Plasmodium falciparum Histidine-Rich Protein 2 and 3 Gene Deletions in Strains from Nigeria, Sudan, and South Sudan

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Deletion of histidine-rich protein genes *pfhrp2/3* in *Plasmodium falciparum* causes infections to go undetected by HRP2-based malaria rapid diagnostic tests. We analyzed *P. falciparum* malaria cases imported to Australia (n = 210, collected 2010–2018) for their *pfhrp2/3* status. We detected gene deletions in patients from 12 of 25 countries. We found >10% *pfhrp2*-deletion levels in those from Nigeria (13.3%, n = 30), Sudan (11.2%, n = 39), and South Sudan (17.7%, n = 17) and low levels of *pfhrp3* deletion from Sudan (3.6%) and South Sudan (5.9%). No parasites with *pfhrp2/3* double deletions were detected. Microsatellite typing of parasites from Nigeria, Sudan, and South Sudan revealed low relatedness among gene-deleted parasites, indicating independent emergences. The gene deletion proportions signify a risk of false-negative HRP2-RDT results. This study's findings warrant surveillance to determine whether the prevalence of gene-deleted parasites justifies switching malaria rapid diagnostic tests in Nigeria, Sudan, and South Sudan.

During 2000–2015, global malaria incidence and death rates were reduced by more than half (*1*). Malaria control efforts are credited with increasing life expectancy by 5% globally and by 12.3% in sub–Saharan Africa, where ≈90% of the disease burden is located (*2*). Gains in malaria control have been attributed primarily to the implementation of key intervention measures including insecticide-treated netting, indoor residual spraying, combination medicines, and diagnostic tests. Malaria decline has been more gradual, or has stalled, in endemic regions with limited access to these interventions (*3*).

Rapid diagnostic tests (RDTs) are recommended, and have become essential, for malaria case management in many regions because they meet the challenges for remote and low-resource settings. These tests are affordable, easy to transport and store, and less skill- and resource-demanding than microscopy, but they offer comparable sensitivity to quality microscopy (*4*). These RDTs are used, along with Giemsa-stained blood films, for diagnosis of imported malaria in pathology laboratories in Australia (including those of the Australian Defence Force) (*5*). A preliminary diagnosis using RDTs is made and the diagnosis confirmed by stained thick and thin films. False-negative tests from RDTs will result in delayed treatment, which may affect the patient's clinical outcomes.

Histidine-rich protein 2 (HRP2)–based RDTs are largely preferred for detecting *P. falciparum* malaria because of their superior sensitivity and heat-stability profile over *Plasmodium* lactate dehydrogenase (pLDH) or aldolase (*6*). HRP2-based tests detect the HRP2 antigen (and, to a lesser extent, HRP3, because of cross-reactivity) at levels as low as ≈1 ng/mL blood; however, in practice, the detection limit of HRP2-based tests is reportedly comparable with that of quality microscopy (≈200 parasites/μL) (*7*). This level is adequate for case management but much less sensitive than molecular methods. RDTs have been reported to have failed to detect a substantial proportion of asymptomatic infections (*8*).

Parasite deletion of the genes *pfhrp2* and *pfhrp3* has been implicated in false-negative results using

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DOI: https://doi.org/10.3201/eid2702.191410

HRP2-based RDTs. There are recent reports of *pfhrp2*-deleted parasites in several countries in Africa (*9*–*15*), as well as India (*16*), China and Myanmar (*17*), and countries in South America, including Peru (*18*). Single *pfhrp2* gene deletions represent an increased risk for RDT failure, especially in cases of low parasitemia or inferior RDTs (*19*). In the instance of a double deletion of *pfhrp2* and *pfhrp3,* the parasite is undetectable with HRP2-based RDTs (*20*). Because RDTs are the mainstay diagnostic tool for many endemic countries, loss of effectiveness constitutes a public health emergency and poses a major challenge to *P. falciparum* control and elimination efforts. For countries reliant on RDTs, gene-deletion prevalence data are needed to inform case management policy.

The World Health Organization has estimated a threshold of 5% of parasites lacking HRP2 as the point at which false negatives from lack of antigen expression would likely exceed the rate of false negatives observed using alternative RDTs and, as such, the point at which HRP2-based tests are no longer recommended for that location (*21*). Therefore, surveillance is critical to estimate whether the prevalence of parasites with gene deletions has reached the threshold for switching RDTs and is recommended to focus primarily on locations or nearby locations where gene deletions have been detected. Imported cases of malaria are a resource to detect gene deletions in countries of origin and the outcomes can prompt large-scale surveillance.

