf3D7_1102500 (p-value < 2.2e-16) (Supplementary Table 3, Extended data).

Next, we evaluated the potential of protection against malaria by responses to PHISTb antigens by comparing antibody levels...
Figure 1. Antibody responses to PHISTb antigen by malaria hyper-immune sera. Pooled hyper-immune sera was used as a positive control and serum from malaria naïve adults from Europe (UK06, EU35 and SWE03) served as a negative control, each included in duplicate. Antibody seropositivity was determined as the cutoff above the mean plus three standard deviations of three malaria naïve adult sera. Antibody responses were measured at a wavelength of OD\textsubscript{492nm}.

Figure 2. Anti-PHIST antibody responses across different malaria endemic regions in Africa. The figure shows antibody responses to schizont and gametocyte extract and PHISTb antigens across three different geographical locations with varying malaria transmission intensities. Sukuta in The Gambia is predominantly a low malaria transmission area, while Takaungu and Siaya have moderate and high transmission intensities, respectively. On the y-axis is the antibody response for each recombinant PHISTb antigen, schizont and gametocyte extracts, while the x-axis shows the geographical location. A, B, C, D and E represent antibody responses to crude schizont extract, gametocyte antigen, Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500 respectively. Boxes indicate the median and interquartile ranges and the mean antibody responses is shown in a red circle.
between children who had tested positive for *P. falciparum*, and those who were negative despite living within the same endemic region (Figure 3). We observed increased level of responses to various PHIST antigens in infected children relative to those who tested negative, but the differences were not statistically significant. However, we observed similar trends in antibody response for Pf3D7_1401600 and crude schizont extracts, whereby low antibody responses were observed for individuals positive for *P. falciparum* infection.

Anti-PHIST responses were analysed in parasite positive individuals compared to parasite negative. Boxes indicate the median and interquartile ranges, whiskers indicate maximum and minimum values. *P. falciparum* (Pf) infection status indicates responses in parasite positive (1) vs parasite negative (0). No significant difference is evident in individuals tested positive and negative for *P. falciparum* infection across all screened antigens.

Seroprevalence of PHISTb antigens in different Age Groups

Next, we investigated whether the difference in antibody responses against the PHISTb antigens was correlated with the natural acquisition of antimalaria immunity. We categorized children into four age class categories (1–2, 3–5, 6–8,9–11 years) as previously done in Snow *et al.* and evaluated the anti-PHISTb responses in these children. Children aged below 2 years had the lowest antibody titres; however, significant correlations between age and antibody responses were not observed (Figure 4).

In these analyses, there was a correlation in antibody response between the crude schizont extract and Pf3D7_0532400 (Table 1). These findings suggest that acquisition of anti-PHIST antibodies is not dependent on frequency of parasite exposure but rather other intrinsic features related to the parasites found in low malaria endemic regions.

**Discussion**

Blood stage parasite antigens have been targets for several leading malaria vaccine studies. Merozoite and mature schizont surface proteins are some of the most commonly studied antigens. However, none of these have reached advanced stages of vaccine development due to a number of reasons, the key being that they are a wide repertoire of genes (about 5400 genes per genome), providing enough opportunity for the parasite to develop enormous diversity through genetic polymorphisms, and redundancy of key pathways required for invasion and immune escape. For example, in our recent work parasites in low malaria transmission areas were observed to have undergone evolution by investing more resources into the expression of genes that support further transmission into...
Figure 4. Antibody responses in children by age classes against crude schizont and gametocyte extract and recombinant PHISTb antigens. A, B, C, D and E denote anti-PHIST responses to crude schizont extract, gametocyte antigen, Pf3D7_0532400, Pf3D7_1102500 and Pf3D7_1401600 respectively in different age categories. Boxes indicate the median and interquartile ranges, whiskers indicate maximum and minimum OD values. Filled red circles indicate the mean OD values.

Table 1. A comparison of analysis based on Pf infection status and age.

| PHISTb antigens | Coefficient | Std Error | t (test statistic) | p>−t (significance level) | [95% Confidence Interval] |
|-----------------|-------------|-----------|-------------------|--------------------------|--------------------------|
| PF3D7_0532400   | 0.001469    | 0.000558  | 2.63              | 0.009                    | 0.000368 - 0.002569      |
| AGE (months)    | -0.02073    | 0.051914  | -0.4              | 0.690                    | -0.12322 - 0.081758      |
| _cons (constant)| 0.330171    | 0.04873   | 6.78              | <0.001                   | 0.23397 - 0.426373       |
| PF3D7_1102500   | -0.00028    | 0.000418  | -0.66             | 0.507                    | -0.0011 - 0.000548       |
| AGE (months)    | 0.030883    | 0.038924  | 0.79              | 0.429                    | -0.04596 - 0.107726      |
| _cons (constant)| 0.213335    | 0.036536  | 5.84              | <0.001                   | 0.141205 - 0.285464      |
| PF3D7_1401600   | -0.00026    | 0.000177  | -1.44             | 0.150                    | -0.0006 - 9.37E-05       |
| AGE (months)    | 0.01203     | 0.016473  | 0.73              | 0.466                    | -0.02049 - 0.04455       |
| _cons (constant)| 0.15826     | 0.015462  | 10.24             | <0.001                   | 0.127735 - 0.188785      |
| SCHIZONT        | 0.002691    | 0.001024  | 2.63              | 0.009                    | 0.00067 - 0.004712       |
| AGE (months)    | -0.0392     | 0.095308  | -0.41             | 0.681                    | -0.22735 - 0.148956      |
| _cons (constant)| 0.639576    | 0.089462  | 7.15              | <0.001                   | 0.462963 - 0.81619       |

