RESEARCH ARTICLE

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

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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

This article is included in the KEMRI | Wellcome Trust gateway.

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**Author roles:** Isebe TI: Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; Bargul JL: Supervision, Writing – Review & Editing; Gichuki BM: Formal Analysis, Writing – Review & Editing; Njunge JM: Formal Analysis, Writing – Review & Editing; Tuju J: Formal Analysis, Investigation, Supervision, Writing – Review & Editing; Rono MK: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

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

**Grant information:** This work was supported by The Third World Academy of Sciences (TWAS) [16-194 RG/BIO/AF/AC_I – FR3240293352] and The Royal Society FLAIR fellowship [FLR_R1_190497] awarded to MKR. TII is supported by the DELTAS Africa Initiative [DEL-15-003]. The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)’s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa’s Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust [107769] and the UK government. JLB is supported by DELTAS Africa Initiative grant [DEL-15-011] to THRIVE-2. The views expressed in this publication are those of the author(s) and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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**How to cite this article:** Isebe TI, Bargul JL, Gichuki BM et al. Molecular characterization of *Plasmodium falciparum* PHISTb proteins as potential targets of naturally-acquired immunity against malaria [version 1; peer review: awaiting peer review] Wellcome Open Research 2020, 5:136 https://doi.org/10.12688/wellcomeopenres.15919.1

**First published:** 10 Jun 2020, 5:136 https://doi.org/10.12688/wellcomeopenres.15919.1
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 Africa1, with *P. falciparum* infections accounting for most malaria deaths2. 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 will 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 countries1. 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 factor3.

Among the *PHIST* genes that prominently featured in parasite adaptation in low transmission areas 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 CD364. Pf3D7_1102500 is a gametocyte export protein and involved with cytoadherence and plasmodium translocon of exported proteins5, while Pf3D7_1401600 function is largely unknown apart from an association with placental malaria6. The importance of these PHISTs 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 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 infancy7,8.

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 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 number18080085). 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 data9), supplemented with 2.5mM MgCl2 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 data9) 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 data9). 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 data9), supplemented with 2.5mM MgCl2 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 benzonase 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 R930-25, 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 50 mM Tris-HCl (Sigma-Aldrich, United States) containing 8 M urea (Sigma-Aldrich, United States), pH 8. Subsequently, the denatured proteins were reduced with 40 mM dithiothreitol (Sigma-Aldrich, United States) at room temperature with shaking for 1 hour and alkylated in the dark for 1 hour with 80 mM 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 6 M urea in 50 mM 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 protocol9. 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 protocol10. 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 (150 mM 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 repeated13.

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 OD 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 North and Kilifi South, respectively. 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
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 triplicate. 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}.

Antibody responses to malaria parasite antigens in children increased with disease endemicity\(^{15}\). 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\(^9\)).

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 (Supplementary Figure 2, Extended data\(^9\)). 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 \textit{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 (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 (Supplementary Table 3, Extended data\(^9\)). We observed similar trends in antibody response for Pf3D7\_1401600 and crude schizont extracts, whereby low antibody responses were observed for individuals positive for \textit{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\(^{18}\). 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
Figure 2. Anti-PHIST antibody responses across different malaria endemic regions in Africa. The figure shows antibody responses to schizont 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 and schizont extract, while the x-axis shows the geographical location. Boxes indicate the median and interquartile ranges and the mean antibody responses is shown in a red circle.

Figure 3. Analysis of antibody levels and \textit{P. falciparum} infection status in children from malaria endemic regions in Africa. 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 and filled circles show outliers. \textit{P. falciparum} (Pf) infection status indicates responses in parasite positive vs parasite negative. No significant difference is evident in individuals tested positive and negative for \textit{P. falciparum} infection across all tested antigens.

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\(^3\). 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\(^1\). Notably, several genes from the \textit{Plasmodium} PHIST family were upregulated in parasites from low transmission areas\(^3\).

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, using archived samples we could still confirm the malaria prevalence profile using anti-crude 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_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 Kamuya et al., who reported low antibody levels for one PHISTb antigen Pf3D7_1401600. 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 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. Age is a critical factor in determining protection against clinical malaria. 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.

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 et al. showed no relation between age and transmission-reducing activity in antigens investigated. 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. Studies focusing on Pf6230 and Pf54845, two proteins expressed in gametocytes, showed no correlation between age and induced antibody responses. 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.

**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. [https://doi.org/10.17605/OSF.IO/DRWA6](https://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. [https://doi.org/10.17605/OSF.IO/DRWA6](https://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)
- 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


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).

Archived code at time of publication: http://doi.org/10.17605/OSF.IO/DRWA625
License: GNU General Public License version 2 (GPL-2.0)