RESEARCH ARTICLE

Molecular characterization of *Plasmodium falciparum* PHISTb proteins as potential targets of naturally-acquired immunity against malaria [version 1; peer review: 3 approved with reservations]

Tony I. Isebe¹,², Joel L. Bargul²,³, Bonface M. Gichuki¹, James M. Njunge¹, James Tuju¹,⁴, Martin K. Rono¹,⁵

¹KEMRI-Wellcome Trust Research Programme, Centre for Geographic Medicine Research, P.O. Box 230-80108, Kilifi, Kenya
²Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00200, Nairobi, 00200, Kenya
³Animal Health Theme, International Centre of Insect Physiology and Ecology, P.O. Box 30772-00100, Nairobi, Kenya
⁴Department of Chemistry and Biochemistry, Pwani University, P.O Box 195-80108, Kilifi, Kenya
⁵Pwani University Bioscience Research Centre, Pwani University, P.O Box 195-80108, Kilifi, Kenya

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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 in order 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:** Our findings show that children and adults from malaria-endemic regions recognized the three PHISTb proteins. Responses against the 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 the Gambia, as compared to Siaya, a high transmission region in western Kenya, unlike Pf3D7_0532400. Anti-PHIST responses show a negative correlation between antibody levels and malaria transmission intensity for two PHIST antigens, Pf3D7_1102500 and Pf3D7_1401600. However, we report a correlation in antibody responses between schizont extract and Pf3D7_0532400 (p=0.00582). Acquisition of anti-PHIST antibodies was correlated with exposure to malaria for PHISTb protein Pf3D7_0532400 (p=0.009) but not the other PHIST antigens 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, but the responses 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 potential for these parasite antigens 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

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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\(^1\), with *P. falciparum* infections accounting for most malaria deaths\(^1\). 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\(^2\). A key component in designing new intervention strategies will rely on our understanding of the parasite biology to identify metabolic processes 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\(^3\).

Among the *PHIST* genes that prominently featured in parasite adaptation in low transmission areas were *PHIST*b 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\(^4\). *Pf3D7_1102500* is a gametocyte export protein and involved with cytoadherence and plasmodium translocon of exported proteins\(^5\), while *Pf3D7_1401600* function is largely unknown apart from an association with placental malaria\(^6\). The importance of these *PHIST*s in disease transmission and natural acquired immunity to malaria is still puzzling despite our transcriptome data suggesting their importance to parasites in low transmission areas in Africa.

In this study we investigated the role of *PHIST*b antigens *Pf3D7_0532400*, *Pf3D7_1401600* and *Pf3D7_1102500* as targets of naturally acquired immunity. Recombinant *PHIST*b antigens were expressed and evaluated for antibody responses in a cohort of African children who have shown clinical protection against malaria during infancy\(^7\)\(^8\).

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 *PHIST*b 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 number18080085). 2µL of the cDNA was subsequently used as template to amplify *PHIST*b 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*\(^9\)), 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*\(^9\)) 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 TOP10chemically-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*\(^9\)). 2µL of plasmid DNA with PHISTb gene inserts was used to amplify genes by PCR using 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*\(^9\)), 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) Plasmid preparations were made from the positive colonies and used to transform *E. coli* BL21 (DE3) pLysS for protein expression. Transformed bacterial cells were grown in 500ml of auto-induction media at 37°C/150rpm for protein production for 24 hours containing 100µg/ml ampicillin and 34µg/ml chloramphenicol. The cultures were spun at 13000rpm for 30mins and the supernatant discarded. The bacterial pellets of individual samples were lysed in 2.5ml of Bugbuster lysis solution (Novagen), supplemented with 250U/ml 0.1µl of benzozene nuclease (Novagen) and incubated at 4°C for 20 minutes under gentle rotation. The lysate was spun at 13000rpm for 30 minutes at 4°C to separate the pellet from the supernatant fraction. The expressed His-tagged proteins were purified from the pellet fraction under denaturing conditions (8M urea, 200mM NaCl) and checked for purity by SDS-PAGE electrophoresis, followed by a western
blot analysis. The 6x-His Tag Mouse Monoclonal Antibody (3D5) from Thermo Fisher Scientific (Catalog number R930-2S, RRID AB_2556553) was used in the assay (anti-his) and recognized the histidine tag present in the recombinant PHISTb proteins.

