Am. J. Trop. Med. Hyg., 109(2), 2023, pp. 273–276
doi:10.4269/ajtmh.22-0715

Plasmodium falciparum with *pfhrp2/3* Deletion Not Detected in a 2018–2021 Malaria Longitudinal Cohort Study in Kinshasa Province, Democratic Republic of the Congo

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Abstract. Histidine-rich protein 2– (HRP2-) based rapid diagnostic tests (RDTs) are widely used to detect *Plasmodium falciparum* in sub-Saharan Africa. Reports of parasites with *pfhrp2* and/or *pfhrp3* (*pfhrp2/3)* gene deletions in Africa raise concerns about the long-term viability of HRP2-based RDTs. We evaluated changes in *pfhrp2/3* deletion prevalence over time using a 2018–2021 longitudinal study of 1,635 enrolled individuals in Kinshasa Province, Democratic Republic of the Congo (DRC). Samples collected during biannual household visits with ≥ 100 parasites/μL by quantitative real-time polymerase chain reaction were genotyped using a multiplex real-time PCR assay. Among 2,726 *P. falciparum* PCR-positive samples collected from 993 participants during the study period, 1,267 (46.5%) were genotyped. No *pfhrp2/3* deletions or mixed *pfhrp2/3*-intact and -deleted infections were identified in our study. *Pfhrp2/3*-deleted parasites were not detected in Kinshasa Province; ongoing use of HRP2-based RDTs is appropriate.

INTRODUCTION

Progress toward malaria control and elimination in Africa requires prompt diagnosis and treatment with effective antimalarial drugs. Rapid diagnostic tests (RDTs) are widely used to identify individuals infected with *Plasmodium*; their deployment has enabled significant improvements in malaria diagnostic testing across the continent over the past decade.[1,2] The majority of RDTs used to diagnose falciparum malaria in sub-Saharan Africa detect *Plasmodium falciparum* histidine-rich protein 2 (HRP2) and its paralog HRP3, encoded by the *pfhrp2* and *pfhrp3* genes, respectively.[1] Histidine-rich protein 2–based RDTs are generally more sensitive and heat stable than RDTs detecting other antigens.[3] However, test-and-treat strategies that rely upon HRP2-based RDTs are threatened by the emergence of *P. falciparum* strains that escape detection owing to deletion of the *pfhrp2* and/or *pfhrp3 (pfhrp2/3)* genes.[1,4] High prevalence of *pfhrp2/3*-deleted parasites in Eritrea,[5] Ethiopia,[6,7] Djibouti,[8,9] and surrounding countries recently prompted changes in malaria diagnostic testing policies. Reports from sub-Saharan countries outside of the Horn of Africa, however, indicate lower prevalence.[10]

The Democratic Republic of the Congo (DRC) has one of the highest malaria burdens in the world, accounting for 12% of global malaria cases and deaths.[1] In the DRC, we previously reported 6.4% *pfhrp2* deletion prevalence in samples from a 2013–2014 nationally representative cross-sectional survey of asymptomatic children under 5 years of age.[11] However, no *pfhrp2/3* deletions were observed in our 2017 cross-sectional study of symptomatic children and adults across three DRC provinces.[12] These studies were both cross-sectional and did not provide information about how the prevalence of deletions may be changing over time. This study aimed to estimate *pfhrp2/3* deletion prevalence and changes over time in Kinshasa Province, the most populous province in the DRC.

MATERIALS AND METHODS

This study includes samples collected as part of a 2018–2021 longitudinal study of malaria conducted at seven sites across Kinshasa Province, DRC. A total of 1,635 participants were enrolled in one urban neighborhood, three peri-urban villages, and three rural villages (Figure 1). These study sites experience varying malaria endemicities, reflective of the heterogeneity in malaria transmission observed in the DRC and other sub-Saharan African countries. Study visits were conducted as part of twice-yearly household surveys in the dry and rainy seasons (active surveillance) and as part of routine care at local health centers (passive surveillance) as previously described.[13,14] At each visit, a comprehensive questionnaire on malaria symptoms and treatment and bed net usage was administered. A finger- or heel-prick sample was obtained at each visit for RDT (SD Bioline Ag P.f./Pan RDT [05FK60], Alere, Gyeonggi-do, Republic of Korea, or CareStart Malaria Pf HRP2 Ag [02571], Access Bio, Somerset, NJ) and dried blood spot (DBS) preparation for future molecular investigation. Rapid diagnostic test–positive patients were treated according to national guidelines.

