Pfhrp2-Deleted Plasmodium falciparum Parasites in the Democratic Republic of the Congo: A National Cross-sectional Survey

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BACKGROUND

Rapid diagnostic tests (RDTs) account for more than two-thirds of malaria diagnoses in Africa. Deletions of the Plasmodium falciparum hrp2 (pfhrp2) gene cause false-negative RDT results and have never been investigated on a national level. Spread of pfhrp2-deleted P. falciparum mutants, resistant to detection by HRP2-based RDTs, would represent a serious threat to malaria elimination efforts.

METHODS

Using a nationally representative cross-sectional study of 7,137 children under five years of age from the Democratic Republic of Congo (DRC), we tested 783 subjects with RDT-/PCR+ results using PCR assays to detect and confirm deletions of the pfhrp2 gene. Spatial and population genetic analyses were employed to examine the distribution and evolution of these parasites.

RESULTS

We identified 149 pfhrp2-deleted parasites, representing 6.4% of all P. falciparum infections country-wide (95% confidence interval 5.1–8.0%). Bayesian spatial analyses identified statistically significant clustering of pfhrp2 deletions near Kinshasa and Kivu. Population genetic analysis revealed significant genetic differentiation between wild-type and pfhrp2-deleted parasite populations (GST = .046, p ≤ .00001). DNA variation was found in roughly 2% of 1,000 unrelated children in the DRC.

CONCLUSIONS

Pfhrp2-deleted P. falciparum is a common cause of RDT-/PCR+ malaria among asymptomatic children in the DRC and appears to be clustered within select communities. Surveillance for these deletions is needed, and alternatives to HRP2-specific RDTs may be necessary.

KEYWORDS

rapid diagnostic tests; false-negative; diagnostic resistance; histidine-rich protein 2; pfhrp3; hrp2; hrp3; RDT; deletion; malaria.

BACKGROUND

Malaria-related mortality has fallen by 60% in Africa since 2000 [1]. One of the cornerstone strategies of malaria control programs is the use of RDTs instead of traditional microscopy for diagnosis. From 2008 to 2014, annual sales of RDTs increased from 46 million to 314 million units, representing a $151 million annual investment funded largely by multilateral and bilateral donors [2]. In 2014, RDTs accounted for 71% of all diagnostic testing employed to evaluate suspected malaria cases in Africa [1]. In the Democratic Republic of the Congo (DRC) and throughout Africa, RDTs have become the primary mode of malaria diagnosis.

Most widely used RDTs rely on the detection of histidine-rich protein 2 (HRP2), an antigen specific to Plasmodium falciparum. HRP2-based RDTs are known to generate false-positive results in the setting of persistent circulating HRP2 antigen after antimalarial treatment and false-negative results in individuals with low levels of parasitemia beneath the assay’s threshold for detection, typically around 200 parasites/µL for most commercially available RDTs [2–4]. Recently, however, false-negative results have been reported in individuals infected with P. falciparum parasites harboring a deletion of the pfhrp2 gene [5–15]. Several of these reports also identified co-existing deletions of the P. falciparum histidine-rich protein 3 (pfhrp3) gene, which produces an antigen that exhibits some cross-reactivity with select HRP2-based RDTs. Understanding the distribution and evolution of these mutant parasites is a priority for the World Health Organization, which recently hosted a Technical Consultation on P. falciparum hrp2/3 gene deletions and drafted interim guidance for investigating false-negative RDTs [16, 17]. It is unknown whether reliance on HRP2-based RDTs to guide treatment is exerting evolutionary pressure favoring the spread of this mutation.
Such a finding could have significant implications for malaria control and elimination efforts in Africa. To date, pfhrp2-deleted mutant parasites have not been investigated using country-wide population-based surveys. Here, we use samples from a large, nationally representative cross-sectional study of children younger than 5 years of age in the DRC to determine the prevalence, spatial distribution, and population genetics of these deletions.