t is the t-test statistic value, df is the degrees of freedom, p-value is the significance level of the t-test (p-value), conf.int is the confidence interval of the correlation coefficient at 95%, sample estimates is the correlation coefficient (Cor.coeff).
new hosts\(^3\). At the molecular level these changes were orchestrated by the AP2-G gene, which is a master regulator of gametocytes, the transmissible form of the parasites\(^2\). Notably, several genes from the *Plasmodium* PHIST family were upregulated in parasites from low transmission areas\(^1\).

PHIST proteins belong to a multigene family and are involved in remodeling of infected red blood cells. However, data on host antimalaria immune responses to these proteins, which could be crucial for vaccine development (including malaria transmission blocking), is largely missing. In this study, we selected three PHISTb proteins that were differentially expressed in parasites from low malaria endemic regions and investigated their role in development of antimalaria immunity in a cohort of children from malaria endemic regions of Africa. Although this cohort was established over two decades ago\(^1\), using archived samples we could still confirm the malaria prevalence profile using anti-crude schizont and gametocyte ELISA analysis, which is a proxy for malaria transmission intensity. The most endemic region was Siaya in Kenya, while Sukuta in the Gambia was least endemic. Our analysis of PHISTb antigens showed that children previously exposed to malaria had significant antibody responses to these parasite proteins. We confirmed that these responses were specific to malaria exposure since they were not observed in malaria naïve individuals from Europe. However, antibody levels were low in children, with Pf3D7_1401600 reporting the lowest immune response compared to the schizont extract, and Pf3D7_0532400 and Pf3D7_1102500 with moderate levels of response. Our findings were consistent with a previous study by Kamuyu *et al.*, who reported low antibody levels for one PHISTb antigen Pf3D7_1401600\(^7\). Although the responses were low for this PHISTb antigen, they showed association with protection, as evidenced by reduced odds of clinical episodes of malaria. Our data and other studies have shown Pf3D7_1401600 as a possible vaccine candidate as it provides potential protection against malaria infection. But these observations need further analysis of Pf3D7_1401600 to confirm its localization on infected cells. Surface expression/display of parasite proteins provides opportunity for host immunity to interrupt parasite development; however, it is not clear if indeed Pf3D7_1401600 finds its way to the iRBC surface.

Siaya has a higher proportion of children with malaria parasites compared to Takaungu and Sukuta. A comparison of antibody responses to schizont and gametocyte extract and recombinant PHISTb antigens confirms an increase in levels for parasite positive individuals; however, the difference is not statistically significant. Whether or not the responses to PHISTb antigens are associated with protection against *P. falciparum* infection is a factor that should be probed further. There is limited knowledge regarding the function of antibodies required in the mediation of immunity or which serve as a correlate of immunity\(^8\). Age is a critical factor in determining protection against clinical malaria\(^8\). The development of protective immune responses is based on repeated exposure to malaria, and consequently, older people living in endemic regions have higher antibody levels, suggestive of enhanced protection against malaria infection\(^8\).

We investigated whether there was a relation between age and antibody responses against PHISTb antigens. There was no relationship between age and antibody responses against the investigated PHISTb antigens. We hypothesize that this may be due to different reasons. First, it could be due to poor immunogenicity of PHISTb antigens that were investigated. Low antibody responses could be the result of poor memory that is generated by the PHISTb antigens. Moreover, anti-PHISTb responses in children may not have been involved in acquisition of immunity possibly because of variability of PHISTb antigens and low level of exposure during this limited duration of time. Studies conducted by Boudin *et al.* showed no relation between age and transmission-reducing activity in antigens investigated\(^7\). Here, we see no direct evidence of age and acquisition of antibody responses. Secondly, it could be associated with the dynamics of antibody development. In Premawansa *et al.* it has been shown that development of antibodies could be affected by frequency of infection\(^8\). Studies focusing on Pf6230 and Pf648/45, two proteins expressed in gametocytes, showed no correlation between age and induced antibody responses\(^8\). Therefore, the development of an immune response against the PHISTb antigens could similarly be associated with gametocyte factors rather than high rates of exposure/transmission, which also correlates well with our previous observations at the transcription level for PHISTb transcripts\(^1\). Another possible reason for inconsistencies in antibody responses by age could be due to variations in intensity and stability of malaria transmission.

