Molecular surveillance reveals the presence of pfhrp2 and pfhrp3 gene deletions in Plasmodium falciparum parasite populations in Uganda, 2017–2019

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Abstract

Background: Histidine-rich protein-2 (HRP2)-based rapid diagnostic tests (RDTs) are the only RDTs recommended for malaria diagnosis in Uganda. However, the emergence of Plasmodium falciparum histidine rich protein 2 and 3 (pfhrp2 and pfhrp3) gene deletions threatens their usefulness as malaria diagnostic and surveillance tools. The pfhrp2 and pfhrp3 gene deletions surveillance was conducted in P. falciparum parasite populations in Uganda.

Methods: Three-hundred (n = 300) P. falciparum isolates collected from cross-sectional malaria surveys in symptomatic individuals in 48 districts of eastern and western Uganda were analysed for the presence of pfhrp2 and pfhrp3 genes. Presence of parasite DNA was confirmed by PCR amplification of the 18s rRNA gene, msp1 and msp2 single copy genes. Presence or absence of deletions was confirmed by amplification of exon1 and exon2 of pfhrp2 and pfhrp3 using gene specific PCR.

Results: Overall, pfhrp2 and pfhrp3 gene deletions were detected in 29/300 (9.7%, 95% CI 6.6–13.6%) parasite isolates. The pfhrp2 gene was deleted in 10/300 (3.3%, 95% CI 1.6–6.0%) isolates, pfhrp3 in 9/300 (3.0%, 95% CI 1.4–5.6%) while both pfhrp2 and pfhrp3 were deleted in 10/300 (3.3%, 95% CI 1.6–6.0%) parasite isolates. Proportion of pfhrp2/3 deletions was higher in the eastern 14.7% (95% CI 9.7–20.0%) compared to the western region 3.1% (95% CI 0.8–7.7%), p = 0.001. Geographical location was associated with gene deletions aOR 6.25 (2.02–23.55), p = 0.003.

Conclusions: This is the first large-scale survey reporting the presence of pfhrp2/3 gene deletions in P. falciparum isolates in Uganda. Roll out of RDTs for malaria diagnosis should take into consideration the existence of pfhrp2/3 gene deletions particularly in areas where they were detected. Periodic pfhrp2/3 surveys are recommended to inform future decisions for deployment of alternative RDTs.

Keywords: Malaria rapid diagnostic tests, Plasmodium falciparum, Histidine rich protein 2, Histidine rich protein 3, Gene deletion, Deoxyribonucleic acid, Microscopy

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Background

In 2018, the World Health Organization (WHO) estimated there were 228 million cases and 405,000 deaths globally due to malaria. The WHO African Region...
continues to contribute a disproportionately high share of the global burden (93% of malaria cases and 94% of malaria deaths) in 2018 alone [1]. Nearly all malaria cases in the region are caused by *Plasmodium falciparum*. Uganda is ranked among the six highest burden countries [1]. The 2018 and 2014 Uganda national malaria indicator surveys have reported overall malaria parasite prevalence of 9 and 19%, respectively [3, 4]. *Plasmodium falciparum* is the most predominant parasite in Uganda, accounting for >95% of malaria infections [3, 5].

The WHO recommends parasitological confirmation of malaria in all suspected cases prior to treatment with artemisinin-based combination therapy (ACT) [6, 7]. The Uganda National Malaria Control Division adopted this policy and shifted from clinical to parasite-based diagnosis with microscopy or rapid diagnostic tests (RDTs) in 2011 [6, 7]. Since the introduction of RDTs in late 2000s, over 800 million RDTs have been used for malaria testing in Uganda which has led to increased access to parasite-based diagnosis [2, 8]. A similar increase has been seen particularly in the African region, where large volumes of histidine rich protein 2 (HRP2)-based RDTs are used due to the predominance of *P. falciparum* species in this region [1, 2]. However, RDTs must remain effective and accurate in detecting the presence of parasites in order to be useful in supporting diagnostic and surveillance programmes [9–11]. There are several documented factors that have been known to affect the accuracy and functionality of RDTs that range from product design, transport or storage conditions, parasite factors due to gene deletions, operator-related factors and host parasite densities [12, 13]. Many endemic countries in collaboration with the WHO and the manufacturers have instituted quality assurance systems to address the possible causes of false RDT results [14, 15], however, parasite gene deletions have not been studied at a wider scale in many parts of Africa and evidence remains limited [12, 16]. Studies have suggested the possibility of evolution of gene-deleted parasities by a genetic event due to selective pressure resulting from long-term use of HRP2-based RDTs [17]. Failure of the parasite to express the HRP2 target antigen, alteration in the HRP2 protein sequence or pattern of histidine repeats and variation in the number of repeats have been known to affect the sensitivity of HRP2-based RDTs [18–20]. Although investigation of other causes of false negative RDTs was outside the scope of this study, several studies have provided possible explanations for their occurrences. They include variation in the composition of *pfhrp2* repeat sequence, number of repeat types and the amino acid composition of the HRP2 all of which may have impact on RDT sensitivity [19, 21].