**METHODS**

**Study Population**

Samples were collected from children under 5 years of age as part of the 2013–2014 DRC Demographic and Health Survey (DHS) [18]. The majority of children were asymptomatic at the time of sample collection. As previously described, heel- or finger-prick blood from each participant was analyzed by light microscopy for parasites, applied to an RDT targeting the *P. falciparum* HRP2 antigen (SD BIOLINE Malaria Ag Pf., Standard Diagnostics, Gyeonggi-do, Republic of Korea), and used to prepare dried blood spots (DBS) [18, 19]. The SD BIOLINE Malaria Ag Pf. RDT is *P. falciparum*-specific and meets current procurement criteria recommended by the World Health Organization, with high panel detection scores of 95 and 99 (on a 100-point scale) at 200 and 2,000 parasites/µL, respectively [2]. Three diagnostic methods were performed for each child: RDT, microscopy, and polymerase chain reaction (PCR). After extracting DNA from the DBS using established methods [20], a real-time PCR assay with a limit of detection of 100 parasites/µL was employed to identify *P. falciparum* infection by amplifying a region of the single-copy *pfldh* gene as previously described [19]. This study was approved by the University of Kinshasa School of Public Health and the University of North Carolina Institutional Review Boards. Informed consent was obtained from a parent or responsible adult for all subjects.

**Detection of pfhrp2 Deletions**

We performed PCR to identify the presence or absence of *pfhrp2* deletions among the subset of *P. falciparum* parasites with RDT-/PCR+ results. We included both microscopy-positive and -negative samples because excluding samples with insufficient parasite density for microscopic diagnosis could lead to an underestimate of the true *pfhrp2*-deleted parasite prevalence. We employed PCR assays targeting *pfhrp2* exon 1 and exon 2, using both 72°C and 60°C elongation temperatures based on reports of improved PCR sensitivity for AT-rich targets using lower elongation temperatures [21]. Additionally, we tested RDT+/PCR+ samples from select provinces to evaluate the occurrence of RDT-positivity due to HRP3 alone (expressed by *pfhrp2*-deleted parasites with an intact *pfhrp3* gene). PCR methods are described in the Supplementary Material.

**Confirmation of *P. falciparum* Infection**

For the subset of samples in which PCR assays failed to amplify either region of the *pfhrp2* gene, we performed additional PCR assays targeting other single-copy genes to confirm that sufficient *P. falciparum* DNA persisted in each sample for amplification, including the *pfhrp3* gene as described below and/or the *P. falciparum* beta tubulin (*Pf β-tubulin*) gene (see Supplementary Materials). Samples with successful amplification of *pfhrp3* or *Pf β-tubulin* were deemed to contain *pfhrp2*-deleted *P. falciparum*. The presence of amplifiable DNA was further confirmed for a subset of samples using PCR assays for multiple flanking microsatellite loci as described below.

**Detection of Coexisting *pfhrp3* Deletions**

We performed PCR assays to detect co-existing complete *pfhrp3* deletions among *pfhrp2*-deleted *P. falciparum* parasites. We employed assays targeting *pfhrp3* exon 2, followed by assays for exon 1 as described in the Supplementary Material. Samples with negative results for these assays were deemed to contain coexisting *pfhrp2* and *pfhrp3* deletions.

**Prevalence and Geographical Distribution of *pfhrp2*-Deleted *P. falciparum***

We calculated the prevalence of *P. falciparum* infections among children younger than 5 years of age, using PCR-positive infections (determined previously by the initial real-time PCR assay targeting *pfldh*) as the numerator and the total number tested as the denominator. We calculated the proportion of *pfhrp2*-deleted *P. falciparum* infections overall and by province, using *pfhrp2* deletions as the numerator and total PCR-positive *P. falciparum* cases as the denominator. All calculations utilized sample weights generated by the DHS to account for complex sampling design. Locations of *pfhrp2*-deleted isolates were mapped using R software (R Core Team, Vienna, Austria) and assessed for clustering by province using a Bayesian clustering technique as previously described [22]. We allowed for 20% of the study population to be included in the scanning window, consistent with other scan-based cluster methods. For the prior distributions for the null and non-null configurations, we specified conjugate gamma priors, such that null relative risks ranged between 0.95 and 1.05, and non-null relative risks ranged between 0 and 4 with 95% probability. Finally, we specified a prior probability of no clusters as 0.95 (consistent with a null hypothesis of no clustering). See Supplementary Material for details.