**Conclusion**

This study reveals that recombinant PHISTb antigens are targets of naturally acquired immunity against malaria. Evaluation of antibody responses in three locations with varying malaria transmission intensities indicates variable levels of immunity. An assessment with pooled hyper-immune sera shows PHISTb antigens had a dose-dependent response to antibody levels, as compared with serum obtained from malaria naïve individuals. Further, a positive correlation between some PHISTb antigens and schizont extract and the high conservation of PHISTb sequence confirms the potential of these antigens as serological markers for *P. falciparum* infection. In addition, we observed an interesting correlation between immune responses to PHISTb antigens Pf3D7_1401600 and Pf3D7_1102500 and low malaria transmission, and in our previous analysis, high transcript abundance and gametocyte development. All these lead to our conclusion these two PHIST antigens have potential for further consideration as potential targets for disrupting gametocyte development and malaria transmission.

**Data availability**

Underlying data
Open Science Framework: Molecular characterization of *Plasmodium falciparum* PHISTb proteins as potential targets
of naturally-acquired immunity against malaria. http://doi.org/10.17605/OSF.IO/DRWA6

This project contains the following underlying data:
- Raw ELISA data in CSV format
- Raw mass spectrometry data in XLSX format
- Original unedited gel and western blot image files in TIF format

Extended data
Open Science Framework: Molecular characterization of Plasmodium falciparum PHISTb proteins as potential targets of naturally-acquired immunity against malaria. http://doi.org/10.17605/OSF.IO/DRWA6

This project contains the following extended data:
- Table 1.docx (Gene-specific PCR primers for amplifying PHIST genes from cDNA)
- Table 2.docx (Results of mass spectrometry analysis)
- Table 3.docx (Spearman’s rank correlation test between schizont extract and PHISTb antigens)
- Supplementary Figure 1.tif (Colony PCR for selection of successful transformants and SDS-PAGE western blot analysis for PHISTb proteins)
- Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

Code availability
Reproducible code is available at: https://github.com/Tisebe/PHISTb_Research

Archived code at time of publication: http://doi.org/10.17605/OSF.IO/DRWA6

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Eizo Takashima
Division of Malaria Research, Proteo-Science Center, Ehime University, Matsuyama, Japan

The authors, Isebe et al., have revised their manuscript, but it still needs some minor corrections.

The definitions of malaria are not clear. Clinical episodes and clinical malaria are the same, but they seem to be defined differently.

In addition, is protection against clinical malaria the same as protection against malaria parasitic infection? This needs to be clarified to avoid confusion.

Amino acid positions of the expressed proteins should be indicated in the main text. I have checked the corresponding primer sequences, and the reverse primer for Pf3D7_1401600 (TATGGTCACATATCTTTGGAC) doesn't match the sequence available in malaria genome databases. Please recheck this.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Parasitology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 01 June 2021

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Stephen J. Rogerson

Department of Medicine, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Vic, Australia

Isebe et al. have made some useful changes to their manuscript, including addition of new data and some new analyses. There are some inaccuracies that have crept in in places that need some final clarifications and corrections.

In the abstract they present correlation coefficients with p values- which should be presented to two significant figures, and then write CI=95%; they need to include these 95% confidence intervals, after the p values. Given these correlations are not significant or close to significant I would say that the antibody responses are NOT correlated. (The authors might find it helpful to plot these antibody responses against each other which provides a visual representation of their (possible lack of) relatedness).

In the paragraph on PHIST protein across different species it is not clear when they turn their focus specifically to *P. falciparum*.

Under data analysis, for comparing different sites was a rank sum test used? Wilcoxon test is for paired data which these are not.

The definitions added regarding malaria episodes are confusing. I would take “clinical malaria” and a “clinical episode” to be the same thing but they are differently defined. One option would be to call the higher parasitemia event clinical malaria, and the other event possible clinical malaria.

In Figure 2 it seems that the red dot which is supposed to be the mean (and was previously distinct from the median) is now indicating the median. Please revise the figure or the legend.

Figure 3 needs more context than just “children from malaria endemic regions in Africa”. Which data were included in this analysis?

Table 1: as with the abstract, these data would be best displayed to (generally) two significant figures rather than up to 5. Also the table footnotes do not refer to a regression analysis as presented in the table so please correct this.

Given the authors included the correlation data in the abstract I am surprised they do not present these as a Table in the manuscript proper. I tried to find them in Supplementary Table 3 but could not track this down.

Minor comments

In the introduction (Page 3 second column first paragraph) text should read “cerebral” not cerebral.

In the same paragraph but previous column the species should be *P. reichenowi*.

**Competing Interests:** No competing interests were disclosed.
Reviewer Expertise: Malaria immunity.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Version 1

Reviewer Report 26 August 2020

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Eizo Takashima
Division of Malaria Research, Proteo-Science Center, Ehime University, Matsuyama, Japan

In this the study Isebe et al. found a negative correlation between human antibodies against 2 PHISTb proteins and malaria transmission intensity. While this is an interesting finding, the authors need to give a more detailed background on this family of proteins, including the regions or the domains assessed. The following items need to be addressed.