Due to the predominance of *P. falciparum* in Uganda, the national policy recommends exclusive use of HRP2-based RDTs for malaria diagnosis [7]. The principal target recognized by HRP2-based RDTs are HRP2 antigens although, due to similarity in amino acid sequences, antibodies cross-react with HRP3 [5, 7]. These antigens are not expressed in malaria parasites in some parts of Africa due to the absence of the *pfhrp2* and *pfhrp3* genes [12, 16, 17, 19, 22–28]. When *P. falciparum* parasites express little or no *pfhrp2/3* target antigens, they are not detected by HRP2-based RDTs, threatening the usefulness of HRP2-based RDTs as a diagnostic test [11, 12, 25]. This poses a public health threat as a large number of infected patients will go untreated, leading to increased risk of malaria morbidity, mortality and transmission [12, 16, 17, 22].

The WHO recommends routine surveillance of *pfhrp2* and *pfhrp3* gene deletions in malaria parasites in countries that are neighbouring areas where deletions have been confirmed or where there are reports of false negative RDTs [9–11, 16]. Surveillance data on parasite gene deletions could potentially inform national malaria diagnostic policies regarding choice of RDTs [11, 12]. A policy switch to more effective, alternative RDTs is recommended when the prevalence of false negative RDT results due to *pfhrp2/3* gene deletions exceeds 5% [10, 11]. A threshold of 5% was selected because it is somewhere around this point that the proportion of cases missed by HRP2 RDTs due to non-HRP2 expression may be greater than the proportion of cases that would be missed by less-sensitive pLDH-based RDTs [29].

Parasite *pfhrp2/3* gene deletions have been reported in areas neighbouring Uganda, including Kenya, Democratic Republic of Congo (DRC), Rwanda, and Eritrea [17, 28, 30, 31], however data on their occurrence and distribution in Uganda are limited. Only one study in Uganda reported the existence of *pfhrp2/3* gene deletions in nine (9/416) PCR-confirmed parasite isolates, however its scope was limited to archived samples in one district [32]. The magnitude, extent of spread and the possible factors associated with the *pfhrp2/3* gene deletions in Uganda is poorly understood. To improve understanding of the extent and spectrum of *pfhrp2* and *pfhrp3* gene deletions in Uganda on a wider scale, surveillance of *pfhrp2* and *pfhrp3* gene deletions in *P. falciparum* parasite populations in 48 districts of Uganda.

**Methods**

**Study design and setting**

This was a cross-sectional study that analysed samples collected from parasite surveys that were conducted in 48 out of 134 districts of Uganda between 2017 and 2019. The primary objective of the surveys was to evaluate the
effect of piperonyl butoxide (PBO) long-lasting insecticide-treated nets (LLINs) on parasite prevalence [33, 34] and covered nearly half of the country and a wide range of epidemiological settings. Details of the PBO study have been published elsewhere [33, 34]. Malaria is endemic in 95% of the country, and transmission occurs throughout the year with two peak transmission seasons between June to July and November to December [5, 33, 34]. The parasite surveys were conducted at 6-month intervals and coincided with the two peak transmission seasons.

Study population
Details of sampling, participant selection and enrolment have been described and published under the PBO studies [33, 34]. Briefly, a total of 104 clusters (Health Sub-Districts) across 48 districts were selected and randomized to receive different LLINs. Fifty (n = 50) households were randomly selected from each cluster. In selected households, children aged 2–10 years were assessed for presence of fever (based on axillary temperature of >37.5 °C) before enrolment. Enrolled children were tested for malaria using RDTs and by malaria microscopy [33, 34]. Additionally, dried blood spots (DBS) were collected and stored for molecular testing of parasites. Written consent was obtained from all participants prior to study procedures commencing.

This study utilized the DBS samples from the PBO parasite surveys to assess the presence of pfhrp2 and pfhrp3 gene deletions. Included in the study were samples that were RDT negative but microscopy positive for malaria RDT−/microscopy+ (n = 222) and a random sample set of 15% (n = 140) from those that were both RDT and microscopy-positive (RDT+/microscopy+). The additional inclusion criteria were if the participants were aged 2–10 years, had a DBS filter paper sample available and provided consent for use of samples for future studies. Samples were excluded if they contained non-Plasmodium falciparum species by DNA PCR and if they had low quality DNA based on failure to amplify the single copy genes merozoite surface protein 1 (MSP1) and merozoite surface protein 2 (MSP2).