**Microsatellite Analysis**

In order to explore whether evolutionary selective pressure favoring *pfhrp2*-deleted parasites was present (e.g. selective treatment based on HRP2-based RDT results), we performed additional testing on a subset of *pfhrp2*-deleted parasites. We chose to test all *pfhrp2*-deleted parasites identified in Kinshasa, North Kivu, and South Kivu Provinces. Additionally, an equal number of randomly
selected, geographically matched HRP2-positive control samples were included in our testing to achieve a 1:1 ratio of deleted to control samples in each province, resulting in a frequency-matched sample that was analyzed without regard to individually matched pairs. We performed PCR to amplify six previously described microsatellite regions flanking the pfhrp2 gene and employed capillary electrophoresis (Eton Biosciences, San Diego, CA) to analyze the amplicons [6]. Primers and reaction conditions are described in Supplementary Table 1. Capillary electrophoresis chromatograms were visualized and scored for amplicon length using GeneMapper v. 4.1 (Applied Biosystems, Foster City, CA). We considered peaks with an intensity of less than 200 relative fluorescent units (RFU) as background signal and analyzed only the dominant peak at each locus. We adjusted amplicon lengths according to the difference between P. falciparum 3D7 controls and their theoretical fragment lengths from the consensus 3D7 (v3) sequence in PlasmoDB [23].

The adjusted amplicon lengths were then used to create haplotypes for each sample consisting of each of the six microsatellite loci. We defined haplotypes in the form of repeats per microsatellite locus rather than fragment lengths. We calculated the number of repeats in each amplicon by dividing the difference between our adjusted amplicon length and the minimum theoretical amplicon length, assuming zero repeats, by the repeat unit length. Only the dominant haplotype for each sample was included in population genetic analyses.