1. Since this manuscript focuses on evaluation of PHIST family of proteins, which has about 24 members, it's important to give a deeper introduction and known facts about these proteins, such as how many, structure, cellular localization, topologies, polymorphisms, etc.

2. A reference to this statement would be helpful. “In our previous work, we used DNA microarrays to investigate parasite adaptation to malaria transmission intensity at the transcript level. Among the key changes we observed was consistent upregulation of Plasmodium helical interspersed subtelomeric (PHIST) genes in low malaria transmission areas.”

3. Which regions - amino acid positions - of the proteins were expressed? This should be presented in the main text as its central to the study.

4. A clear justification describing why focusing on these 3 proteins and/or regions should be considered as representative of the entire PHISTb family of proteins.

5. Since the recombinant proteins are purified and checked for purity by SDS-PAGE electrophoresis, followed by a Western blot analysis, it important to present the CBB data to assess protein purity as it fits well with the title of this manuscript.

6. Generally, but not always, antibody titers against plasmodial proteins correlate with increasing age. Is it possible that the Sukuta samples were drawn from older children
compared to Siaya and Takaungu?

7. In addition to antibody OD values, it will be of interest to show the seroprevalence of different antigens in different age groups and populations.

8. Clear definitions of “clinical malaria”, “clinical episodes”, “clinical protection”, and “protection against clinical malaria”, and “protection against malaria infection” needs to be given as this is used throughout the manuscript.

9. Minor correction. Immunolon -> Immulon  and Coating buffer (15Mm -> mM)

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Parasitology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 May 2021
Tony Imunyo Isebe, KEMRI-Wellcome Trust Research Programme, Centre for Geographic Medicine Research, P.O. Box 230-80108, Kenya

We appreciate this review and the helpful comments and suggestions made to improve the state of the paper. We have included a case-by-case response to the issues raised.

In this the study Isebe et al. found a negative correlation between human antibodies against 2 PHISTb proteins and malaria transmission intensity. While this is an
interesting finding, the authors need to give a more detailed background on this family of proteins, including the regions or the domains assessed. The following items need to be addressed.

The PHIST family forms part of the large number of proteins exported by the parasite to the iRBC. The proteins are found exclusively in the genus *Plasmodium* and are greatly expanded in *P. falciparum* and *P. reichnowi*². The exact number of proteins in different species of malaria is not clear. There are 39 PHIST proteins for *P. vivax*, but studies on the gene family *Pv_fam_e* shows presence of 44 *rad* genes and 21 *phist* genes³. For *P. knowlesi*, Sargeant et al. report the presence of 27 PHIST proteins, while Pain et al. confirm 38 proteins and PlasmoDB lists 39 records.³ There are three PHIST subfamilies including PHISTa, PHISTb and PHISTc⁴. PHISTa proteins amount to 26 different proteins, and possess two conserved tryptophan residues⁴. PHISTb proteins comprise 24 members and have 300-600 residues. They have a unique C-terminal amino acid stretch following the PHIST domain³. All characterized PHISTb proteins localize and interact with the human host cytoskeleton⁴. PHIST proteins localize within the iRBCs and they are implicated in molecular and cellular activities. The proteins are involved in display of PfEMP1, change in cell rigidity, gametocytogenesis and cerebral and pregnancy-associated malaria⁴. The PHISTc subgroup has 18 members and is diverse with the length of amino acids varying from 200-1200⁴. The PHIST domain is located next to the C terminus of the protein, similar to PHISTa⁴.

Since this manuscript focuses on evaluation of PHIST family of proteins, which has about 24 members, it’s important to give a deeper introduction and known facts about these proteins, such as how many, structure, cellular localization, topologies, polymorphisms, etc.

We have provided additional information regarding the PHIST family of proteins as highlighted above.

A reference to this statement would be helpful. “In our previous work, we used DNA microarrays to investigate parasite adaptation to malaria transmission intensity at the transcript level. Among the key changes we observed was consistent upregulation of *Plasmodium* helical interspersed subtelomeric (PHIST) genes in low malaria transmission areas.”

Thank you for identifying this. We have added a reference to the statement.

Which regions - amino acid positions - of the proteins were expressed? This should be presented in the main text as its central to the study.

Plasmid preparations were made from the positive colonies and used to transform *E. coli* BL21 (DE3) pLysS for protein expression targeting the PHIST domain position of 100 to 200 amino acids all of which were Histidine tagged.

A clear justification describing why focusing on these 3 proteins and/or regions should be considered as representative of the entire PHISTb family of proteins.