When case management policies for imported malaria are developed, regional *pfhrp2*/*pfhrp3* deletion levels should also be considered. The lack of clarity regarding the status of many endemic regions has fueled concern on the part of physicians. In settings where only RDTs are used for diagnosis, laboratories need to be aware of the possibility of false negatives when testing samples with >5% rate of HRP2 deletion. Consequently, we investigated the *pfhrp2*/*pfhrp3* status of malaria cases imported from travelers, immigrants, and refugees entering Australia to identify evidence for *pfhrp2* and *pfhrp3* deletions in *P. falciparum* from malaria-endemic countries.

Methods

Sample Collection and DNA Extraction

Malaria cases in Australia require that a blood sample be sent to a regional reference laboratory for confirmation and storage. We determined *Plasmodium* spp. infection and species by microscopy (Giemsa-stained thick and thin smears) and confirmed them by PCR (*22*) at the New South Wales Health Pathology Parasitology Laboratory at Westmead Hospital (Westmead, New South Wales, Australia). We aliquoted whole blood from archived *P. falciparum*–positive samples from imported malaria cases (n = 210) and recorded deidentified patient information. We extracted genomic DNA from whole blood using QIAamp mini DNA kits (QIAGEN, https://www.qiagen.com) according to the manufacturer's directions. We assessed DNA quality by subjecting DNA to agarose gel electrophoresis. We measured DNA concentrations by spectrophotometric analysis using a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher, https://www.thermofisher.com) at 260 nm and 280 nm. We included genomic DNA from *P. falciparum* laboratory reference strains in each PCR assay as experimental controls for various *pfhrp2/pfhrp3* deletion statuses: 3D7 (*pfhrp2* +/*pfhrp3* +), HB3 (*pfhrp2* +/*pfhrp3* −), 3BD5 (*pfhrp2* −/*pfhrp3* −), Dd2 (*pfhrp2* −/*pfhrp3* +), and D10 (*pfhrp2* −/*pfhrp3* +). We stored samples at −20°C before use.

Characterization of *pfhrp2* and *pfhrp3*

We investigated the status (presence/absence) of *pfhrp2* (PlasmoDB gene ID Pf3D7_0831800) and *pfhrp3* (PlasmoDB gene ID Pf3D7_1372200) genes by amplifying across exon 1–exon 2 and exon 2, as previously described (*10*; Appendix Table, https://wwwnc.cdc.gov/EID/article/27/2/19-1410-App1.pdf). Samples were considered to contain the *pfhrp2*- or *pfhrp3*-deleted parasites when there was a negative PCR result for exon 1 or exon 2 of the gene, or both, along with a positive PCR amplifying all 3 single-copy reference genes: merozoite surface protein 1 (*pfmsp1*), merozoite surface protein 2 (*pfmsp2*), and glutamate-rich protein (*pfglurp*). The use of the comparable single copy reference gene assays as a DNA quality control has been observed in several studies reporting *P. falciparum* with and without deletions to show a concordant limit of detection when the genes are present (*23*).

Rapid Diagnostic Testing

We used SD Bioline (Standard Diagnostics, https://www.globalpointofcare.abbott) HRP2-based malaria RDTs according to the manufacturer's instructions to test thawed whole blood samples that had been determined to contain *P. falciparum* with gene deletions (when whole blood was available). We performed additional tests on *pfhrp2/pfhrp3* positive and negative samples at various parasite densities, and we conducted comparative tests using BinaxNOW (Inverness Medical Binax, https://www.globalpointofcare.abbott) and Carestart (AccessBio, https://accessbiodiagnostics.net) HRP2-based malaria RDTs.

Microsatellite Analysis

We conducted microsatellite analysis as described elsewhere (*10*). In brief, for each sample originating from Sudan, South Sudan, or Nigeria, we analyzed 7 neutral microsatellite markers (TA1, PolyA, PfPK2, TA109, 2490, 313, and 383). We amplified markers per PCR conditions and primers listed (Appendix Table). We sized amplicons using an ABI 3100 Genetic Analyzer (Applied Biosystems, https://www.thermofisher.com). We scored alleles manually using Peak Scanner Software version 1.0 (Applied Biosystems), including a minimum peak height of 300 relative fluorescence units (Appendix Figure 1). To exclude artifactual stutter peaks (likely polymerase slippage on extended tandem repeats, which are frequent in *Plasmodium* genomes), we disregarded peaks less than one third of the predominant peak (*24*).