### Table 1. Characteristics of Children Infected With P. falciparum

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All subjects</th>
<th>All P. falciparum infections*</th>
<th>pfhrp2-deleted P. falciparum</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (weighted prevalence [95% CI])</td>
<td>7,137</td>
<td>2,752 (34.8 [32.3 – 37.4])</td>
<td>149 (6.4 [5.1 – 8.0])</td>
<td>—</td>
</tr>
<tr>
<td>Age in months, median (IQR)</td>
<td>32 (19 – 46)</td>
<td>36 (23 – 48)</td>
<td>31 (14 – 45)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Female sex, n (weighted proportion)</td>
<td>3,568 (49.9)</td>
<td>1,340 (49.0)</td>
<td>67 (48.4)</td>
<td>0.92</td>
</tr>
<tr>
<td>Microscopy positive, n (weighted proportion)</td>
<td>1,695 (58.7)</td>
<td>18 (9.7)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Fever in last 2 weeks, n (weighted proportion)**A</td>
<td>2,094 (0.32)</td>
<td>934 (39.8)</td>
<td>27 (18.5)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Province, n (weighted prevalence [95% CI])</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinshasa</td>
<td>362</td>
<td>92 (25.6 [19.9,32.1])</td>
<td>20 (21.9 [13.9,32.8])</td>
<td>+</td>
</tr>
<tr>
<td>Kwango</td>
<td>308</td>
<td>67 (21.9 [13.3,27.4])</td>
<td>9 (12.8 [5.4,27.4])</td>
<td>–</td>
</tr>
<tr>
<td>Kivu</td>
<td>360</td>
<td>70 (18.9 [14.0,24.9])</td>
<td>6 (3.0 [1.1,8.2])</td>
<td>–</td>
</tr>
<tr>
<td>Mai-Ndombe</td>
<td>272</td>
<td>116 (44.0 [31.9,56.8])</td>
<td>4 (9.6 [2.6,29.6])</td>
<td>–</td>
</tr>
<tr>
<td>Kongo Central</td>
<td>321</td>
<td>148 (43.1 [31.6,55.4])</td>
<td>6 (4.2 [1.5,11.7])</td>
<td>–</td>
</tr>
<tr>
<td>Equateur</td>
<td>198</td>
<td>57 (23.6 [11.4,42.5])</td>
<td>3 (2.8 [0.4,18.7])</td>
<td>–</td>
</tr>
<tr>
<td>Mongala</td>
<td>255</td>
<td>74 (28.3 [14.6,47.5])</td>
<td>5 (4.4 [0.9,19.0])</td>
<td>–</td>
</tr>
<tr>
<td>Nord-Ubangi</td>
<td>231</td>
<td>143 (61.5 [46.7,76.4])</td>
<td>5 (5.3 [1.7,15.9])</td>
<td>–</td>
</tr>
<tr>
<td>Sud-Ubangi</td>
<td>276</td>
<td>86 (29.5 [23.6,36.2])</td>
<td>7 (12.4 [5.4,26.1])</td>
<td>–</td>
</tr>
<tr>
<td>Tshuapa</td>
<td>205</td>
<td>56 (26.3 [16.4,39.5])</td>
<td>6 (8.1 [2.2,22.1])</td>
<td>–</td>
</tr>
<tr>
<td>Kasai</td>
<td>310</td>
<td>118 (34.5 [20.9,51.2])</td>
<td>2 (1.3 [0.3,5.1])</td>
<td>–</td>
</tr>
<tr>
<td>Kasai-Central</td>
<td>327</td>
<td>155 (50.9 [42.1,59.7])</td>
<td>3 (4.3 [1.2,9.2])</td>
<td>–</td>
</tr>
<tr>
<td>Kasai-Oriental</td>
<td>255</td>
<td>106 (42.5 [22.5,52.6])</td>
<td>3 (9.2 [0.9,11.1])</td>
<td>–</td>
</tr>
<tr>
<td>Lomami</td>
<td>351</td>
<td>221 (63.6 [52.7,77.3])</td>
<td>5 (1.8 [0.5,5.9])</td>
<td>–</td>
</tr>
<tr>
<td>Sankuru</td>
<td>194</td>
<td>68 (26.4 [12.2,48.0])</td>
<td>6 (18.4 [5.6,33.8])</td>
<td>–</td>
</tr>
<tr>
<td>Haut-Katanga</td>
<td>236</td>
<td>81 (31.7 [19.1,47.8])</td>
<td>3 (1.5 [0.1,10.5])</td>
<td>–</td>
</tr>
<tr>
<td>Haut-Lomami</td>
<td>261</td>
<td>99 (42.8 [34.0,52.9])</td>
<td>3 (1.7 [1.0,12.5])</td>
<td>–</td>
</tr>
<tr>
<td>Lualaba</td>
<td>176</td>
<td>87 (44.9 [30.8,59.9])</td>
<td>2 (1.5 [1.1,25.5])</td>
<td>–</td>
</tr>
<tr>
<td>Tanganika</td>
<td>245</td>
<td>157 (61.7 [45.1,75.9])</td>
<td>3 (1.7 [0.3,9.5])</td>
<td>–</td>
</tr>
<tr>
<td>Maniema</td>
<td>349</td>
<td>182 (50.6 [38.0,63.1])</td>
<td>13 (4.6 [2.1,10.0])</td>
<td>–</td>
</tr>
<tr>
<td>Nord-Kivu</td>
<td>426</td>
<td>72 (12.2 [8.5,17.3])</td>
<td>12 (5.5 [2.7,29.4])</td>
<td>+</td>
</tr>
<tr>
<td>Bas-Uele</td>
<td>180</td>
<td>109 (68.2 [54.9,79.9])</td>
<td>1 (0.2 [0.1,1.8])</td>
<td>–</td>
</tr>
<tr>
<td>Haut-Uele</td>
<td>170</td>
<td>87 (57.5 [46.6,67.7])</td>
<td>0 (0.0 [NA])</td>
<td>–</td>
</tr>
<tr>
<td>Ituri</td>
<td>255</td>
<td>122 (43.7 [30.6,57.8])</td>
<td>9 (11.5 [6.4,19.7])</td>
<td>+</td>
</tr>
<tr>
<td>Tshopo</td>
<td>225</td>
<td>106 (45.3 [36.3,54.6])</td>
<td>6 (4.5 [1.5,12.3])</td>
<td>–</td>
</tr>
<tr>
<td>Sud-Kivu</td>
<td>389</td>
<td>73 (14.2 [9.8,20.0])</td>
<td>8 (18.3 [7.1,39.9])</td>
<td>+</td>
</tr>
</tbody>
</table>