The PHIST family forms part of the parasite exportome and are exclusively found in parasites that infect primates and are greatly expanded in *P. falciparum* and *P. reichnowi*². The exact number of proteins in different species of plasmodium parasites is not clear.
There are 39 PHIST proteins in *P. vivax*, but studies on the gene family *Pv_fam_e* show presence of 44 rad genes and 21 phist genes. For *P. knowlesi*, Sargeant et al. report the presence of 27 PHIST proteins, while Pain et al. confirm 38 proteins and PlasmoDB lists 39 proteins. The PHIST family of proteins is defined by a conserved domain of approximately 150 amino acids that have been predicted to form four consecutive alpha helices.

Depending on presence and position of conserved tryptophan residues within the PHIST domain, the family has been divided further into three subgroups including PHISTa, PHISTb and PHISTc. PHISTa proteins amount to 26 different proteins, and possess two conserved tryptophan residues. PHISTb proteins comprise of 24 members and have 300-600 amino acid residues. They have a unique C-terminal amino acid stretch following the PHIST domain. Some PHISTb proteins localize and interact with the human host cytoskeleton. PHIST proteins localize within the iRBCs and have been implicated in molecular and cellular function of the infected cells. These include surface display of PfEMP1, change in cell rigidity, gametocytogenesis and cerebral and pregnancy-associated malaria. The PHISTc subgroup has 18 members and exhibits the most diversity in length of amino acids varying from 200-1200. The PHIST domain is located next to the C terminus of the protein, similar to PHISTa.

PHIST genes have been associated with parasite adaptation to malaria transmission. Among them were PHISTb genes Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500.

Pf3D7_0532400 is a lysine-rich membrane-associated protein that directly associates to infected red blood cell (iRBC) cytoskeletons and enhances cytoadherence to Cd36. Pf3D7_1102500 is a gametocyte export protein and interacts with the cytoskeleton, while Pf3D7_1401600 contains an MEC domain, its function is largely unknown apart from its association with placental malaria and is upregulated in sexually committed parasites.

Recent reports by Rono et al., identified some PHISTb genes linked to parasite adaptation to local malaria transmission forces. The expression of PHIST genes Pf3D7_1401600 and Pf3D7_1102500 were strongly correlated with malaria parasites from low transmission regions. Pf3D7_0532400 has been implicated in PfEMP1 surface display on infected erythrocytes and involves the interactions between the PHIST protein and ATS domain of PfEMP1. The role of PHISTb proteins in cyto-adherence to iRBCs and the differential expression of the proteins in high versus low malaria transmission areas establishes the need to study them further, and assess them as potential targets of naturally acquired immunity against malaria.

In this study we investigated the role of PHISTb antigens Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500 as targets of naturally acquired immunity. Recombinant PHISTb antigens were expressed and evaluated for antibody responses in a cohort of African children who have shown clinical protection against malaria during infancy.

Since the recombinant proteins are purified and checked for purity by SDS-PAGE electrophoresis, followed by a Western blot analysis, it is important to present the CBB data to assess protein purity as it fits well with the title of this manuscript.

While we acknowledge this, we determined the purity of our PHISTb proteins using LC/MS and the data is available in the supplementary section. Table 2.docx (Results of mass spectrometry analysis)
Generally, but not always, antibody titers against plasmodial proteins correlate with increasing age. Is it possible that the Sukuta samples were drawn from older children compared to Siaya and Takaungu?

While we acknowledge the statement on correlation of antibody response with increasing age, we did not identify this in these PHISTb proteins. As is the case for the other sites, Sukuta samples were drawn from children aged below 9 years in all the study sites.

In addition to antibody OD values, it will be of interest to show the seroprevalence of different antigens in different age groups and populations.

Next, we investigated whether the difference in antibody responses against the PHISTb antigens was correlated with the natural acquisition of antimalaria immunity. We categorized children into four age class categories (1-2, 3-5, 6-8,9-11 years) as previously done in Snow et al. 15 and evaluated the anti-PHISTb responses in these children. Children aged below 2 years had the lowest antibody titres; however, significant correlations between age and antibody responses were not observed (Figure 4). These findings suggest that acquisition of anti-PHIST antibodies is not dependent on frequency of parasite exposure but rather other intrinsic features related to the parasites found in low malaria endemic regions.

Clear definitions of “clinical malaria”, “clinical episodes”, “clinical protection”, and “protection against clinical malaria”, and “protection against malaria infection” needs to be given as this is used throughout the manuscript.

A clinical episode is defined as the body temperature above 37.5 °C with ≥ 2500 parasites per microlitre of blood 12. Clinical malaria reflects a history of fever that is accompanied by peripheral parasitemia ≥100/μL13 14. Clinical protection can be defined as a very low incidence of clinical malaria13. Protection against malaria is defined as objective evidence of a low risk of clinical disease reflected by absence of fever with parasitemia and lower densities of parasitemia15. Protection against malaria infection is defined as reduced parasite replication in infected red blood cells or enhanced clearance of infected erythrocytes16-18.

