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The Search for *Plasmodium falciparum* histidine–rich protein 2/3 Deletions in Zambia and Implications for *Plasmodium falciparum* histidine-rich protein 2-Based Rapid Diagnostic Tests

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Abstract. We attempted to identify *Plasmodium falciparum* histidine–rich protein 2/3 (*pfhrp2/3*) deletions among rapid diagnostic test (RDT)–negative but PCR- or microscopy-positive *P. falciparum*–infected individuals in areas of low transmission (Choma District, 2009–2011) and high transmission (Nchelenge District, 2015–2017) in Zambia. Through community-based surveys, 5,167 participants were screened at 1,147 households by *P. falciparum* histidine-rich protein 2 (PfHRP2)-based RDTs. Slides were made and dried blood spots were obtained for molecular analysis. Of 28 samples with detectable *P. falciparum* DNA, none from Nchelenge District were *pfhrp2/3* negative. All eight samples from Choma District had detectable *pfhrp3* genes, but *pfhrp2* was undetectable in three. DNA concentrations of *pfhrp2*-negative samples were low (< 0.001 ng/μL). These findings suggest that PfHRP2-based RDTs remain effective tools for malaria diagnosis in Nchelenge District, but further study is warranted to understand the potential for *pfhrp2/3* deletions in southern Zambia where malaria transmission declined over the past decade.

The availability of *Plasmodium falciparum* histidine-rich protein (PfHRP) 2–based rapid diagnostic tests (RDTs) has dramatically improved parasitological confirmation of suspected malaria cases in resource-limited settings. Recently, numerous studies reported *P. falciparum* parasites lacking *pfhrp2* and *pfhrp3* genes in Africa, including the Democratic Republic of the Congo, Eritrea, Ghana, Kenya, Mali, Mozambique, Rwanda, and Senegal.[1–3] The *pfhrp3* gene is highly homologous to *pfhrp2*,[4] and parasites lacking both *pfhrp2* and *pfhrp3* genes, or substantial parts of these genes, do not express functional proteins and are not detected by PfHRP2-based RDTs.[1]

In Zambia, the National Malaria Control Center introduced PfHRP2-based RDTs in 2005 and achieved national-level scale-up in 2009.[5] Zambia has made significant progress in reducing malaria transmission and aims to eliminate malaria by 2021. To achieve this goal, the use of PfHRP2-based RDTs has expanded beyond the diagnosis of suspected cases to also be used to screen individuals residing in proximity to symptomatic index cases through reactive case detection.[6] Despite the expanded use of PfHRP2-based RDTs, the extent of *pfhrp2/3* deletions in Zambia is unknown. We attempted to identify *pfhrp2/3* deletions using blood samples collected during community-based, active case detection in Choma and Nchelenge districts, Zambia.

Choma District in the Southern Province has seasonal malaria transmission, and PfHRP2-based RDTs were introduced in 2007.[7] As malaria transmission has declined, Choma District is considered a pre-elimination setting (malaria prevalence by RDT < 1%).[8,9] By contrast, malaria transmission in Nchelenge District, Luapula Province, is high with little seasonal fluctuation. Malaria control interventions have been only modestly effective and malaria prevalence by RDT is approximately 50%.[9,10] Luapula Province is one of the four focus provinces where the National Malaria Elimination Program supports additional malaria control efforts, including school-based bed net distribution, expansion of community case management, and training of health providers in the management of severe malaria.[6]