Laboratory analysis
Rapid diagnostic tests (RDTs)
As part of the PBO parasite surveys, a HRP2-based P. falciparum-specific RDT (SD Bioline Malaria Ag Pf 05FK120; Standard Diagnostics, Gyeonhhi-do, South Korea) was used to test for malaria in febrile patients with a history of fever (based on axillary temperature of >37.5 °C). The test is designed to exclusively detect P. falciparum infections only. RDT testing was done as per the manufacturer’s instructions. The RDT results were obtained from the PBO study database.

Blood smear microscopy
In addition to the RDT test in the field, a thick blood smear was collected, shipped and read at the Infectious Diseases Research Collaboration (IDRC) reference laboratory in Kampala during the PBO parasite surveys. At the reference laboratory, blood smears were stained with 2% Giemsa for 30 min. Each blood slide was read independently by two competent (WHO competency assessment level 1) laboratory scientists. The slide readers were blinded to each other’s results and were not aware of patients’ RDT results. Thick blood smears were evaluated for the presence of parasites (axial forms) and gametocytes following standard WHO methodology [35]. Parasitaemia was determined by counting the number of parasites per 200 white blood cells (WBC), or 500 WBCs for low-density infections, on thick smears (assuming a standard of 8000 WBC per µl in accordance with WHO methods) [35]. Smears were considered negative if no parasites were seen in 200 oil-immersion fields (1000×) in a thick blood film. All blood smear results were obtained from the PBO study database. Blood smears were only retrieved for cross-checking and quality control purposes.

Parasite DNA extraction
All DBSs for the pfhrp2 and pfhrp3 studies were shipped to the Australian Defence Force Malaria and Infectious Disease Institute (ADFMIDI) where molecular testing was conducted. From each DBS sample, three discs of DBS were punched into 1.5-mL microfuge tubes. DNA was extracted using QIAamp DNA Mini Kits and a QIAcube Robot (QIAGEN, Crawley, UK) according to the manufacturer’s instructions. Samples were eluted into a volume of 100 µl with AE buffer. A P. falciparum-positive control DBS spot was extracted and processed in each run alongside samples. Details of the QIAamp DNA Mini Kits and a QIAcube Robot extraction method have been described and published elsewhere [12, 17, 25, 36, 37].

Confirmation of Plasmodium falciparum parasite DNA
Presence of different Plasmodium spp. was confirmed by amplification of the 18S ribosomal RNA (18S rRNA) gene using multiplex PCR. Presence of P. falciparum infection was further confirmed by P. falciparum-specific PCR and amplification of the msp1 and msp2 single copy genes. Gel electrophoresis using 2% agarose was used to confirm the presence of bands. The detailed procedure for the controls, primers and the PCR conditions used have been described previously and widely published [12, 17, 25, 36, 37].
Amplification of pfhrp2 and pfhrp3 parasite genes
All samples that were confirmed as *P. falciparum* positive and in which *msp1* and *msp2* were detected, the exon 1 and exon 2 of the *pfhrp2* and *pfhrp3* genes were amplified to investigate the presence or absence of *pfhrp2* and *pfhrp3* genes. PCR controls using laboratory lines DD2, 3BD5, HB3, and 3D7 with known *pfhrp2/3* status and human negative controls were included in each PCR run. PCR runs were only considered valid if all controls were amplified and resulted in bands of expected size on gel electrophoresis. The detailed procedures, primers used and PCR conditions have been well described and published elsewhere [12, 17, 25]. In all cases, samples were considered gene deleted if they had a positive *P. falciparum* DNA PCR and confirmed presence of *msp1* and *msp2* single copy genes but failed to amplify exon 1 or exon 2 of the *pfhrp2* or *pfhrp3* genes.

Quality control
As part of quality control, all slides were read in a blinded manner by WHO-certified level 1 microscopists. In addition, a random sample of 20% of the slides were re-read by two level 1 WHO-certified microscopists and a third level 1 expert resolved any discrepant readings (differences between two microscopy readings including > 20% difference in parasite counts, or between RDTs and smears). All three slide readers were independent from an external laboratory. The research laboratory in Australia where molecular analysis of samples was done is a WHO Collaborating Centre for malaria, a member of the WHO *pfhrp2/3* gene deletion detection laboratory network and participates in the WHO nucleic acid amplification tests (NAAT) external quality assurance programme.

Ethical approval
The study was approved by the Makerere University School of Medicine Research and Ethics committee (#REC REF 2017-111), the Uganda National Council of Science and Technology (Ref No: HS271ES), and the Australian Department of Defence and Veterans’ Affairs Human Research Ethics Committee (DDVA HREC 096-18). In the primary surveys from where samples were collected, participants were enrolled after providing consent following a detailed explanation about the use of samples for future research studies.