Minor correction. Immunolon -> Immulon and Coating buffer (15Mm -> mM)

Briefly, Dynex 4HBX Immunolon plates (Dynex Technologies Inc.) were coated with 100 µL of 0.05 µg/mL recombinant PHISTb antigen diluted in coating buffer (15mM Na₂CO₃, pH 9.4) and 100µL of crude schizont extract diluted 1:6000 as described previously 12 .

Competing Interests: No competing interests.

Reviewer Report 26 August 2020

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Stephen J. Rogerson
Department of Medicine, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Vic, Australia

This study examines antibody responses to three recombinant PHISTb proteins in children from 3 areas, a higher and lower transmission area in Kenya and an area in The Gambia. Responses to these proteins are compared to those to schizont extract, which is a generally suitable indicator of malaria exposure and antibody acquisition. While antibody to one of the three antigens, Pf3D7_0532400, shows a moderate correlation with antibody to schizont extract, antibody to the other two does not, but they do correlate with each other. Surprisingly, antibody to none of the PHISTb antigens seems to develop in a clearly age dependent manner, nor to be significantly influenced by infections, and the authors do not offer a strong explanation for these findings. The authors’ contention that these antigens may be vaccine candidates seems very premature given our limited knowledge about them. One would like to know how conserved they are, and whether antibody is associated with protection rather than just exposure.

The observation that antibodies to two of these antigens are higher in a lower transmission area than a higher transmission area remains largely unexplained as we know little about the specific biological functions of these proteins including their roles in gametocyte development of function.

Specific comments

1. In presenting correlations (in both the abstract and main text) it is always more informative to present the correlation coefficients than the P values alone. This indicates the strength of the association. The coefficients are good, moderate correlations, but they are hidden away in a supplementary table. Similarly when comparing antibody levels between groups P values should be supported by relevant measures of the magnitude of the difference between groups.

2. The legend for Figure 1 is not clear. It reads as through the variable is concentration of antigen used, but this is not consistent with the methods. If it is concentration of antibody then we need details of how this was determined, even if it is contained in previous publications using these samples. It is not clear if the malaria immune globulin was used (which might have yielded a concentration) rather than the PHIS. The figure would also be more informative if error bars for each data point were included.

3. Also relating to Figure 1, it seems the OD for Pf3d7_141600 is really quite low and that this ELISA could have been better optimised to give a better range of responses.

4. While the authors make a point of stating that a cut off for positivity was defined (data analysis), none of the data are presented as positive or negative. They also state that the malaria immune globulin was used to allow conversion of OD data to relative (“Arbitrary” units), but none of the results are presented this way. Finally, in data analysis they state that samples were classified as high or low based on the median, but again none of the analysis is presented this way. This part of the methods seems superfluous in this case, although these additional analyses if included would strengthen the interrogation of the data.

5. By contrast, when they show individual patient data (Figures 2 and 3) showing the non-exposed controls and positive control would have been helpful.
6. In the discussion (P 5), the size of the parasite genome is not a valid or logical reason for the lack of success of merozoite surface protein based vaccines.

Minor comments:
1. The authors’ use of terms like high and low transmission and high and low endemicity is rather loose. If parasite prevalence in Sukuta was 37% this would be defined as moderate endemicity by WHO criteria.

2. In different places in the methods the authors state they used 12 non-malaria exposed controls, and only three are shown in Figure 1. Please clarify this.

3. If one group of samples came from Kilifi why are the graphs labelled Takaungu? Where is this?

4. I note the authors have yet to correct the incorrect Figure 3 legend pointed out by the first reviewer.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Malaria immunity.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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Tony Imunyo Isebe, KEMRI-Wellcome Trust Research Programme, Centre for Geographic
We would like to extend our sincere gratitude to the reviewer for the comments that have been key in improving the manuscript. We have included our responses to each of the comments raised.

This study examines antibody responses to three recombinant PHISTb proteins in children from 3 areas, a higher and lower transmission area in Kenya and an area in The Gambia. Responses to these proteins are compared to those to schizont extract, which is a generally suitable indicator of malaria exposure and antibody acquisition. While antibody to one of the three antigens, Pf3D7_0532400, shows a moderate correlation with antibody to schizont extract, antibody to the other two does not, but they do correlate with each other. Surprisingly, antibody to none of the PHISTb antigens seems to develop in a clearly age dependent manner, nor to be significantly influenced by infections, and the authors do not offer a strong explanation for these findings. The authors’ contention that these antigens may be vaccine candidates seems very premature given our limited knowledge about them. One would like to know how conserved they are, and whether antibody is associated with protection rather than just exposure.