* Diagnosed by real-time PCR targeting the pfldh gene during initial screening, as previously described [19].

** Comparing pfhrp2-deleted to non-deleted P. falciparum.

**Fever in the last 2 weeks based on survey response.

+ Provinces with geographical clustering of pfhrp2-deleted P. falciparum by Bayesian spatial cluster analysis.

Abbreviations: CI, confidence interval; IQR, interquartile range; NA, not applicable.
Population Genetic Analysis

The unbiased expected heterozygosity by locus for pfhrp2-deleted and non-deleted parasites was calculated using GenAlEx version 6.5 and plotted in R [24, 25]. To visualize potential relationships between individual parasites, we generated a matrix of pairwise genetic distances between parasites using GenAlEx for haploid single-sequence repeats, with missing data interpolated by inserting the average genetic distances for each population-level pairwise contrast [24, 25]. We then created a neighbor-joining tree using the APE package in R and visualized the tree using Cytoscape [26, 27].

To explore genetic differentiation between parasite populations, we calculated the observed G_{ST} for pfhrp2-deleted parasites versus controls overall and by province. G_{ST}, a measure of genetic distance between populations that can incorporate multiple genetic markers, was calculated using the formula of Nei at each locus before averaging over all loci [28]. We used permutation testing to determine the statistical significance of observed G_{ST} values; population labels (pfhrp2-deletion vs. positive control) were permuted 99,999 times and the position of the observed value in this ordered list was converted to an empirical p-value.

Data Analysis

We made comparisons using Fisher’s exact test for categorical variables, the Wilcoxon rank-sum test for non-normally distributed continuous variables, and the t-test for normally distributed continuous variables. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC) and R software.

RESULTS

Prevalence and Geographical Distribution of pfhrp2-Deleted P. falciparum

We identified 149 P. falciparum isolates with a deletion of the pfhrp2 gene, representing a country-wide prevalence of 6.4% (95% CI 5.1 – 8.0) among the 2,752 children diagnosed with P. falciparum infection by real-time PCR and 19.0% of the 783 RDT-/PCR+ parasites tested. Figure 1 and Supplementary Figure 1 outline the outcome of PCR assays employed to detect deletions of the pfhrp2 gene and to confirm the presence of P. falciparum DNA. Of the 149 pfhrp2-deleted P. falciparum isolates, only 5 (3.4%) had co-existing complete pfhrp3 deletions (Supplementary Figure 1). We did not identify any pfhrp2 deletions among the 107 RDT+/PCR+ parasites detected in children from Kinshasa, North Kivu, or South Kivu Provinces. The characteristics of children diagnosed with P. falciparum infection by PCR and the subset with pfhrp2-deleted P. falciparum are described in Table 1. We include previously published results of the initial P. falciparum ldh (pfldh) real-time PCR for reference. [18, 19] Pfhrp2-deleted parasites had lower parasite densities than wild-type parasites (p < .0001), with mean pfldh cycle threshold values of 36.1 (standard deviation [SD] 3.2) and 32.7 (SD 3.6), respectively. Children infected with pfhrp2-deleted P. falciparum were significantly younger than those infected with wild-type parasites (p = .0007) and more likely to be microscopy-negative (p < .0001) and afebrile (p = .0004). There was no significant difference in gender between the groups (p = .92).