Genetic Diversity Phylogenetic Analysis

We produced a predominant haplotype for each sample based on the sizes of the 7 microsatellite markers. We used PHYLOViZ software (*25*) using a minimum spanning tree approach to compare the genetic diversity and genetic relatedness of the Sudan, South Sudan, and Nigeria cohorts within this study and to compare with parasites from Eritrea and Peru (haplotypes characterized in a previous study [*10*]). We standardized values for the microsatellite marker sizes against the *P. falciparum* 3D7 reference strain. We used FSTAT to calculate microsatellite allele frequencies at each locus, average number of alleles, and expected and observed heterozygosity (*26*).

Results

Patient Data Analysis

This study included parasite samples from persons from 25 countries, with most (194/210) originating from countries in Africa. A large proportion of the patient cohort traveled to Australia from Nigeria (n = 30) or Sudan (n = 39); for all other countries of origin, n<20. The clinical state, when known, was predominantly symptomatic travelers who came to the hospital; however, the cohort included ≥15 potentially asymptomatic samples collected during refugee screening (n = 8 within the cohort from South Sudan). The study population was composed of 149 male patients, 53 female patients, and 8 patients with unknown gender; age range was 6 months to 79 years at the time of infection (median age 42 years). Of the samples collected, 75.2% had a parasitemia ranging from 0.01% (≈500 parasites/μL) to 30.1% (1,505,000 parasites/μL), with a mean of 1.34 ± 3.00%, 67,000 parasites/μL; 24.8% had a parasitemia <0.01%. Only 48% of patients (when reported) had used chemoprophylaxis (doxycycline, artemether/lumefantrine, or mefloquine), and instances of concurrent conditions were low (reported in <5% of cases, most commonly dengue fever; Appendix Table 2).

Presence/Absence of *pfhrp2* and *pfhrp3*

We observed *pfhrp2* or *pfhrp3* deletion (together with positive *pfmsp1*, *pfmsp2*, and *pfglurp* results) in 24 of 210 parasite samples from 12 of 25 countries of origin (Table 1). Results from assays amplifying exon 2 of *pfhrp2* and *pfhrp3* matched the findings from assays amplifying across exon 1–2, suggesting whole rather than partial gene deletion. We observed *pfhrp2*-deleted parasites in 3 samples from Nigeria (3/30, 10%), 4 samples from Sudan (4/39, 10.26%), and 4 samples from South Sudan (4/17, 17.65%) (Figure 1). We observed a single sample with *pfhrp2*-deletion in specimens originating from Ghana (1/17, 5.88%), Kenya (1/18, 5.55%), Mali (1/3, 33.33%), Togo (1/1, 100%), and Zambia (1/5, 20%). We found 3 samples (3/27) of unknown African origins to be *pfhrp2*-deleted.

We observed a single sample with *pfhrp3* deletion per origin in parasites from Sudan (1/39, 2.56%), South Sudan (1/17, 5.88%), Tanzania (1/4, 25%), Sumatra (1/2, 50%), and Peru (1/1, 100%). No parasites were observed to have both the *pfhrp2* and *pfhrp3* gene deletion.

Rapid Diagnostic Test Results

We tested 20 gene deletion blood samples with HRP2 RDTs (18 *pfhrp2* deleted, 2 *pfhrp3* deleted). Of these, 16 samples produced a positive Pf band using HRP2-based SD BioLine malaria RDTs (14 *pfhrp2* deleted, 2 *pfhrp3* deleted). Of the 16 gene deletion parasites detected by HRP2 RDT, 10 samples had a parasitemia ≥1000/μL. Four of 18 *pfhrp2*-deleted parasites failed to be detected by HRP2 RDTs; 3 of these 4 cases had a parasitemia level <500/μL (Table 2). Only 9 of 20 samples gave a positive pan band; 8 of the 9 had a parasitemia level ≥2,000/μL.