While we note the interesting findings that none of the PHISTb antigens do not develop in an age-dependent manner, we have provided additional information which may support the observation. Moreover, anti-PHISTb responses in children may not have been involved in acquisition of immunity possibly because of variability of PHISTb antigens and low level of exposure during this limited duration of time. Another possible reason for inconsistencies in antibody responses by age could be due to variations in intensity and stability of malaria transmission. Furthermore, we have provided additional information regarding the PHIST family of proteins, and how they are conserved. The PHIST family of proteins is defined by a conserved domain of approximately 150 amino acids that have been predicted to form four consecutive alpha helices. Depending on presence and position of conserved tryptophan residues within the PHIST domain, the family has been divided further into three subgroups including PHISTA, PHISTb and PHISTc. PHISTA proteins amount to 26 different proteins, and possess two conserved tryptophan residues. PHISTb proteins comprise 24 members and have 300-600 residues. They have a unique C-terminal amino acid stretch following the PHIST domain. All characterized PHISTb proteins localize and interact with the human host cytoskeleton. PHIST proteins localize within the iRBCs and they are implicated in molecular and cellular activities. The proteins are involved in display of PfEMP1, change in cell rigidity, gametocytogenesis and cerebral and pregnancy-associated malaria. The PHISTc subgroup has 18 members and is diverse with the length of amino acids varying from 200-1200. The PHIST domain is located next to the C terminus of the protein, similar to PHISTA. Indeed, the determination of whether antibody responses are associated with protection rather than exposure is key. However, in our work, we focused on exposure, but the design of an experiment to inform on association with protection would be key. Previous studies on one of the PHISTb antigens (PF3D7_1401600) have however shown association between antibody responses and protection. The determination of whether or not antibody
responses elicited by PHISTb antigens elicit immune responses would inform further on the function of these PHIST family of proteins.

The observation that antibodies to two of these antigens are higher in a lower transmission area than a higher transmission area remains largely unexplained as we know little about the specific biological functions of these proteins including their roles in gametocyte development of function. We acknowledge that the functions of these proteins are not entirely known, hence our first interest in determining if they can elicit an immune response using samples with varied malaria transmission intensity. Based on literature, some roles of these proteins have been illustrated and duly cited in the manuscript.

In presenting correlations (in both the abstract and main text) it is always more informative to present the correlation coefficients than the P values alone. This indicates the strength of the association. The coefficients are good, moderate correlations, but they are hidden away in a supplementary table. Similarly, when comparing antibody levels between groups P values should be supported by relevant measures of the magnitude of the difference between groups. We have included the Spearman's correlation test and a table on relevant measures.

The legend for Figure 1 is not clear. It reads as through the variable is concentration of antigen used, but this is not consistent with the methods. If it is concentration of antibody then we need details of how this was determined, even if it is contained in previous publications using these samples. It is not clear if the malaria immune globulin was used (which might have yielded a concentration) rather than the PHIS. The figure would also be more informative if error bars for each data point were included.

We determined the optimal antigen concentration using PHIS (as the positive control), UK06, SWE03 and EU35 (as the negative controls). We have modified the graph to show that for Figure 1.

Also relating to Figure 1, it seems the OD for Pf3d7_141600 is really quite low and that this ELISA could have been better optimised to give a better range of responses. We confirm in our experimental results that responses to Pf3d7_1401600 were very low. While the optimization of the ELISA may have provided a better range of responses, our hypothesis is the differences in the nature of the antigen, specifically the structure and conformation may have contributed to these low antibody responses.

While the authors make a point of stating that a cut off for positivity was defined (data analysis), none of the data are presented as positive or negative. They also state that the malaria immune globulin was used to allow conversion of OD data to relative (“Arbitrary” units), but none of the results are presented this way. Finally, in data analysis they state that samples were classified as high or low based on the median, but again none of the analysis is presented this way. This part of the methods seems superfluous in this case, although these additional analyses if included would strengthen the interrogation of the data.

We have included a graph that defines a cut off for positivity.
By contrast, when they show individual patient data (Figures 2 and 3) showing the non-exposed controls and positive control would have been helpful. Indeed, it would have been more helpful to include the patient data, but these samples were drawn from a previous study (Snow et al., 1997), which has details of both patient characteristics and malaria transmission intensity. Moreover, in Figure 1, we included findings for non-exposed controls, serum drawn from malaria naïve individuals and pooled hyper-immune serum from individuals in malaria endemic regions.

In the discussion (P 5), the size of the parasite genome is not a valid or logical reason for the lack of success of merozoite surface protein based vaccines. We have amended this statement to read: However, none of these have reached advanced stages of vaccine development due to a number of reasons, including presence of a wide repertoire of genes (about 5400 genes per genome), providing enough opportunity for the parasite to develop enormous diversity through genetic polymorphisms, and redundancy of key pathways required for invasion and immune escape.

The authors’ use of terms like high and low transmission and high and low endemicity is rather loose. If parasite prevalence in Sukuta was 37% this would be defined as moderate endemicity by WHO criteria. Regarding this, we used historical data which at the time 37% malaria prevalence rate was considered as low compared to Siaya (83%) and Kilifi South which is the modern day Takaungu to be 74%.

In different places in the methods the authors state they used 12 non-malaria exposed controls, and only three are shown in Figure 1. Please clarify this. Thank you for pointing this out. We have amended the statement to this; ‘Antibody sero-positivity was defined as three standard deviations above the mean of responses from 3 malaria naïve individuals done in duplicate’.