Statistical analysis
The aim was to estimate the proportion of *pfhrp2/pfhrp3* gene deletion in the parasite isolates to within 5 percentage points (absolute precision) of the true value with 95% confidence. The assumption that the prevalence of deletions in the *P. falciparum* isolates was unlikely to exceed 5% and a design effect of 1.5 were considered in order to estimate the minimum required sample.

As part of data management, demographics and predictor variables linked to the DBS samples were extracted from the primary PBO study database. All data were entered and managed in one central Excel database. Data quality checks were done to check for and correct any inconsistencies. Data analysis was done with STATA Ver 14, College Station, TX, USA: StataCorp LP). Descriptive analysis was done to describe the baseline characteristics with respect to the predictor variables. ArcGIS software version 10.8, Environmental Systems Research Institute (ESRI), CA, USA) was used to map the locations from where all blood samples were collected and where the *pfhrp2* and *pfhrp3* gene deletions actually occurred. Bivariate analysis was performed to relate *pfhrp2/3* gene deletions and each of the independent variables. The exact binomial test was used to assess if the observed proportions of deletions were different from the 5%. As appropriate, the Chi square or Fisher’s exact test were used to compare proportions of deletions. Lastly, multivariate analysis was done with logistic regression to determine the factors associated with *pfhrp2/3* gene deletions. The 95% confidence interval was estimated for all estimates while *p* < 0.05 was considered significant.

Results
Characteristics of the study population
Out of 7276 participants enrolled and tested for malaria in the PBO surveys, 2058 (28.3%) had a positive blood smear. Of the 2058, 10.8% (222/2058) had a negative RDT despite a positive blood smear and were considered for the *pfhrp2/3* study. In addition, a random sample of 140 (i.e., 15%) of the RDT-positive/microscopy-positive samples (RDT+/microscopy+) were included for *pfhrp2/3* deletion study. Sixty-two samples (57 in the RDT-/micro+ and 5 in the RDT+/micro+) were excluded from gene deletion analysis due to contamination (n = 3), absence of parasite DNA (n = 27), and non-*P. falciparum* species (n = 32), leaving 300 samples for *pfhrp2/3* gene deletion analysis. The distribution of study samples
(RDT−/microscopy+ and RDT+/microscopy+) across the study sites is shown in Fig. 1 and the study profile in Fig. 2. The majority of participants studied were male (52.3%) and were aged >5 years (59.3%). Most participants were from the eastern region of Uganda 56.7% (50.9–62.4%). A majority of samples had parasite density ≥ 1000/µl Table 1.

Overall, the pfsr2 and pfsr3 genes were deleted in 9.7% (29/300) of the P. falciparum isolates (95% CI 6.6–13.6%). The specific proportions of gene deletions were 3.3% (95% CI 1.6–6.0%) for pfsr2−/pfsr3+, 3.0% (95% CI 1.4–5.6%) for pfsr2+/pfsr3− and 3.3% (95% CI 1.6–6.0%) for pfsr2−/pfsr3−. The pfsr2 and pfsr3 genes were present and detected in 62.0% (186/300) of the P. falciparum isolates (95% CI 55.9–67.2%).
Fig. 2  *pfhrp2* and *pfhrp3* study profile. RDT in this case means samples tested with HRP2 rapid diagnostic tests. PCR is the polymerase chain reaction for parasite detection and speciation. *pfhrp2* and *pfhrp3* PCRs are the polymerase chain reactions for amplification of exon 1 and exon 2 of *P. falciparum* histidine-rich protein 2 and histidine-rich protein 3 genes. *pfhrp2/pfhrp3* PCR negative are samples in which *pfhrp2/3* genes were missing despite presence of parasite DNA and *msp1* and *msp2* single copy genes. Low quality DNA means samples that were DNA PCR positive but could not amplify two single copy genes (*msp1* and *msp2*) as indicator of quality of DNA.
Table 1 Baseline characteristics of samples (n = 300)

| Variable | Frequency | Proportion (%) |
|----------|-----------|---------------|
| Gender   |           |               |
| Male     | 156.90    | 52.30         |
| Female   | 143.10    | 47.70         |
| Age (years) |         |               |
| < 5      | 122.10    | 40.70         |
| ≥ 5      | 177.90    | 59.30         |
| Region   |           |               |
| Eastern  | 170.10    | 56.70         |
| Western  | 129.90    | 43.30         |
| Endemicity |         |               |
| Low transmission | 195.90 | 65.30 |
| Moderate transmission | 104.10 | 34.70 |
| Parasite density (μL) |         |               |
| < 1000   | 117.00    | 39.00         |
| ≥ 1000   | 183.00    | 61.00         |

< 5 means children under 5 years of age; ≥ 5 means children above 5 years of age. Low transmission means P. falciparum prevalence of < 10% (< 10% PPR), moderate transmission means (10–35% PPR) based on WHO surveillance guidelines for malaria epidemiological stratification [38]; < 1000 and ≥ 1000 are parasite quantification counted per microlitre of blood.