DNA was extracted from DBS using Chelex-100 (Sigma-Aldrich, St Louis, MO) and saponin or Tween (Bio-Rad Laboratories, Hercules, CA) as previously described.[15,16] Quantitative real-time polymerase chain reaction (qPCR) targeting the *P. falciparum lactate dehydrogenase* (*pfldh*) gene was used to estimate *P. falciparum* parasitemia, using serial dilutions of DNA extracted from a mock DBS made with cultured *P. falciparum* 3D7 or FCR3 strain parasites at known parasite density. Samples with ≥ 100 parasites/μL were selected for *pfhrp2/3* deletion identification using a multiplex real-time PCR assay that detects *pfldh*, *pfhrp2*, *pfhrp3*, and human *beta-tubulin* (*HumTuBB*).[17] We used this parasite density threshold to reduce the risk of unintentional misclassification of deletions in the setting of low DNA concentrations.[18,19] Positive calls required cycle threshold (C_t) values < 35. Samples positive for *HumTuBB* and

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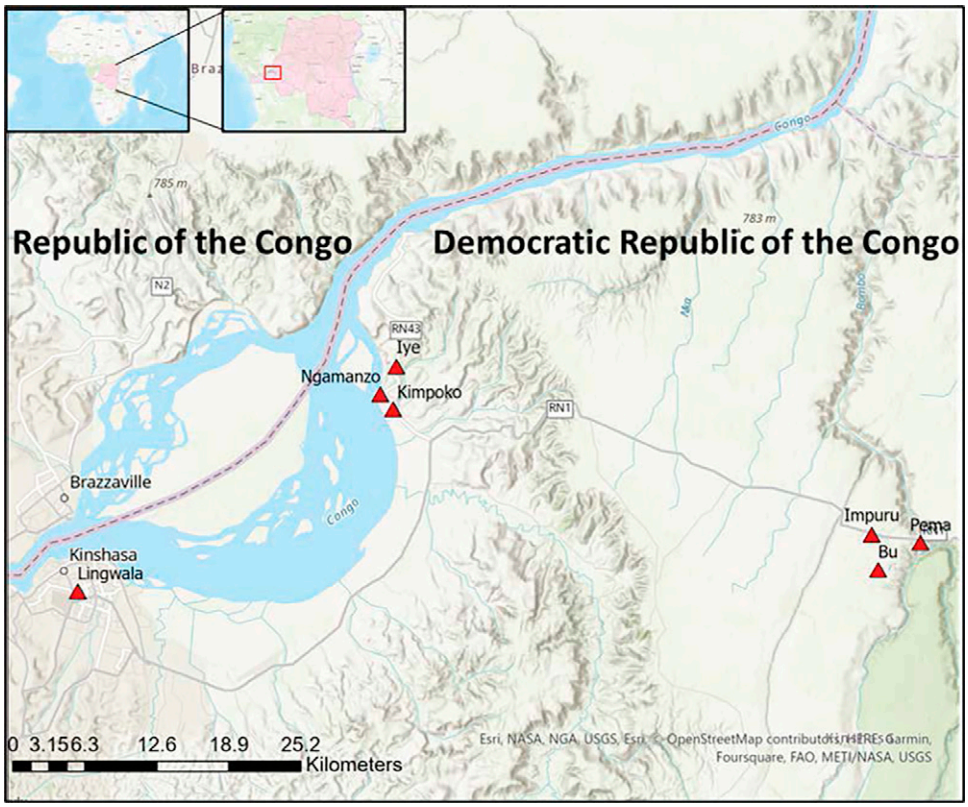

FIGURE 1. Study sites in Kinshasa Province, Democratic Republic of the Congo.

pfldh but negative for *pfhrp2* or *pfhrp3* were subjected to a confirmatory real-time PCR targeting the *P. falciparum beta-tubulin* (*PfBtubulin*) gene. Deletion calls were limited to samples positive for *HumTuBB* and both single-copy *P. falciparum* genes (*pfldh* and *pftubulin*), but negative for *pfhrp2* and/or *pfhrp3*. Mixed infections of *pfhrp2/3*-intact and -deleted strains were defined conservatively as samples in which (*pfhrp2* C_t − *pfldh* C_t) > 3 or (*pfhrp3* C_t − *pfldh* C_t) > 3.[17] All assays included *P. falciparum* 3D7, DD2, and HB3 strain DNA as wildtype, *pfhrp2*-deleted, and *pfhrp3*-deleted controls, respectively. All participants provided informed assent/consent. Ethical approval for this study was granted by the Institutional Review Boards of the University of North Carolina-Chapel Hill and the Kinshasa School of Public Health.

RESULTS

A total of 1,267 samples collected from 649 individuals in 179 households between 2018 and 2021 were included in this study (Figure 2). Among these, the median number of *P. falciparum* infections with ≥ 100 parasites/μL detected per participant was 2.0 (interquartile range [IQR]: 1–3). The median age at enrollment in 2018 was 9 years (IQR: 5–15 years of age); 48.8% reported female gender. At the time of enrollment, 48.7% reported malaria in the preceding 6 months, and 42.7% of those reported more than one episode in that 6-month period. The median household size was 8 (IQR: 6–10), with high bed net coverage across the study population (90.2%). The baseline characteristics of the study population are summarized in Table 1.