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% H₂O, 1% acetonitrile, 0.1% formic acid).

The validation of PHISTb proteins was conducted following established protocol11. 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 protocol11. Briefly, Dynex 4HBX Immunoln 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 previously12. An overnight incubation was done at 4°C followed blocking for 5 hours at room temperature with 1% skimmed milk diluted in 1x 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 1x 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 Na₂HPO₄, 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 as a standard to allow conversion of OD reading to antibody concentration relative to those in MIG13. 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 repeated14.

Data analysis

Statistical analysis was performed in R Studio (version 1.2.1335)14. Antibody sero-positivity was defined as three standard deviations above the mean of responses from 12 malaria naïve individuals. 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 Kenya15. 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 controls15. Details of samples used for the study are described elsewhere15. 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 between1985 and 2004 across Africa16. 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, respectively8.

Results

Expression 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 data9. 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 but not malaria naïve adults from Europe (Figure 1).

Antibody responses to PHISTb varies with malaria endemicity in Kenya and The Gambia

Antibody levels against crude schizont extracts have been used as a marker of malaria exposure and transmission intensity17. 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 and schizont 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 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 (Supplementary Figure 2, Extended data). 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. To further evaluate the responses to PHISTb antigens, we compared antibody levels between children who had tested positive for P. falciparum, and those who were negative (Figure 3). There was an increase in the level of responses to various antigens in infected children relative to those who tested negative. However, the difference between these two groups was not statistically significant (Pf3D7_0532400; p=0.9, Crude schizont extract; p=0.83, Pf3D7_1102500; p=0.33, Pf3D7_1401600; p=0.40). 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.

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 new hosts. 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. Notably, several genes from the *Plasmodium* PHIST family were upregulated in parasites from low transmission areas.

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\textsuperscript{14}, using archived samples we could still confirm the malaria prevalence profile using anti-cruce schizont 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\textsubscript{1401600} reporting the lowest immune response compared to the schizont extract, and Pf3D7\textsubscript{0532400} and Pf3D7\textsubscript{1102500} with moderate levels of response. Our findings were consistent with a previous study by Kamuya \textit{et al.}, who reported low antibody levels for one PHISTb antigen Pf3D7\textsubscript{1401600}\textsuperscript{46}. 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\textsubscript{1401600} as a possible vaccine candidate as it provides potential protection against malaria infection. But these observations need further analysis of Pf3D7\textsubscript{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\textsubscript{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 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 \textit{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\textsuperscript{21}. Age is a critical factor in determining protection against clinical malaria\textsuperscript{22}. 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\textsuperscript{11}.

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. Studies conducted by Boudin \textit{et al.} showed no relation between age and transmission-reducing activity in antigens investigated\textsuperscript{25}. 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 \textit{et al.} it has been shown that development of antibodies could be affected by frequency of infection\textsuperscript{15}. Studies focusing on Pf6230 and PfS48/45, two proteins expressed in gametocytes, showed no correlation between age and induced antibody responses\textsuperscript{29}. 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\textsuperscript{7}.

**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 \textit{P. falciparum} infection. In addition, we observed an interesting correlation between immune responses to PHISTb antigens Pf3D7\textsubscript{1401600} and Pf3D7\textsubscript{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 \textit{Plasmodium falciparum} PHISTb proteins as potential targets of naturally-acquired immunity against malaria. https://doi.org/10.17605/OSF.IO/DRWA6\textsuperscript{9}

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 \textit{Plasmodium falciparum} PHISTb proteins as potential targets of naturally-acquired immunity against malaria. http://doi.org/10.17605/OSF.IO/DRWA6\textsuperscript{9}

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)
- Supplementary Figure 2.tif (Antibody responses in children by age classes against crude schizont extract and recombinant PHISTb antigens)

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

References


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

License: GNU General Public License version 2 (GPL-2.0)

Acknowledgements
We would like to thank patients who contributed samples for the study. We would also wish to thank staff from the KEMRI-Wellcome Trust Research Programme laboratories for their contribution in facilitating experimental work. This work is published with permission from the Director of Kenya Medical Research Institute (KEMRI).
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.

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.

[Reviewer Report 22 June 2020](https://doi.org/10.21956/wellcomeopenres.17461.r39046)

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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

**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.