Pfhrp2-deleted parasites were present in nearly all provinces but were significantly clustered in the provinces surrounding the capital Kinshasa in the southwest and Lake Kivu in the northeast (Table 1 and Figure 2). They were especially common in Kinshasa, where they were responsible for more than one of every five P. falciparum infections. A Bayesian spatial cluster analysis supported significant geographical clustering of pfhrp2-deleted parasites in Kinshasa Province (posterior probability of cluster membership = 0.99) and three provinces in the northeast near Lake Kivu (posterior probabilities of 0.69 in South Kivu, 0.70 in North Kivu, and 0.69 in Ituri Provinces [Supplementary Figure 2]). A sensitivity analysis using different prior probabilities for no clustering (0.50) and different scanning windows (up to 50% of the study population) did not change the results meaningfully (Supplementary Figures 3 and 4, Supplementary Material). The pattern of deletions (i.e. complete deletion of the gene, partial deletion of exon 1, or partial deletion of exon 2) is depicted in Supplementary Figure 5. Because spatial clustering suggests the possibility of person-to-person spread and/or expansion of the mutant parasite population, we attempted to obtain genetic evidence for spread and selective pressures using microsatellite analyses.

Genetic Origins of pfhrp2-Deleted P. falciparum

Failure to amplify microsatellite loci 1.4kb and 2.5kb downstream in pfhrp2-deleted parasites suggests that the predominant gene deletion pattern spans at least several kilobases and includes regions downstream from the pfhrp2 gene (Figure 3). This pattern of amplification failure was most common
in pfhrp2-deleted parasites from Kinshasa, but it was also observed in a subset of parasites from North and South Kivu. These findings, in conjunction with the distribution of partial versus complete gene deletions (Supplementary Figure 5), suggest that there are multiple deletion genotypes circulating in the DRC.

We did not observe clear evidence of a selective sweep (Figure 3), although there appeared to be loss of heterozygosity at a single microsatellite locus 15kb downstream from the pfhrp2 gene (unbiased expected heterozygosity of 0.66 among pfhrp2-deleted parasites and 0.93 among controls). Amplification failure among pfhrp2-deleted parasites prevented similar analyses at the two proximal downstream loci.

Initial analysis of haplotypes using an unrooted neighbor-joining tree revealed three distinct clusters of pfhrp2-deleted parasites, one containing primarily isolates from Kinshasa Province, another containing a mixture of isolates from North and South Kivu, and a third containing a mixture of isolates from all three provinces (Figure 4). Because analysis of individual loci did not provide sufficient discriminatory power to distinguish parasite populations (Supplementary Table 2), we explored the genetic relatedness of pfhrp2-deleted parasites and non-deleted controls using a permutation test based on the statistic $G_{ST}$. This analysis revealed significant genetic differentiation between the two populations ($G_{ST} = .046, p \leq .00001$, Figure 5). Inclusion of the parasites' geographical origins in the analysis confirmed significant differentiation between pfhrp2-deleted and non-deleted parasites in both Kinshasa ($G_{ST} = .079, p \leq .00012$) and Kivu ($G_{ST} = .06, p \leq .0004$).

**Relationship Between pfhrp2-Deleted Mutants and P. falciparum Prevalence**

P. falciparum infections are presumed to be more polyclonal in areas of higher prevalence [29, 30]. Accordingly, coinfections with a more diverse population of wild-type parasites could increase the probability of a positive RDT and mask the presence of pfhrp2-deleted parasites. We observed a decline in the frequency of presumed monoclonal infections by parasites harboring a pfhrp2 deletion at higher parasite prevalence, consistent with this hypothesis (Figure 6 and Supplementary Material).
DISCUSSION

This is the first nationally representative study to demonstrate the presence and estimate the prevalence of malaria caused by \( pfhrp2 \)-deleted \( P. falciparum \) in asymptomatic children. Our findings suggest that nearly one of every 15 children with \( P. falciparum \) malaria in the DRC are infected by a \( pfhrp2 \)-deleted mutant. Because most RDTs employed in the DRC are HRP2-based, they will fail to detect these parasites.