Random sampling based on satellite imagery was used to select households for participation in community-based surveys in between 2009 and 2011 in Choma District, and between 2015 and 2017 in Nchelenge District. Briefly, satellite images of the catchment area were used to create a sampling frame from which households were randomly selected for participation in community-based, serial cross-sectional surveys. Trained local field-workers used global positioning system coordinates to locate selected households for initial notification visits and data collection. All household residents older than the age of 3 months present at the time of the visit were eligible for enrollment, and written informed consent was obtained from all adults or caregivers of children who agreed to participate. Tympanic temperature was taken, and participants were tested for malaria by a PfHRP2-based RDT (Choma District: ICT Malaria *P. falciparum* [ICT Diagnostics, Cape Town, South Africa]; Nchelenge District: SD Bioline Malaria Ag *P. falciparum* [Standard Diagnostics, Inc., Gyeonggi-do, Republic of Korea]). Different RDTs were deployed at the two sites based on a change in the Zambian Ministry of Health guidelines in 2013. Both RDTs met World Health Organization procurement criteria and reliably detect parasite densities of 200 parasites/μL or higher.[11,12] Using finger-prick blood, dried blood spots (DBS) were prepared for molecular analysis. All RDT-positive participants were offered treatment according to the guidelines of the Zambian Ministry of Health.

In Choma District, 2,183 residents from 414 households agreed to participate in the study. In Nchelenge District, 2,984

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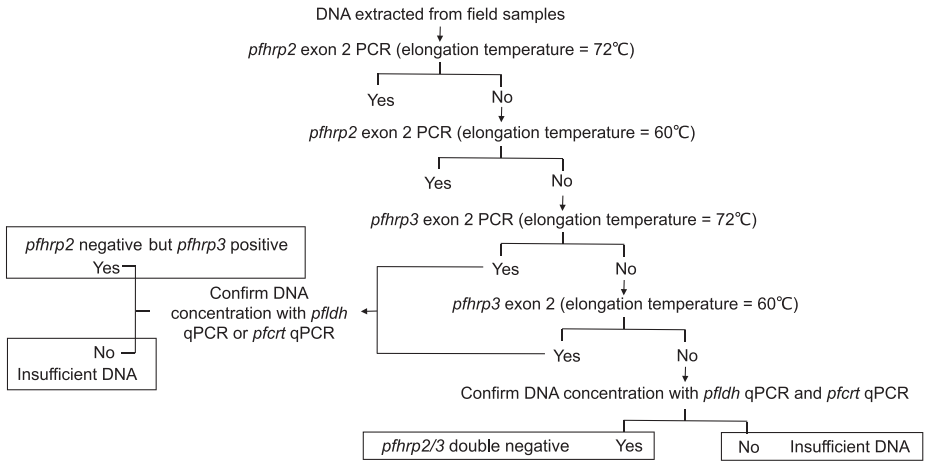

FIGURE 1. Workflow for detecting *Plasmodium falciparum* histidine–rich protein 2/3 (*pfhrp2/3*)-negative parasites.

residents from 733 households agreed to participate. Samples were transported to laboratories at Macha Research Trust in Choma District or the Tropical Disease Research Centre in Ndola. DNA was extracted from the DBS and screened using a nested-PCR targeting the mitochondrial cytochrome b gene (*cytb*), which is conserved among the major human *Plasmodium* species.[8,12] To exclude non–*P. falciparum* malaria infection, which results in a negative PfHRP2-based RDT, all *cytb* PCR-positive and RDT-negative samples from Choma District were further screened by species-specific qPCR.[8,13] For samples from Nchelenge District, species were confirmed by trained microscopists. Rapid diagnostic test–negative but PCR- or microscopy-positive *P. falciparum* samples from both sites were analyzed by a series of PCRs targeting *pfhrp2* or *pfhrp3* as previously described.[14] Briefly, RDT-negative but *cytb* PCR- or microscopy-positive samples were tested by PCR to amplify exon two of *pfhrp2*. Samples that failed amplification were assayed at lower elongation temperatures (Figure 1). Samples that failed *pfhrp2* amplification at lower temperatures were subjected to *pfhrp3* PCR. Samples that failed to amplify *pfhrp3* were then assayed at lower elongation temperatures. DNA concentrations were determined by *pfldh* qPCR and those that failed to amplify *pfhrp2* or *pfhrp3* genes were further assayed using an additional single-copy gene qPCR-targeting *pfcrt*.[15]