The proportion of gene deletions were significantly higher in RDT−/microscopy+ samples 14.5% (95% CI 9.5–20.9%) compared to the RDT+/microscopy+ samples 3.7% (95% CI 1.2–8.4%), p = 0.001. An important observation to note is that parasite densities were significantly lower in the RDT-/microscopy+ compared to the RDT+/microscopy+ samples (median: 520.0 (119.5–1086.5 vs 8400 (3628.5–29,600.0), p = 0.001.

The proportions of P. falciparum isolates with detectable pfhrp2 and pfhrp3 genes were significantly higher in the RDT+/microscopy+, 91.9% (95% CI 85.9–95.9%) compared to the RDT−/microscopy+ samples, 37.0% (95% CI 29.6–44.8%) p = 0.001.

Overall, pfhrp2 and pfhrp3 gene deletions were higher in parasite isolates collected from eastern region, 14.7% (95% CI 9.7–20.9%) compared to western region of Uganda 3.1% (95% CI 0.8–7.7%), p = 0.001. The difference in this distribution was more marked when the parasites had both pfhrp2 and pfhrp3 deletions (pfhrp2−/pfhrp3−), 5.3% vs 0.8%, p = 0.047 for eastern and western regions, respectively (Table 2).

All 29 P. falciparum isolates with pfhrp2/3 gene deletions were mapped based on latitude and longitude

Table 2 Proportion of pfhrp2 and pfhrp3 gene deletion overall, by RDT−/microscopy+/PCR+, RDT+/microscopy+/PCR+ and by geographical location

| Gene deletion | Overall proportions and stratified by RDT/microscopy results | Deletions by geographical location |
|---------------|-------------------------------------------------------------|-----------------------------------|
|               | Total (N = 300), Proportion n (%, 95% CI) | RDT-/microscopy+/PCR+ N = 165 n (%, 95% CI) | RDT+/microscopy+/PCR+ N = 135 n (%, 95% CI) | Prevalence ratio (RDT-/RDT+) n (95% CI) | p value |
| Any deletion  | 29 (97, 6.6–13.6) | 24 (14.5, 9.5–20.9) | 5 (3.7, 1.2–8.4) | 3.9 (1.5–10.0) | 0.002 |
| pfhrp2−/pfhrp3+ | 10 (3.3, 1.6–6.0) | 9 (5.5, 2.5–10.1) | 1 (0.7, 0.0–4.1) | 7.4 (1.0–57.4) | 0.21 |
| pfhrp2+/pfhrp3− | 9 (3.0, 1.4–5.6) | 5 (3.0, 1.6–6.0) | 4 (3.0, 0.8–7.4) | 1.02 (0.3–3.7) | 1.00 |
| pfhrp2−/pfhrp3− | 10 (3.3, 1.6–6.0) | 10 (6.1, 2.9–10.9) | 0 (0.0, 0.0–2.7) | N/A | 0.04 |
| pfhrp2+/pfhrp3+ | 185 (61.7, 55.9–67.2) | 61 (37.0, 29.6–44.8) | 124 (91.9, 85.9–95.9) | 0.4 (0.3–0.5) | 0.001 |

Parasite isolates were categorized as those in which the pfhrp2 gene was deleted but pfhrp3 gene present (pfhrp2−/pfhrp3+), the pfhrp3 gene deleted but pfhrp2 gene present (pfhrp2+/pfhrp3−), those in which both genes were deleted (pfhrp2−/pfhrp3−) and those where both pfhrp2 and pfhrp3 genes were present (pfhrp2+/pfhrp3+). Any deletion means total (overall) number of samples where deletion of any type was detected (summation of pfhrp2−/pfhrp3+, pfhrp2+/pfhrp3− and pfhrp2−/pfhrp3−).
Fig. 3  Mapping the exact locations of \textit{pfhrp2} and \textit{pfhrp3} gene-deleted \textit{Plasmodium falciparum} isolates. Exact location of collection sites for 29 gene-deleted \textit{P. falciparum} parasites by latitude and longitudes coordinates. \textit{pfhrp2}−/\textit{pfhrp3}+ (indicated by red dots), \textit{pfhrp2}+/\textit{pfhrp3}− (indicated by green circles) and \textit{pfhrp2}−/\textit{pfhrp3}− (indicated by the purple hexagons).
coordinates to determine their exact location in the study area Fig. 3. There was pronounced clustering of the pfhrp2−/pfhrp3− gene deleted isolates in mid-eastern Uganda and near the Uganda-Kenya border. The pfhrp2−pfhrp3+ isolates in western Uganda were mainly clustered along the Ugandan border with DRC (Fig. 3).