Several provinces had a markedly higher prevalence of \( pfhrp2 \)-deleted mutants. The high prevalence observed in Kivu and Kinshasa is especially concerning because RDTs appear to have been introduced in these regions prior to introduction elsewhere. HRP2-based RDTs have been deployed in humanitarian interventions in Kivu since at least 2005, whereas they were deployed in most remaining regions in approximately 2011 [31–33]. In Kinshasa, HRP2-based RDTs were deployed on a limited basis by at least 2009 and their availability increased 10-fold by 2013 [34]. Geographic and genetic clustering of \( pfhrp2 \)-deleted mutants raises concern for evolutionary selection in the setting of selective treatment of RDT-positive \( P. falciparum \) parasites.

There are several lines of evidence confirming that our inability to detect the \( pfhrp2 \) gene was not simply due to insufficient DNA, an important consideration in this type of study [35]. The presence of amplifiable DNA in \( pfhrp2 \)-deleted samples was confirmed by PCR assays for multiple single-copy genes (\( pfldh \), \( pfhrp3 \), and/or \( Pf \beta\)-tubulin) and, for a subset, six microsatellite loci flanking the \( pfhrp2 \) gene. We successfully amplified \( pfhrp3 \) in 96.1% of \( pfhrp2 \)-deleted \( P. falciparum \) isolates with sufficient sample remaining for testing and multiple flanking microsatellite loci in all \( pfhrp2 \)-deleted parasites included in the population genetic analysis. For those considering similar analyses, useful recommendations for streamlining the process of identifying \( pfhrp2 \)-deleted \( P. falciparum \) are now available [36].

Figure 4. Neighbor joining tree. Unrooted neighbor joining tree depicting the relationship between dominant haplotypes of \( pfhrp2 \)-deleted \( P. falciparum \) isolates (yellow) and HRP2-positive controls (gray). Shapes indicate the geographical origin of each isolate: Kinshasa (circle), North Kivu (triangle), or South Kivu (diamond).
Only a small fraction (3.4%) of pfhrp2-deleted parasites had coexisting pfhrp3 deletions, a finding that contrasts with reports from South America [36]. Because pfhrp3 produces an antigen (HRP3) that shares epitopes with HRP2, pfhrp2-deleted parasites with an intact pfhrp3 gene can, in theory, trigger a positive HRP2-based RDT. This phenomenon may afford select RDTs a “failsafe” against pfhrp2-deleted parasites. By examining RDT-/PCR+ parasites, it is possible that we may have underestimated the true prevalence of pfhrp2-deleted parasites. However, we expect this effect to be small given that we did not detect any additional pfhrp2-deleted parasites during testing of RDT+/PCR+ parasites from Kinshasa, North Kivu, and South Kivu Provinces.

We observed a notable improvement in our ability to amplify pfhrp2 exon 1/2 using a lower extension temperature during PCR cycling. This finding likely reflects impaired Taq polymerase extension of the AT-rich intron at higher temperatures due to DNA melting [21]. Future assays employed to detect pfhrp2-deleted parasites, especially those that target the AT-rich intron between exons 1 and 2, should be tested at lower extension temperatures to avoid unintentional misclassification based on false-negative PCR results.

Population genetic analyses of flanking microsatellites confirmed that parasites with a pfhrp2 deletion were genetically distinct from wild-type parasites in both Kinshasa and Kivu. While we did not observe evidence of a selective sweep, the loss of heterozygosity observed at a single locus downstream raises the possibility of a “soft selective sweep” in the population, with multiple genetic origins of the deletion due to multiple breakpoints. Soft selective sweeps have been observed in analyses of pfmdr1 duplication, implicated in antimalarial drug resistance, in Southeast Asia [37]. Additionally, recent analyses of beneficial copy number variation mutations in P. falciparum did not reveal the typical long flanking haplotypes required to produce “hard” selective sweeps, which are usually observed around point mutations [38].