Twenty-nine RDT-negative but *P. falciparum*–specific qPCR-confirmed samples from Choma District and 40 RDT-negative but PCR-positive and microcopy-confirmed *P. falciparum* samples from Nchelenge District were screened for *pfhrp2/3* deletions (Table 1). The prevalence of fever among participants with RDT-negative but *cytb* PCR- or microscopy-positive results (Choma District = 3.4%; Nchelenge District = 0%) did not differ from the background population prevalence of fever on the day of the study visit (Choma District = 1.3%, $P = 0.33$; Nchelenge District = 3.6%, $P = 0.22$). All samples considered to have potential *pfhrp2/3* deletions were additionally screened using a single-copy *pfldh* qPCR assay to ensure there was more than 0.0001 ng/μL *P. falciparum* DNA, the detection limit of the *pfhrp2* and *pfhrp3* assays.[16] Samples with less than 0.0001 ng/μL parasite-specific DNA, the detection limit of the *pfldh* qPCR and the *pfhrp2/3* PCR, were considered to have insufficient parasite DNA and were excluded from further analysis. Of those samples with sufficient DNA (Choma District = 8, Nchelenge District = 28), three samples from Choma District failed to amplify *pfhrp2* (Table 1). All three samples with undetectable *pfhrp2* were *pfhrp3* PCR positive. Among these three samples with undetectable *pfhrp2* but detectable *pfhrp3* from Choma District, the presence of parasite DNA was confirmed using PCR targeting *pfcrt*. None of the RDT-negative but PCR-positive samples from Nchelenge District had evidence of *pfhrp2* or *pfhrp3* deletions.

To our knowledge, this is the first report suggesting the possibility of *pfhrp2* deletions in Zambia, although the level of parasite DNA was lower than those recommended by Parr et al.[16] and limited our ability to make deletion calls. In our study, the range of DNA concentrations of *pfhrp2*-negative samples was 0.0002–0.0006 ng/μL (approximately 8–24 genomes/μL). However, all PCR protocols used to confirm the DNA concentration and subsequently amplify *pfhrp2/3* had a detection limit of 0.0001 ng/μL (~4 genomes/μL),[16] and, therefore, the failure to amplify *pfhrp2* in three samples was not likely because of insufficient DNA alone. It seems plausible that *pfhrp2*-deleted parasites are present in Choma District, Zambia, but further investigation is needed to confirm this observation. Genes encoding PfHRP2 and PfHRP3 share high homology,[4] and detection of the D10 strain of *P. falciparum*

TABLE 1

Summary of screening results to detect *Plasmodium falciparum* histidine–rich protein 2/3 (*pfhrp2/3*)-negative parasites in northern and southern Zambia

Study area	Year	RDT positivity in the community, % (95% CI)	Prevalence of fever in the community, % (95% CI)	Samples considered for screening	Prevalence of fever among sample, % (95% CI)	Samples with *P. falciparum* DNA concentration > 0.0001 ng/uL	*pfhrp2*-negative, number (%; 95% CI)	*pfhrp2/3* double negative, number (%; 95% CI)
Choma	2009–2011	0.7 (0.4, 1.1)	1.3 (0.8, 1.8)	29	3.4 (0.1, 18)	8	3 (38; 8.5, 76)	0 (0; 0, 37)*
Nchelenge	2015–2017	45 (43, 47)	3.6 (2.9, 4.3)	40	0 (0, 8.9)*	28	0 (0; 0, 14)*	0 (0; 0, 14)*

RDT = rapid diagnostic test.
* One-sided, 97.5% CI.