Nine different types of pfhrp2 and pfhrp3 gene deletion patterns were observed in the 29 pfhrp2/3-deleted isolates (Table 3). Out of 29 P. falciparum parasite isolates identified with gene deletions, 8 (27.6%) had complete deletion of the pfhrp2/3 exon1 and exon2. The rest of the isolates had deletions of either exon1 or exon2 of pfhrp2 and pfhrp3 genes.

Additional data was obtained on predictor variables and statistical testing performed to determine if any were possibly associated with the P. falciparum gene deletions. The predictor variables included endemicity, geographical location, age, gender, parasite density of the samples per microlitre of blood (Table 4).

Overall, deletions were more likely to occur in the eastern compared to western regions of Uganda, aOR 6.25 (95% CI 2.02–23.55), p = 0.003. When stratified the pfhrp2−/pfhrp3+ gene deleted parasites were still more detectable in samples collected from eastern Uganda aOR 6.84 (1.50–48.30), p = 0.022.

In Uganda, malaria transmission is epidemiologically stratified according to the WHO surveillance guidelines into low (≤ 10% PfPR), moderate (10–35% PfPR) and high (≥ 35% PfPR) transmission based on population-based parasite surveys [38]. The pfhrp2−/pfhrp3+ gene deletions were less likely to occur in parasite isolates collected from moderate compared to low transmission settings aOR 0.19 (95% CI 0.03–0.88), p = 0.049. In this study, parasite density and gene deletions were not associated, aOR 0.97 (0.42–2.16), p = 0.943 as deletions occurred in both low and high parasite density samples.

| Table 3 | Pattern of deletions in the pfhrp2 and pfhrp3 genes in the 29 deleted Plasmodium falciparum isolates (n = 29) |
|---------|----------------------------------------------------------------------------------|
| **Pf DNA PCR** | **msp1** | **msp2** | **pfhrp2 Exon1** | **pfhrp2 Exon-2** | **pfhrp3 Exon1** | **pfhrp3 Exon-2** | **Sample (%)** |
| +       | +       | +       | −         | −         | −         | −         | 8 (27.6%) |
| +       | +       | +       | −         | −         | +         | −         | 5 (17.2%) |
| +       | +       | +       | −         | −         | +         | 2 (6.9%)  | 1 (3.4%)  |
| +       | +       | +       | +         | −         | +         | +         | 2 (6.9%)  |
| +       | +       | +       | −         | +         | −         | 1 (3.4%)  | 3 (10.3%) |
| +       | +       | +       | +         | +         | −         | 1 (3.4%)  | 1 (3.4%)  |
| +       | +       | +       | −         | +         | +         | 2 (6.9%)  | 2 (6.9%)  |
| +       | +       | +       | +         | +         | +         | 6 (20.7%) | 6 (20.7%) |

PCR amplification and detection results for the 29 deleted samples. Positive and negative PCR results are represented by (+) and (−) respectively.
This is the first large-scale survey reporting the presence of pfhrp2 and pfhrp3 gene deletions in P. falciparum parasite isolates in Uganda. The methods used in the study are adopted from the WHO-recommended protocol for investigation of pfhrp2 and pfhrp3 gene deletions [11]. Samples were confirmed for the presence of parasite DNA and gene deletion classifications were made following the WHO recommended procedure, i.e., quality assured DNA quality by amplifying single copy genes msp1 and msp2 before performing the pfhrp2 and pfhrp3 gene specific PCRs [11, 12]. These methods have been used and widely published in many studies [12, 16, 17, 25].

The study objectives were to determine the proportion of pfhrp2/3 gene deletions in the parasite isolates, extent of spread and investigate the possible factors associated with these deletions. Overall, it was observed that the gene deletions were present in 9.7% (95% CI 6.6–13.6) of the P. falciparum parasite isolates in the exon1 and exon2 of pfhrp2/3 genes. The gene deletions occurred in both surveyed regions but were disproportionately higher in eastern Uganda 14.7% (9.7–20.9), \( p = 0.001 \). The specific gene deletions were \( pfhrp2(-)/pfhrp3(+) \) 3.3% (CI

### Table 4 Factors associated with pfhrp2/3 deletions (overall)

| Variable                      | Univariable | Multivariable |
|-------------------------------|-------------|---------------|
|                               | OR (95% CI) | p-value       | aOR (95% CI) | p-value |
| Gender                        |             |               |             |         |
| Male                          | 1.00        |               | 1.00        |         |
| Female                        | 1.20 (0.55–2.60) | 0.646        | 1.24 (0.55–2.80) | 0.598    |
| Age (years)                   |             |               |             |         |
| < 5                           | 1.00        |               | 1.00        |         |
| \( \geq 5 \)                  | 1.34 (0.61–3.10) | 0.477        | 1.52 (0.68–3.61) | 0.321    |
| Geographical location         |             |               |             |         |
| Eastern                       | 5.43 (2.04–18.81) | 0.002        | 6.25 (2.02–23.55) | 0.003    |
| Western                       | 1.00        |               | 1.00        |         |
| Endemicity                    |             |               |             |         |
| Low transmission              | 1.00        |               | 1.00        |         |
| Moderate transmission         | 1.88 (0.86–4.08) | 0.109        | 0.78 (0.32–1.91) | 0.579    |
| Parasite density (μL)         |             |               |             |         |
| <1000                         | 1.12 (0.50–2.41) | 0.782        | 0.97 (0.42–2.16) | 0.943    |
| \( \geq 1000 \)               | 1.00        |               | 1.00        |         |