Microsatellite Analysis

We amplified and scored 7 microsatellite loci for each sample from Sudan, South Sudan, and Nigeria (n = 86), finding 88 unique haplotypes. Two samples shared a haplotype, and we observed 2 instances of multiple haplotype infection. All 7 microsatellite markers were found to be polymorphic. We found a mean of 11 alleles per locus, a range from 6 (microsatellite markers TA109 and 2490) to 16 (microsatellite marker 383) distinct alleles. We found the genetic

relatedness of *P. falciparum* populations to correspond weakly with country of origin (represented by small clusters of 2–3 haplotypes), as compared with the population structure of parasites from Eritrea (Figure 2, panel A). Unlike large clustering of *pfhrp2/3*-deleted parasites in Eritrea, *pfhrp2*- or *pfhrp3*-deleted parasites within the cohorts from Sudan, South Sudan, and Nigeria were not found to be more closely related to each other than to *pfhrp2/pfhrp3*-positive parasites within their cohort (Figure 2, panel B). The expected heterozygosity of populations (by country and by deletion status) did not exceed the observed heterozygosity for any cohort.

Discussion

Increasing availability and use of HRP2-based malaria RDTs in Africa has been pivotal to improving case management over the past 20 years (*27*). Evaluation of compliance to RDT outcomes in sub-Saharan Africa found that protocols often varied among healthcare workers, particularly in the case of negative RDT results (*28*). Increased rates of RDT false-negative results may undermine confidence in adherence to World Health Organization guidelines (*29*) and would threaten the recent gains in malaria control.

Several countries relying on HRP2-based malaria RDTs lack molecular data on parasite *pfhrp2* and *pfhrp3* deletion. Sudan and South Sudan had no previously reported data regarding *pfhrp2* and *pfhrp3* status. We observed the presence of both *pfhrp2* and *pfhrp3*-deleted parasites in this study, although no double deletions were detected. The presence of these parasites is not altogether unexpected given the low level of *pfhrp2*- and *pfhrp3*-deleted parasites previously found in natural *P. falciparum* populations (*30*) and the presence of these parasites in neighboring endemic regions (*10*). The levels of *pfhrp2* deletion raise concerns: 10.3% observed from Sudan (mean collection date 2016), and 17.5% from South Sudan (mean collection date 2017–2018) (Figure 1). Mathematical modeling predicts rapid (≈3 years) selection for widespread *pfhrp2*-deletion within a population subjected to HRP2-based RDT use, with a baseline *pfhrp2*-deletion level lower than what we observed

Table 1. Summary of *pfhrp2/pfhrp3* gene deletion screening results showing *pfhrp2/pfhrp3* status for *Plasmodium* spp. isolates, by parasite country of origin, Australia*

Source	Country/strain name	No. cases	pfhrp2/pfhrp3 status, no. (%)			% Symptomatic	% Refugee
			+/–	–/+	–/–		
Africa	Cameroon	3	0	0	0	66.6	33.3
	Gambia	5	0	0	0	100	0
	Ghana	17	0	1 (0.06)	0	100	0
	Ivory Coast	2	0	0	0	100	0
	Kenya	18	0	1 (0.06)	0	100	0
	Madagascar	1	0	0	0	100	0
	Malawi	6	0	0	0	33.3	66.6
	Mali	3	0	1 (33.3)	0	100	0
	Nigeria	30	0	4 (13.3)	0	100	0
	Sierra Leone	13	0	0	0	92.3	7.7
	South Africa	2	0	0	0	100	0
	South Sudan	17	1 (5.9)	3 (17.6)	0	52.9	47.1
	Sudan	39	1 (2.6)	4 (10.3)	0	100	0
	Sumatra	2	1 (50)	0	0	100	0
	Togo	1	0	1 (100)	0	100	0
	Tanzania	4	1 (25)	0	0	100	0
	Uganda	2	0	0	0	100	0
	Zambia	5	0	1 (20)	0	100	0
	Zimbabwe	1	0	0	0	100	0
	Unknown†	27	0	3 (11.1)	0	100	0
Asia	Cambodia	1	0	0	0	100	0
	India	3	0	0	0	100	0
	Indonesia	1	0	0	0	100	0
	Papua New Guinea	5	0	0	0	100	0
	Thailand	1	0	0	0	100	0
South America	Peru	1	1 (100)	0	0	100	0
Laboratory strains	3D7	1	0	0	0	NA	NA
	3BD5	1	0	0	1 (100)	NA	NA
	D10	1	0	1 (100)	0	NA	NA
	Dd2	1	0	1 (100)	0	NA	NA
	HB3	1	0	1 (100)	0	NA	NA

*NA, not applicable.
†Cases in which clinical notes state African origins but do not specify a country.