If one group of samples came from Kilifi why are the graphs labelled Takaungu? Where is this? Indeed, present day Kilifi County is categorized into Kilifi South (Takaungu). However, we have made changes to the manuscript text to reflect this.

I note the authors have yet to correct the incorrect Figure 3 legend pointed out by the first reviewer. We have made corrections in the legend to read: Anti-PHIST responses were analysed in parasite positive individuals compared to parasite negative. Boxes indicate the median and interquartile ranges, whiskers indicate maximum and minimum values. *P. falciparum* (Pf) infection status indicates responses in parasite positive (1) vs parasite negative (0). No significant difference is evident in individuals tested positive and negative for *P. falciparum* infection across all tested antigens. A, B, C, D and E represent anti-PHIST to crude schizont extract, gametocyte antigen, Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500 respectively.
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This study on three members of the PHISTb family has attempted to gain valuable insight into the immune response to this family in distinct geographical regions and their links to malaria intensity and transmission. While the study was generally scientifically sound I believe altering the study design to include gametocyte extract in the assays could improve the paper. The study found an interesting negative correlation between two of the PHISTs to malaria intensity (schizont extract) by showing these two PHISTs had increased recognition of recombinant proteins by immune sera from the low transmission area. While this is an interesting finding it is also an area of the study that needs potentially more work to characterise this finding.

The three PHISTb proteins chosen for this study were PF3D7_0532500 (LyMP) a known exported asexual stage protein, and PF3D7_1102500 (GEXP02) and PF3D7_1401600. In the introduction it was mentioned that PF3D7_1102500 could be involved with cytoadherence and the export translocon, however the interaction with the translocon is due to PF3D7_1102500 being an exported protein and there is no evidence that the function of PF3D7_1102500 directly involves the translocon. The cytoadherence is based on a yeast two hybrid prediction with a recent study showing this protein is expressed largely in the gametocyte stages where it interacts with the cytoskeleton and being a sexual stage exported protein it is unlikely to have a significant role in cytoadhesion in asexual stages. For PF3D7_1401600 while the exact function is not well characterised, it has been shown to affect iRBC membrane rigidity and is upregulated in sexually committed parasites. The connection of PF3D7_1102500, and possibly even PF3D7_1401600, with sexual stages points to the possible increase of sexual stages in the low transmission area, a theory mentioned in the discussion and it is my opinion that this paper would be significantly improved if this idea was given a larger focus.

This comes to the experimental design where schizont extract was used and while this is an accepted proxy, given that two of the PHISTs are potentially linked to the sexual stages it would be of great interest to also use the immune sera against gametocyte extract, if available, to see if a positive correlation to gametocyte recognition is seen for PF3D7_1102500 and PF3D7_1401600. Alternatively, if possible in these historic sample sets, to correlate the changes in gametocyte numbers in the samples from different areas to the changes in PHIST recognition, however this
could only be done if there is sufficient/any sample material to measure gametocyte numbers or if this information was originally taken when sampling. By using samples from infected individuals in the same assays against gametocyte extract would give more support to the idea that these proteins would be useful for research into transmission and by looking reactivity to gametocyte extract and comparing it to schizont extract the study would also provide important insight into the possibility of the parasites reaction to low transmission is to increase gametocyte production.

Finally one minor correction should also be included in Figure 3 the figure legend mentions that the filled circles show outliers but all circles in the graphs are filled.

References
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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular parasitology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
This study on three members of the PHISTb family has attempted to gain valuable insight into the immune response to this family in distinct geographical regions and their links to malaria intensity and transmission. While the study was generally scientifically sound I believe altering the study design to include gametocyte extract in the assays could improve the paper. The study found an interesting negative correlation between two of the PHISTs to malaria intensity (schizont extract) by showing these two PHISTs had increased recognition of recombinant proteins by immune sera from the low transmission area. While this is an interesting finding it is also an area of the study that needs potentially more work to characterise this finding.

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Thank you for the insightful comments. In response to this, we screened the available immune sera against the gametocyte antigens. Our findings show a similar profile in antibody responses to schizont extracts in different geographical locations with Siaya and Sukuta recording high and low responses respectively. We have included figures to support this.

Finally, one minor correction should also be included in Figure 3 the figure legend mentions that the filled circles show outliers but all circles in the graphs are filled. We have made corrections in the legend to read:

Anti-PHIST responses were analysed in parasite positive individuals compared to parasite negative. Boxes indicate the median and interquartile ranges, whiskers indicate maximum and minimum values. *P. falciparum* (Pf) infection status indicates responses in parasite positive (1) vs parasite negative (0). No significant difference is evident in individuals tested positive and negative for *P. falciparum* infection across all tested antigens. A, B, C, D and E represent anti-PHIST responses to crude schizont extract, gametocyte antigen, Pf3D7_0532400, Pf3D7_1401600 and Pf3D7_1102500 respectively.

**Competing Interests:** No competing interests.