### pfhrp2−/pfhrp3+ gene deletions

| Variable                      | Univariable | Multivariable |
|-------------------------------|-------------|---------------|
|                               | OR (95% CI) | p-value       | aOR (95% CI) | p-value |
| Gender                        |             |               |             |         |
| Male                          | 1.00        |               | 1.00        |         |
| Female                        | 1.10 (0.30–4.04) | 0.881        | 0.89 (0.23–3.39) | 0.862    |
| Age (years)                   |             |               |             |         |
| < 5                           | 1.00        |               | 1.00        |         |
| \( \geq 5 \)                  | 1.62 (0.44–7.65) | 0.489        | 1.64 (0.43–7.92) | 0.493    |
| Geographical location         |             |               |             |         |
| Eastern                       | 3.16 (0.78–21.18) | 0.15         | 6.84 (1.50–48.30) | 0.022    |
| Western                       | 1.00        |               | 1.00        |         |
| Endemicity                    |             |               |             |         |
| Low transmission              | 1.00        |               | 1.00        |         |
| Moderate transmission         | 0.46 (0.07–1.88) | 0.333        | 0.19 (0.03–0.88) | 0.049    |
| Parasite density (μL)         |             |               |             |         |
| <1000                         | 0.66 (0.14–2.43) | 0.555        | 0.61 (0.13–2.30) | 0.488    |
| \( \geq 1000 \)               | 1.00        |               | 1.00        |         |
mapping of parasite locations showed clustering of the parasites in Uganda. However, the presence of non-
gene deletions was observed in samples that were RDT negative but microscopy positive (RDT+/microscopy+), 14.5% (9.5–20.9%), p = 0.001. GIS mapping of parasite locations showed clustering of the gene deletions close to the Uganda-Kenya border in eastern Uganda and near the Uganda-DRC border in western Uganda (Fig. 3). Overall, a significant proportion of this P. falciparum parasite population contained the pfhrp2 and pfhrp3 genes 62.0% (55.9–67.2), p = 0.001.

The relatively low proportions of gene deletions observed in this study suggests that most parasite isolates were able to express HRP2 antigen (185/300) 62.0% and therefore HRP2-based RDTs will still be useful for malaria diagnosis in these areas. However, the fact that a proportion (24/300) of the P. falciparum isolates lacked the pfhrp2/3 genes and evaded detection and subsequent treatment is of concern. In view of the fact that the HRP2-based RDTs are widely deployed in Uganda, the occurrence and confirmation of pfhrp2/3 gene deletions in P. falciparum parasites may have implications for malaria case management and surveillance, particularly in areas where they have been mapped and located. It is important to conduct follow-up surveys to monitor their prevalence as recommended by the WHO [9, 11]. However, the proportion of gene deletions observed in Ugandan parasite isolates is lower than what was reported in Eritrea and Rwanda [17, 28]. It is however higher than the levels reported in Kenya, Tanzania, DRC, Ghana, and Mali [22–24, 26, 27, 30, 31, 39, 40]. The specific gene deletions of pfhrp2−/pfhrp3+, pfhrp2+/pfhrp3− and pfhrp2−/pfhrp3− were generally lower compared to what has been reported in neighbouring countries. An important point to note however is that the comparison pfhrp2 and pfhrp3 findings across studies in Africa is challenging due to the wide variations in methods and computations of proportions using different denominators [12, 16]. Harmonization of methods for investigation of gene deletions based on WHO-recommended protocol will allow better comparison between studies [11, 16].

