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Limited genetic variations of the Rh5-CyRPA-Ripr invasion complex in *Plasmodium falciparum* parasite population in selected malaria-endemic regions, Kenya

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The invasion of human erythrocytes by *Plasmodium falciparum* merozoites requires interaction between parasite ligands and host receptors. Interaction of *PfRh5*-CyRPA-Ripr protein complex with basigin, an erythrocyte surface receptor, via *PfRh5* is essential for erythrocyte invasion. Antibodies raised against each antigen component of the complex have demonstrated erythrocyte invasion inhibition, making these proteins potential blood-stage vaccine candidates. Genetic polymorphisms present a significant challenge in developing efficacious vaccines, leading to variant-specific immune responses. This study investigated the genetic variations of the *PfRh5* complex proteins in *P. falciparum* isolates from Lake Victoria islands, Western Kenya. Here, twenty-nine microscopically confirmed *P. falciparum* field samples collected from islands in Lake Victoria between July 2014 and July 2016 were genotyped by whole genome sequencing, and results compared to sequences mined from the GenBank database, from a study conducted in Kilifi, as well as other sequences from the MalariaGEN repository. We analyzed the frequency of polymorphisms in the *PfRh5* protein complex proteins, *PfRh5*, *PCyRPA*, *PfRipr*, and *PfP113*, and their location mapped on the 3D protein complex structure. We identified a total of 58 variants in the *PfRh5* protein complex. *PfRh5* protein was the most polymorphic with 30 SNPs, while *PCyRPA* was relatively conserved with 3 SNPs. The minor allele frequency of the SNPs ranged between 1.9% and 21.2%. Ten high-frequency alleles (>5%) were observed in *PfRh5* at codons 147, 148, 277, 410, and 429 and in *PfRipr* at codons 190, 255, 259, and 1003. A SNP was located in protein-protein interaction region C203Y and F292V of *PfRh5* and *PfCyRPA*, respectively. Put together, this study revealed low polymorphisms in the *PfRh5*
Background

The World Health Organization (WHO) estimates the latest global malaria health burden statistic at 627,000 deaths resulting from 241 million malaria infection cases (1). The scourge’s heaviest health and economic burden is borne by the developing countries in Sub-Saharan Africa, where an estimated 90% of all malaria deaths occur, with children under five accounting for 78% of all deaths (2). The emergence of multi-drug resistance Plasmodium falciparum (P. falciparum) resistant strains and insecticide resistance mosquitos remains a significant challenge in treating and eliminating malaria (3, 4). The lack of an effective vaccine remains one of the most critical gaps in the strategies developed to eliminate P. falciparum malaria (5).

The development of an effective P. falciparum vaccine focuses on targeting pre-erythrocytic or erythrocytic stages for parasite development and malaria pathogenesis in humans or the parasite development within the mosquito vector (6). Symptoms elicited by parasite infection originate from the erythrocytic stage of malaria infection. At this stage, the merozoite invades the erythrocytes, where at initial recognition of the human erythrocytes, the merozoite orients itself such that the apical region comes to direct contact with the host’s erythrocyte membrane, followed by irreversible attachment of merozoites to erythrocytes (7). Ring-like moving junction mediates complete parasite internalization to formation of an intracellular parasitophorous vacuole (8). The whole erythrocyte invasion process is mediated by multiple merozoite proteins mainly expressed on the surface, or in the apical organelles such as rhoptry and microneme (9). Since these proteins are essential for invasion and are exposed to host immune system, they are considered ideal targets for blood stage vaccines (BSV) (10–12). However, the exposure of candidate BSV antigens to human immune system during natural infections subjects them to selective pressure, that may result to high levels of polymorphisms (13). This presents a significant challenge for allele-specific immune responses as an ideal vaccine must be able to protect against multiple genetic variants of parasites (14, 15).

The P. falciparum reticulocyte binding homolog 5 complex (PRh5) is a primary vaccine target for developing an effective malaria vaccine. The PRh5 complex comprises four interacting proteins: Pf- reticulocyte binding homolog 5 (Rh5), Pf- interacting protein (PfRipr), Pf-Cysteine-rich protective antigen (CyRPA), and Pf-P113 protein (16). PRh5 proteins bind to erythrocytes via the host receptor basigin, while the other three proteins interact within the complex to initiate erythrocyte invasion. PfCyRPA binds directly to PRh5, while PfRipr interacts with PfCyRPA; thus, PfCyRPA forms the contact sites for PRh5 and PfRipr. Studies have shown that PfP113 interacts with PRh5 protein on the N-terminal, providing a releasable mechanism for anchoring PRh5 to basigin (11, 17). The genes encoding for these proteins are highly maintained, as shown in gene knockout experiments suggesting they are vital for parasite survival (18, 19). Antibodies against PRh5, PfRipr, and PfCyRPA have been shown to inhibit parasite erythrocyte invasion in non-human primates and mice, while antibodies against PfP113 protein have been associated with protection against clinical malaria in vivo [20, 21]. These studies suggest that all proteins of the PRh5 complex can be considered potential BSV targets.