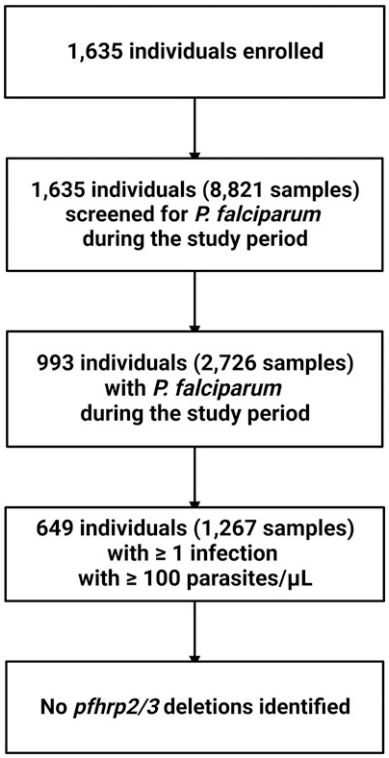

FIGURE 2. Flow diagram for the detection of *pfhrp2/3* deletion in 1,267 samples from 649 participants during household visits of the longitudinal study (2018–2021) in Kinshasa Province, Democratic Republic of the Congo.

TABLE 1
Baseline characteristics of the study population included in this analysis

	Participants ($N = 649$)
Age in years, median (IQR)	9 (5–15)
Age strata, *n* (%)	
< 1 year	15 (2.3)
1–5 years	166 (25.6)
6–10 years	191 (29.5)
11–15 years	119 (18.4)
16–25 years	76 (11.7)
> 25 years	81 (12.5)
Sex, *n* (%)	
Female	317 (48.8)
Male	332 (51.2)
Malaria in previous 6 months, *n* (%)	
No	311 (51.3)
Yes–once	169 (27.9)
Yes–many	126 (20.8)
Slept under bed net previous night, *n* (%)	
No	47 (9.8)
Yes	432 (90.2)
Household size, median (IQR)	8 (6–10)
Site of residence, *n* (%)	
Bu (rural)	87 (13.4)
Impuru (rural)	123 (19.0)
Pema (rural)	121 (18.6)
Ngamanzo (peri-urban)	108 (16.6)
Iye (peri-urban)	60 (9.2)
Kimpoko (peri-urban)	122 (18.8)
Lingwala (urban)	28 (4.3)
Number of samples per participant, median (IQR)	2 (1–3)

IQR = interquartile range.
A total of 1,267 samples with ≥ *100 P. falciparum* parasites/μL from 649 participants during enrollment in the Kinshasa longitudinal study.

All 1,267 samples had detectable human DNA, as indicated by amplification of *HumTuBB* with $C_t < 35$. Multiplex PCR confirmed *P. falciparum* parasitemia in all but two samples that failed to amplify *pfldh*. Both *pfhrp2* and *pfhrp3* were negative in one sample ($C_t > 35$ for both gene targets). However, *pfldh* was negative in the multiplex assay and *Pftubulin* was negative in follow-up testing, indicating that the original *pfldh* qPCR result was a false-positive. Thus, this sample did not meet criteria to be considered a *pfhrp2/3*-deleted parasite. No mixed infections of *pfhrp2/3*-intact and -deleted strains were identified in our study population.

DISCUSSION

Overall, we did not find evidence of *pfhrp2/3* deletion in parasites sampled during our longitudinal cohort study in Kinshasa Province. Our conservative approach to calling *pfhrp2/3* deletions prevented us from detecting low-density infections and thus could underestimate the true prevalence of these strains. These study results differ from our previous DRC *pfhrp2/3* deletion prevalence estimates; this discrepancy could be attributed to spatial heterogeneity in prevalence or incorrect deletion calls in low-density samples during our previous study. The multiplex real-time PCR assay and conservative calling approach used here reduced the risk of incorrect deletion calls. Our finding of no deletions is in line with our more recent study of symptomatic individuals and a similar study of asymptomatic and symptomatic school-aged children that showed no to little evidence of *pfhrp2/3* deletion in the DRC.[12,20] Together, these results support the continued use of HRP2-based RDTs for the diagnosis of malaria in the DRC.

Received November 16, 2022. Accepted for publication April 13, 2023.

Published online June 20, 2023.

Acknowledgments: We thank the study participants and field teams who conducted study visits. We also express our gratitude to the late Prof. Steven Meshnick for mentorship and his role in the longitudinal study upon which this analysis was based. The following reagents were obtained through BEI Resources, National Institute of Allergy and Infectious Diseases, NIH: Genomic DNA from *P. falciparum* strain 3D7, MRA-102G, contributed by Daniel J. Carucci; *P. falciparum* strain HB3, MRA-155G, contributed by Thomas E. Wellems; and *P. falciparum* strain Dd2, MRA-150G, contributed by David Walliker.

Financial support: This study was funded by grant no. R01AI132547 to J. J. J. and R. R. D., with partial support from grant nos. R01AI129812 to A. K. T., R01AI139520 to J. A. B., and a supplement to R. F., grant no. T32AI070114 to K. B., grant no. K24AI134990 to J. J. J., and an American Society of Tropical Medicine and Hygiene/Burroughs-Wellcome Fund award to J. B. P.

Disclosure: J. B. P. reports research support from Gilead Sciences, nonfinancial support from Abbott Laboratories, and consulting for Zymeron Corporation outside the scope of this manuscript.

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