As expected, high proportions of gene deletions were observed in samples that were RDT negative but microscopy positive for malaria (RDT−/microscopy−) compared to those that were RDT and microscopy positive (RDT+/microscopy+). This indicates that gene deletions are one of the contributors to false negative RDT results in Uganda. However, the presence of non-P. falciparum species (n = 32) and low parasite densities as indicated by low quality DNA (n = 86) particularly in RDT-/microscopy− samples suggests that the two could have contributed to false negative RDTs. The contribution of gene deletion to false negative RDTs has been observed and reported elsewhere in previous studies [12, 17, 19, 22, 26–28, 30, 31]. The occurrence of fewer deletions in RDT-/micro− samples supports the assumption that the isolates still harbour the pfhrp2 genes and are therefore able to express the HRP2 antigen. The WHO protocol recommends the RDT-/microscopy+ category as the most suitable samples for analysing gene deletions [10, 11]. However, in this study P. falciparum isolates with pfhrp2/pfhrp3 gene deletions were also detected in the RDT+/microscopy− category of samples, 3.7% (95% CI 1.2–8.4%). This observation supports the previous findings suggesting cross-reactivity between the HRP2 and HRP3 [11, 12, 16, 17, 25, 41]. The detection of pfhrp2/pfhrp3 gene deletions in the RDT+/microscopy− category of samples suggests the possibility of underestimation of the true proportions of deletion in studies that limit themselves to the RDT−/microscopy+ samples only.

Despite the occurrence of P. falciparum gene deletions in both surveyed regions, they were significantly higher in eastern Uganda, 14.7% (CI 9.7–20.9), p = 0.001. Using latitude and longitude coordinates, areas where all the P. falciparum gene-deleted isolates occurred were mapped and located. Although the gene-deleted parasites occurred across the two regions, they were more clustered close to the Uganda–Kenya border and in mid-eastern Uganda. The occurrence of gene deletions in the mid-eastern region had been reported previously in one district in 9 isolates (seven pfhrp2 and two pfhrp3 deletions) out of 416 PCR-confirmed samples and this study confirms this finding [32]. Some gene-deletion clustering was also observed near the Uganda-DRC boarder in western Uganda. Geographical clustering of pfhrp2/pfhrp3 gene deletions in the RDT−/microscopy− category of samples confirms the possibility of underestimation of the true proportions of deletion in studies that limit themselves to the RDT−/microscopy+ samples only.

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observed in this study are consistent with what has been reported elsewhere in DRC, India and Eritrea [13, 16, 17, 30]. In Eritrea, pfhrp2/3 deletion varied between hospitals in different locations of the country [17]. In DRC and India, the proportions of gene deletions varied across provinces and states [13, 30]. Geographical clustering of pfhrp2 and pfhrp3 gene-deleted parasites was reported in malaria-endemic regions of eastern DRC and western Kenya suggesting a possibility of cross-border transmission [30, 31]. The gene deletion mapping data obtained in this study could inform better targeting of pfhrp2/3 [30, 31].

Conclusions

This study provides the first evidence on a large scale of the presence of pfhrp2 and pfhrp3 gene deletions in P. falciparum isolates in Uganda. Deletions occurred in both the eastern and western regions of Uganda but were more marked in the east. Proportions of gene deletions...
were higher in (RDT−/microscopy−) samples compared to (RDT+/microscopy+). In view of these findings the roll-out of RDTs for malaria diagnosis will need to take into consideration the pfhrp2/3 gene deletions in these regions. Periodic pfhrp2/3 surveys will be important to inform future decisions for deployment of alternative RDTs in Uganda.

Abbreviations
HFR2: Histidine-rich protein 2; pfhrp2: The Plasmodium falciparum histidine rich protein 2 gene; RDTs: Rapid diagnostic tests; ACT: Artemisinin-based combination therapy; MSP1: Merozoite surface antigen 1; MSP2: Merozoite surface antigen 2; PCR: Polymerase chain reaction; WHO: World Health Organization; ITNs: Insecticide treated mosquito nets; LLINs: Long-lasting insecticide-treated nets; IRS: Indoor residual spraying; GIS: Geographical Information System; DNA: Deoxyribonucleic acid; rRNA: Ribosomal ribonucleic acid; DBS: Dried blood spots; WBC: While blood cells; SNPs: Single nucleotide polymorphisms; NAAT: Nucleic acid amplification tests.

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Authors’ contributions
BBA, MK, QC designed the study. BBA, QC, KA, KG, CP, DS did the sample analysis. BBA, SW, BL, JK, PM, JN, JNK supported the data analysis. BBA drafted the manuscript. BBA, AX, NS, JIN, JO, RN, SG, EA, PM, CSL, CK, JC, JNN all reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Data for this pfhrp2 and pfhrp3 study is available and kept in the study central excel database and in safe custody.

Ethics approval and consent to participate
Ethical approval for the primary study was obtained from the Makerere University School of Medicine Research and Ethics Committee, the Uganda National Council of Science and Technology, the London School of Hygiene & Tropical Medicine Ethics Committee, and the University of California, San Francisco Committee on Human Research. Ethical approval to access and use participants’ samples from the primary study was obtained from the School of Medicine Research and Ethics Committee (SONREC), the Uganda National Council of Science and Technology (UNCST) and the Australian Department of Defence and Veterans’ Affairs Human Research Ethics Committee (DDVAREC).

Consent for publication
All authors read and approved the manuscript for publication.

Competing interests
All authors declare that they have no competing interests.

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