Polymorphisms in all PRh5 complex encoding genes could impede the development of an Rh5 malaria BSV. Like the PRh5 complex, apical membrane antigen 1 (AMA1), once considered a potential malaria vaccine candidate is also essential for invasion. However, AMA1 is highly polymorphic, leading to allele-specific immune responses and limited efficacy in its Phase Ib trials (22). Investigation into polymorphisms on all members of the PRh5 complex, their effects on the protein structure, and their association significant considerations when designing a vaccine. Studies have demonstrated that P. falciparum parasites have a high within host genetic diversity in high transmission regions compared to low transmission settings (23, 24). This is due to the increased probability of recombination between genetically distinct variants in high transmission settings. This extensive genetic diversity is a major hindrance in malaria vaccine development as the host immune responses may fail to recognize all the variants of an antigen (25).

Here, to explore these questions, we analyzed the four genes of the PRh5 complex by whole genome sequencing in a cross-sectional sample of parasites from two high malaria transmission regions in Kenya.

Methods

Sampling, DNA preparation, and whole genome sequencing

Parasite DNA was extracted from archived whole blood samples from patients recruited for a drug resistance surveillance study in invasion complex in the Lake Victoria parasite population. However, the two mutations identified on the protein interaction regions prompts for investigation on their impacts on parasite invasion process to support the consideration of PRh5 components as potential malaria vaccine candidates.

KEYWORDS

genetic variations, Rh5-CyRPA-Ripr invasion complex, malaria, vaccines, erythrocyte (RBC)
local hospitals on four selected islands (Mfangano, Takawiri, Kibuogi, and Ngodzi) in Lake Victoria, a coastal mainland (Ungoye) between July 2014 and July 2016. The study’s approval was obtained from the Kenyatta National Hospital - University of Nairobi (KNH-UoN) ethical review committee (P609/10/2014) and the Mount Kenya University Ethics Review Committee (038/2014). Written consent was obtained from all the participants or guardians, and malaria cases were treated per the national malaria guidelines. The samples re-analyzed here were a subset of these studies which has been extensively described elsewhere (26, 27).

Briefly, to increase the parasitemia, the field P. falciparum parasites were adapted for in vitro culture as previously described (28), and DNA was extracted from short-term cultures (1 month) at the schizont stage using QIAamp DNA mini kit (Qiagen, Valencia, CA). Paired-end sequencing libraries were prepared using Nextera XT DNA library preparation Kit according to the manufactures protocol. (Illumina, USA). Whole genome sequencing was performed on Illumina MiSeq technology (Illumina, USA) at 30X coverage generating reads of length 150 bps. These sequences are archived at the DDBJ BioProject, Accession number PRJDB12148. Quality control checks were performed using the FASTQC (Babraham Institute, UK) toolkit version 0.11.5.

### Comparison of polymorphisms identified with other regions

For comparative analysis, we obtained previously reported whole genome sequences P. falciparum isolates collected from Kilifi, a malaria endemic region in coastal Kenya (29, 30). The mined sequences were generated from two drug trial studies that were conducted between 2005 and 2008, and the sequences deposited in the GenBank repository under accession numbers PfRipr: MW597717-MW597776, PfRh5: MW597550-MW597609, PfCyRPA : MW597610-MW597716, and PfP113: MW597459-MW597549.

We also accessed the catalogue of genetic variation in P. falciparum, of the global MalariaGEN database v6.0, for comparing and validating the SNPs identified from the Lake Victoria sample population. This dataset comprised of genomic variation records of 7,113 Pf. falciparum samples from 28 malaria-endemic countries. The method used to retrieve the data was previously described by Amato et al., 2016. The dplyr v1.0.9 package (Wickham H, François R, Henry L, 2022) in R v4.2.1 was used to filter out the four genes of the PfRh5 complex using their PlasmoDB unique identifiers. SNPs identified were then filtered and analyzed.