Although we cannot draw definitive conclusions about whether selective pressure is responsible for the pfhrp2 deletion, the ability to evade detection by a widespread diagnostic test...
would provide significant evolutionary advantages for the *P. falciparum* parasite. Clonal selection of *pfhrp2*-deleted parasites could jeopardize recent gains in malaria control, already threatened by the rising tide of resistance to artemisinin-based combination therapies in Southeast Asia and to insecticides [1, 39].

*Pfhrp2*-deleted parasites may be easier to detect in areas of low transmission intensity because polyclonal infections are less likely to occur—i.e. coinfection with wild-type parasites could trigger a positive RDT and mask the presence of a *pfhrp2*-deleted parasite [36]. The inverse relationship we observed between the prevalence of *pfhrp2*-deleted mutants and overall *P. falciparum* prevalence is consistent with this hypothesis. If correct, this hypothesis implies that RDT failure due to *pfhrp2* deletions will become more common as transmission declines.

There are several limitations to this study. First, our ability to explore clinical differences between *pfhrp2*-deleted and wild-type *P. falciparum* malaria is restricted by limited clinical data. Ongoing studies of symptomatic malaria in the DRC may provide valuable insights into the clinical manifestations of infection with *pfhrp2*-deleted parasites. In the present study, however, subjects were enrolled in a large, country-wide survey and not selected based on symptoms of malaria. As a result, we cannot draw conclusions about the relative virulence of *pfhrp2*-deleted parasites. While the lower parasite densities by real-time PCR and the lower proportion of microscopy-positivity and fever among *pfhrp2*-deleted *P. falciparum* cases (Table 1) may simply reflect our ability to detect deletions in less complex infections as noted above, these findings also raise the possibility that there may be a fitness cost to the parasite. Our finding that children infected with *pfhrp2*-deleted parasites were younger than those infected with wild-type parasites could reflect the immature immune system of younger children, who may mount a weaker response to these less fit parasites. The function of *pfhrp2* is not well understood, but detoxification of heme and other possibilities have been suggested [40]. While parasite clones containing the *pfhrp3* gene were favored over *pfhrp3*-deleted clones in a genetic cross, the same has not been demonstrated for *pfhrp2* in vitro [41].

Second, our study was restricted to the DRC. While this may raise questions about generalizability, the DRC is a large and geographically diverse country that borders nine other countries in East, West, and Southern Africa, suggesting that our findings have relevance throughout the region.

Independent of concerns about evolutionary selection, our findings represent the first report of geographical clustering of *pfhrp2*-deleted *P. falciparum* parasites in Africa, where HRP2-based RDT use is most common [1]. Malaria is widespread in the DRC; at least 34.8 percent of the children in this cohort were infected with *P. falciparum* parasites. These and other infected African children bear the brunt of malaria’s heavy toll. Rapid and accurate diagnosis of *P. falciparum* infection, especially among clinically ill patients presenting for evaluation and treatment, is essential for curbing its effects. RDTs that detect antigens other than HRP2 such as lactate dehydrogenase (LDH) and aldolase are commercially available, but they are not widely utilized in sub-Saharan Africa due to concerns about heat stability and inferior *P. falciparum* sensitivity, higher costs, and limited global availability compared to HRP2-based RDTs [42, 43]. Multilateral donors currently invest hundreds of millions of dollars in the procurement and distribution of HRP2-based RDTs. Our findings underscore the need for surveillance of *pfhrp2*-deleted mutants and suggest that alternative diagnostics need to be considered in areas where these deletions are common. Use of RDTs that detect multiple antigens (e.g. HRP2 and LDH) or novel *P. falciparum*-specific antigens may be required.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

**Notes**

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