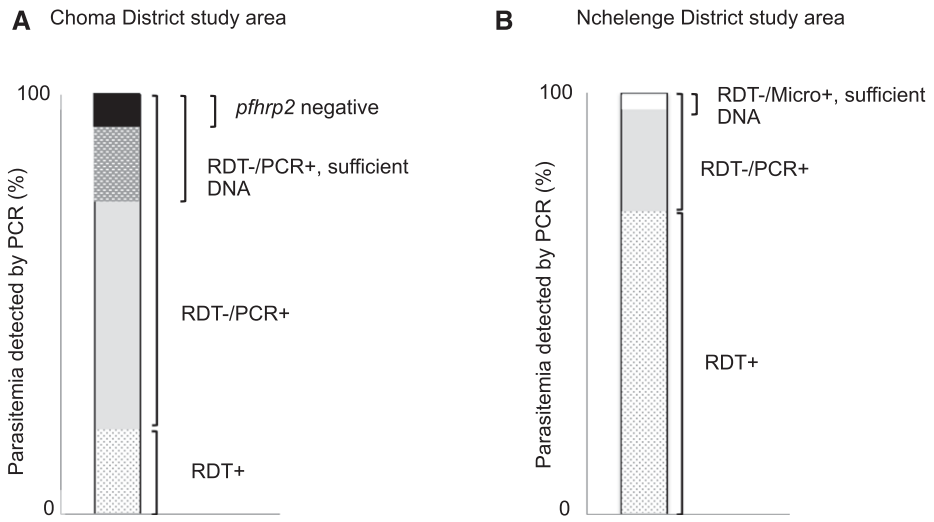

FIGURE 2. Malaria diagnostic testing results for PCR-positive subjects in Choma and Nchelenge districts, Zambia. For study participants with *Plasmodium falciparum* parasitemia detected by PCR in (**A**) the Choma District study area, the black shaded area depicts *P. falciparum* histidine–rich protein 2 (*pfhrp2*)-negative isolates among rapid diagnostic test (RDT)-negative samples with sufficient DNA concentrations for *pfhrp2* PCR testing, and (**B**) the Nchelenge district study area, *pfhrp2* was successfully amplified in all RDT-negative samples with sufficient DNA for *pfhrp2* PCR testing.

(*pfhrp2* negative but *pfhrp3* positive) using PfHRP2-based RDTs at high parasitemia (≥ 1,000 parasites/μL) suggests that PfHRP3 cross-reacts with monoclonal antibodies to PfHRP2 that are used for PfHRP2-based RDTs.[17] As none of three *pfhrp2*-negative parasites from Choma District had evidence of *pfhrp3* deletions, such parasites may still be detected by PfHRP2-based RDTs if parasitemia were high. A previous study in this setting reported that most infected individuals had submicoscopic parasitemia with a low parasite load.[8] These findings suggest that RDT-negative but PCR-positive infections in Choma District are mainly due to low parasitemia, with *pfhrp2* deletions playing only a small role (Figure 2).

It was hypothesized and recently observed that the presence of parasites with *pfhrp2* deletions may be masked in high transmission settings because of co-infection with *pfhrp2* wild-type parasites.[1,14] A prior study within the same study population in Nchelenge District reported that polyclonal infections are common, with a range of three to seven haplotypes present in each infected individual.[18] Because of the high prevalence of polyclonal infections, we cannot exclude the possibility of parasites with *pfhrp2* deletions circulating in Nchelenge District, but the presence of parasite clones with wild-type *pfhrp2* should prolong the use of PfHRP2-based RDTs. Furthermore, the median parasite count of samples by microscopy was 27 parasites/μL (interquartile range = 8–39) in Nchelenge District, less than the detection threshold of commercially available RDTs.[11] Based on this result, we conclude that the false-negative RDTs in Nchelenge District were most likely because of low parasitemia (Figure 2). As malaria transmission in Nchelenge District decreases, parasites with *pfhrp2* deletions may become more important.

In summary, *pfhrp2/3* deletions were not identified in the high transmission setting in northern Zambia, but evidence suggesting the presence of *pfhrp2* deletions was found in the low transmission setting in southern Zambia. Our ability to confirm these gene deletions was limited by low DNA concentrations and small sample volumes. This is the first time parasites with possible *pfhrp2* deletions have been identified in Zambia, but this study highlights the challenges of identifying *pfhrp2/3* deletions using low-parasite density samples by PCR-based assays. *Plasmodium falciparum* histidine rich–protein 2-based RDTs remain effective tools for the diagnosis of malaria in Zambia, but further study is required to ensure their validity.

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