### Read mapping and coverage

Sequence reads were aligned against Plasmodium falciparum 3D7 reference genome (version 8.1) (https://plasmodb.org/common/downloads/release-46/Pfalciparum3D7/fasta/data/) using Burrows-Wheeler Alignment tool (BWA) (31) (http://bio-bwa.sourceforge.net) with default parameters. The resulting alignment was further processed with Samtools (32) and Picard v1.66 (33) to remove duplicates. SNPs were called using Genome Analysis Toolkit (GATK) HaplotypeCaller with the following parameters – genotyping mode DISCOVERY, – output mode EMIT_VARIANTS_ONLY, –stand_emit_conf 10, and –stand_call_conf 30. To improve the quality of variant calling, we further discarded genotyping calls with coverage of <5 reads. The resulting variant call format (VCF) files were then merged into one file using VCF tools (34).

### Variant calling and analysis

The VCF file containing twenty-six samples that passed the quality test from read mapping analysis was used as the input file in VCF tools for variant analysis. High-quality SNPs in four target genes, PfRh5, PfCyRPA, PfRipr, and PfP113, were functionally annotated in the SNPEFF tool (35). Called variants were further analyzed in ARTEMIS software (36). MEGA 7 tool was used to perform multiple sequence alignment and translation of nucleotide sequences to amino acid sequences. Gene variants were identified by aligning the amino acid sequence reads to their corresponding 3D7 reference gene sequence. To test sensitivity of the above approach, we analyzed the sequences at different variant calling parameters to assess the impact of these settings on downstream analysis. Additionally, prior to variant calling, we performed base quality score recalibration to adjust the base quality scores of sequencing reads as well as local realignment around indels to reduce false-positive variant calls resulting from alignment artifacts.

### Population genetics analysis

The population genetic tests for the neutral theory of evolution (37) and Tajima’s D and Fu & Li’s statistics and nucleotide diversity (38) were calculated using DnaSPv6.1 (38). Tajima’s D test statistic calculated the variation between the observed number of singletons and the total number of mutations. Pi was used to test the genetic diversity of each gene of the PfRh5 complex within the parasite population from Lake Victoria region. The P. falciparum adenylosuccinate lyase gene, a housekeeping gene and apical membrane antigen gene were used as control in this analysis. The sequences for these genes were obtained from Lake Victoria parasite population.

### Protein structures

The structure of the Rh5-CyRPA-Ripr complex was retrieved from the Protein Data Bank (http://www.rcsb.org/) under the protein ID 6MPV. The datasets generated from this study were used to map the polymorphic sites of PfRh5 and PfCyRPA protein structures on the Rh5-CyRPA-Ripr complex 3D structure in PyMol (The PyMOL Molecular Graphics System, Version 2.2.0, Schrödinger, LLC) to determine the location of the SNPs in the 3D protein structure and whether the SNPs were localized in the protein-protein interaction regions of the complex.
Results

Genetic variation in the PfRh5 complex genes

Whole genome sequence analysis data for the four genes of the PfRh5 complex were obtained from 26 samples from the Lake Victoria islands. A total of 45, 35, 25, and 3 non-synonymous SNPs were identified within the PfP113, PfRipr, PfRh5, and in the PfCyRPA genes, respectively. The minor allele frequency in the four genes ranged from 0.7% to 24.06%. High-frequency alleles (>5%) were identified in codons Y147, H148, C203, S277, I410, and K429 of the PfRh5 gene and codons V190, M255, Y259, and A1003 of the PfRipr gene (Figure 1). Non-synonymous SNPs identified in PfCyRPA gene at codons S25, D236, and V292 and PfP113 gene at codons L17, E234, Q620, and Q857 occurred at low frequency (Figure 1).

Comparison of polymorphisms identified with the Kilifi population

A total of nine non-synonymous previously not observed from the Kilifi population were identified across the four genes in Lake Victoria isolates (39). PfRipr gene at codon Y226, F236, and T441, PfRh5 at codon S277, PfP113 at codon L17, Q620, and Q857, and PfCyRPA gene at codon S25 and V292 (Figure 1).

Comparison of polymorphisms identified with global MalariaGEN

We further explored the MalariaGEN data to establish whether the variants identified were also present in the global database. We also screened for variants missed due to differences in analysis methodologies. This analysis confirmed that most of the variants observed from the Lake Victoria population had been previously observed elsewhere and deposited in the global MalariaGEN database giving confidence in our analysis methodology on the probability of missing variants (Table 1).