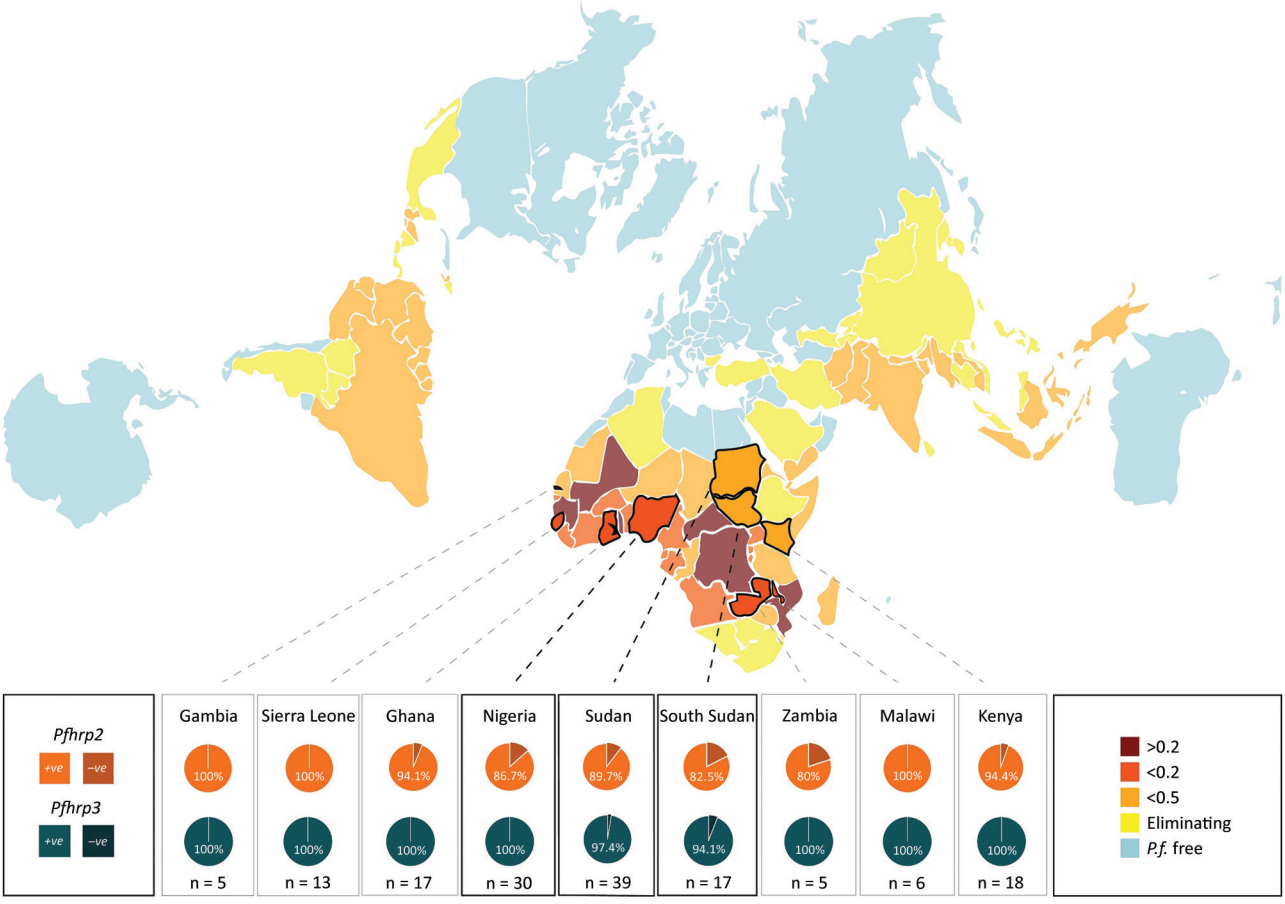

Figure 1. Summary of *pfhrp2* and *pfhrp3* deletion key results showing *pfhrp2* and *pfhrp3* deletion results for *Plasmodium* spp. isolates, by parasite country of origin (where n>4), Australia. National *P. falciparum* endemicity depicted is measured as population-weighted mean *P. falciparum* infection rate of children 2–10 years of age, using data available from the Malaria Atlas Project (http://www.map.ox.ac.uk). Data were mapped using the AuthaGraph world map projection to more truthfully visualize the potential paths of dissemination and adjacency of various endemic zones, as this is considered the most accurate representation of land proportions and relative orientations (https://hrcak.srce.hr/185867). *P.f.*, *P. falciparum*.

in parasites originating from Sudan and South Sudan (*31*). In addition, this region of Africa experiences a great deal of human migration (*32*,*33*), notably in Sudan's neighbor Eritrea (where *pfhrp2*/*pfhrp3* double deletion parasites are prevalent [*10*]), increasing the risk for deletion-parasite dissemination.