Population genetics statistics

A sliding window approach was used to calculate the nucleotide diversity (π) and Tajima’s D statistics. All four genes had a negative neutrality summary statistic. Analysis revealed that the PfP113 gene was the most conserved relative to the other three genes of the PfRh5 complex and the positive control Pfama1 gene, with a Tajima’s D summary statistic of –1.89 and a π of 0.00010 with 4 singleton mutations distributed along the 1578 bp nucleotide sequence. Relative to the negative control Pfadsl gene, the PfRh5 gene was the least conserved, with 25 non-synonymous polymorphisms distributed along the entire 2907 bp nucleotide sequence with a Tajima’s D summary value of -0.56 and a π of 0.00109 (Figures 2, 3). The Fu & Li’s statistics were not significant for PfRh5 and PfRipr genes. PfCyRPA and PfP113 gene had significant Fu & Li’s values, p < 0.05. (Table 2)

Polymorphisms on the PfRh5 protein complex

The polymorphisms established from our dataset were mapped on the PfRh5 protein complex to show whether they occurred within known protein-protein interacting regions and the PfRh5 – basigin interaction region. The previously published Rh5-CyRPA-Ripr invasion complex (16) was superimposed with the basigin structure to show the interaction of PfRh5 with basigin. PfRh5 binds to basigin via His-102 linker, α-2, α-4, and a disulfide loop.
(Cys345–Cys351) (40). The F350 and W447 PfRh5 residues stabilize binding by packing into basigin hydrophobic bonds. Only one SNP corresponding to codon 203 within the PfRh5-basigin interacting region was identified. PfRipr binds to PfcyRPA blade 6 at amino acid residues 281 – 311. One mutation corresponding to this interaction region at codon 292 of PfcyRPA was identified. No mutation corresponding to PfcyRPA and PfRh5 binding regions was identified. Other polymorphisms were localized outside the protein interaction site (Figure 4).

**Discussion**

*Plasmodium falciparum* infects and replicates in human host erythrocytes leading to manifestation of clinical malaria. The invasion process by invasive merozoites involves the interaction of PfRh5 protein and the basigin receptor localized on the erythrocyte membrane (41, 42). However, PfRh5 does not function alone. Upon secretion, it forms a heteromeric complex with two micronemal proteins, PfRipr and PfcyRPA. PfRipr and PfcyRPA proteins do not interact with basigin and have been shown to lack a membrane anchor (11). The rationale for developing a blood-stage malaria vaccine targeting the components of the PfRh5 complex has been supported by *in-vitro* and *in-vivo* studies in non-human primates. Antibodies raised against the PfRh5 proteins have been shown to block erythrocyte invasion by inhibiting its binding to basigin receptor (5, 40, 43–45). Genes coding for proteins of the PfRh5 complex are highly conserved in *P. falciparum*, suggesting their vital role in parasite survival (30, 46). Therefore, an PfRh5-complex-based vaccine would prove effective. In the present study, we identified the genetic variations of the proteins that make up the *P. falciparum* Rh 5 complex and determined the polymorphism’s locus on the protein complex in the parasite population from the Mfangano, Takawiri, Kibuogi, and Ngodhe Islands of Lake Victoria in Western Kenya and compared with Kilifi and global databases. All genes of the PfRh5 complex were relatively conserved, and the negative population genetics statistic suggests the parasite population has limited potential to retain these mutations. The observed negative Tajima’s D statistics from the Lake Victoria population indicated an excess of rare variants and do

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<th>Ref Alt</th>
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<th>Kilifi (n=68)</th>
<th>MalariaGEN global dataset (n=7,113)</th>
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Polymorphisms identified in both populations and the global MalariaGEN dataset are indicated with a plus +, and singleton SNPs are indicated by an asterisk*. Ref refers to the 3D7 *Plasmodium falciparum* strain reference amino acid, while Alt refers to the amino acid variation. The SNPs are classified under S (synonymous or NS (non-synonymous) and frequency of SNPs is expressed as percentages.
not suggest balancing selection (30). Genes with a significant negative Tajima’s D value indicate that the parasites population has a limited potential to retain polymorphisms, especially PfP113 and PfRipr genes (47). These findings are consistent with previous studies of *P. falciparum* in the African population, which showed a majority of genes having a negative Tajima’s D value, suggesting a historical parasite population expansion event (48, 49).
In contrast to other merozoite antigens that are considered potential vaccine candidates such as Apical membrane antigen 1 (AMA1), merozoite surface protein 1 (MSP1), and merozoite surface protein 10 (MSP10) (50, 51), majority of polymorphisms of the PfRh5 complex components were rare variants and did not indicate balancing selection. Recent studies from Nigeria and Kenya reported one non-synonymous mutation on PfRh5 protein at codon C203Y (30, 52). We identified the C203Y mutation in the Lake Victoria population while mutations at codon Y147H, H148D, and K429N were reported in Kilifi samples and MalariaGEN global database as rare variants, which suggests a need for P. falciparum to maintain these mutations across various populations. Mutation at codon S277N observed from Lake Victoria isolates was not reported from the Kilifi population.

Three singleton mutations at codons Y226H, F236V, and T441N of the PfRipr gene were identified in Lake Victoria. Among the three polymorphisms, only the mutation at codon S277N was observed in Uganda (53). Among the SNPs on PfRh5 complex, three were also observed in Uganda, where a negative population statistic on these variants was reported (53).

P. falciparum population from Uganda identified 16 SNPs in the PfRipr gene (53). Among the SNPs on PfRh5 complex identified in our study, three were also observed in Uganda, where a negative population statistic on these variants was reported (53). Considering the geographical proximity between Uganda and Lake Victoria islands, the common variants across the two study sites should be investigated to determine if they affect the functionality of the PfRh5 complex. Mutations on PfCyRPA S25N, V292F, and PfP113 gene L17V, Q620H and Q857E were identified only in Lake Victoria isolates.

We identified two mutations at the basigin-PfRh5 interaction region and PfCyRPA-PfRipr proteins interaction regions. The mutation C203Y on PfRh5 protein was located on the Basigin-PfRh5 interaction region, while the CyRPA F292V mutation is located on blade 6, Ripr – CyRPA proteins interaction region.

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In contrast to other merozoite antigens that are considered potential vaccine candidates such as Apical membrane antigen 1 (AMA1), merozoite surface protein 1 (MSP1), and merozoite surface protein 10 (MSP10) (50, 51), majority of polymorphisms of the PfRh5 complex components were rare variants and did not indicate balancing selection. Recent studies from Nigeria and Kenya reported one non-synonymous mutation on PfRh5 protein at codon C203Y (30, 52). We identified the C203Y mutation in the Lake Victoria population while mutations at codon Y147H, H148D, and K429N were reported in Kilifi samples and MalariaGEN global database as rare variants, which suggests a need for P. falciparum to maintain these mutations across various populations. Mutation at codon S277N observed from Lake Victoria isolates was not reported from the Kilifi population.

Three singleton mutations at codons Y226H, F236V, and T441N of the PfRipr gene were identified in Lake Victoria. Among the three polymorphisms, only the mutation at codon Y226H was reported in MalariaGEN global dataset. The mutations were, however, absent from the Kilifi populations.

P. falciparum population from Uganda identified 16 SNPs in the PfRipr gene (53). Among the SNPs on PfRipr gene identified in our study, three were also observed in Uganda, where a negative population statistic on these variants was reported (53). Considering the geographical proximity between Uganda and Lake Victoria islands, the common variants across the two study sites should be investigated to determine if they affect the functionality of the PfRh5 complex. Mutations on PfCyRPA S25N, V292F, and PfP113 gene L17V, Q620H and Q857E were identified only in Lake Victoria isolates.

We identified two mutations at the basigin-PfRh5 interaction region and PfCyRPA-PfRipr proteins interaction regions. The mutation C203Y on PfRh5 protein was located on the Rh5- basigin interface, while mutation V292F located on blade 6 of PfCyRPA protein which is the region of interaction with PfRipr. Studies have demonstrated that recombinant PfRh5 with the C203Y mutation binds to basigin with the same affinity as the wild type (54). The components of the PfRh5 complex are located in different subcellular locations; thus, the complex only forms during erythrocyte invasion when they are secreted from the rhoptries or micronemes (42). Field studies have demonstrated the PfRh5 complex components exhibit low immunogenicity suggesting the antigens are under limited immune pressure (55). This could explain the limited high-frequency and rare variants observed in this study, as the parasite has a limited need to acquire mutations to escape host immune responses.

Put together, developing an effective malaria vaccine remains a priority among strategies to eliminate and eradicate the disease. One major hindrance to achieving this is the emergence of polymorphisms within the various vaccine antigen targets leading to allele-specific immune responses. Among the PfRh5 complex,
PfRh5 is the most advanced vaccine target. However, the presence of low-frequency mutations raises concerns about immune system evasion. This study recommends functional assay studies to investigate the immunological and biological relevance of the identified mutations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ddbj.nig.ac.jp/, PRJDB12148.

Ethics statement

The study’s approval was obtained from the Kenyatta National Hospital - University of Nairobi (KNH-UoN) ethical review committee (P609/10/2014) and the Mount Kenya University Ethics Review Committee (038/2014). Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin.

Author contributions

HW, BK, and JG conceived and designed the study; JG provided the analysed data sets; HW performed the bioinformatic and statistical analysis, under supervision of JOK and BK; HW and BK wrote the first draft of the manuscript, and the final version included edits from all authors. The final manuscript was read and approved by all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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