Samples originating from Nigeria (n = 30) were collected during 2011–2015 (1 sample from 2011 was, to our knowledge, the earliest reported *pfhrp2*-deleted parasite from Nigeria); however, the proportion of *pfhrp2*-deleted parasites observed (13.3%) is similar to the 17% observed in a 2019 study of contemporary parasites from Nigeria (likewise finding no double deletion) (*14*). Countries in western Africa, such as Nigeria, often make use of exclusively HRP2-based RDTs (no pan–*Plasmodium* spp. antigen target); because reliance on *P. falciparum*–only RDTs would further exacerbate the public health consequences of *pfhrp2*/*pfhrp3*-deleted parasites, ongoing monitoring in these locations is warranted (*31*).

No gene-deleted parasites were observed within the cohorts from several countries (Cameroon, Gambia, Côte d'Ivoire, Madagascar, Malawi, Sierra Leone, South Africa, Sumatra, Uganda, Zimbabwe, and all countries in Asia); in 6 countries in Africa, a single gene-deleted parasite was found (*pfhrp2*: Ghana, Kenya, Mali, Togo, and Zambia; *pfhrp3*: Tanzania). The sample sizes are insufficient to comment on regional proportions. Baselines for these regions are undetermined, although a low level of false-negative results using HRP2-based RDTs has been reported in rural Ghana (*34*), and varying levels (0%–30%) of *pfhrp2* deletion have been observed in regions of Kenya (*12*). *pfhrp2/pfhrp3* double-deleted parasites have been observed within the China–Myanmar border area, where baseline *pfhrp2*-deleted parasite

proportions were as low as 4% (*17*). The observation of a low level of gene-deleted parasites in this study emphasizes the need to monitor *pfhrp2/pfhrp3* status for early detection of emergent double deletions in the countries of origin.

SD BioLine and Carestart HRP2-based tests consistently produced the same outcome, but those results occasionally differed from results from BinaxNOW, which was less sensitive (Table 2). Subjecting the selected samples to testing with HRP2-based SD BioLine malaria RDTs corroborated the hypothesis that infections by *pfhrp2*-deleted parasites may occasionally fail to be detected, particularly in cases of low parasite density (<1,000/μL) and less-sensitive RDT varieties. Indeed, most *pfhrp2*-negative/*pfhrp3*-positive samples tested positive with HRP2-based SD BioLine malaria RDTs when parasitemia was >1000 parasites/μL, which suggests that HRP3 cross-reaction with HRP2-based tests acts as a failsafe, in cases of adequate parasite density (generally observed to be >1,000 parasites/μL [*14*]). *pfhrp2*-deleted parasites, in the absence of a double deletion, may suffer a loss of sensitivity, but these assays remain a viable interim option for remote and low-resource settings.

P. falciparum and pan–*Plasmodium* spp. RDTs failing to detect *pfhrp2/3*-deleted *P. falciparum* through pan–*Plasmodium* spp. pLDH likely reflects the freezing/thawing of whole blood samples, which is reported to degrade the antigen and to cause hemolysis of the blood, leading to sample inhibition (*35*). Freeze-thawing of archived blood is not observed to degrade HRP2 appreciably (*7*).

The main purpose of microsatellite analysis was to compare the genetic relatedness between parasites with gene deletions reported from different areas globally so that we could determine whether parasites with gene deletions were of de novo emergence. For this purpose, we used the same set of microsatellite markers that have been used in other parts of the world, including South America, and included a common control of 3D7 parasite in each run at different laboratories to calibrate outcomes. Microsatellite analyses found high heterogeneity of *P. falciparum* populations within and between Sudan, South Sudan, and Nigeria. The lack of genetic relatedness

Table 2. Assessment of HRP2-based SD BioLine RDT for *pfhrp2/pfhrp3* deletion genotypes for *Plasmodium* spp. isolates, by parasite country of origin, Australia*

Country	Sample ID	Collection year	Parasitemia, % erythrocytes	Parasites/μL blood	Genotype, *pfhrp2/pfhrp3*	BioLine RDT†	
						Pan	*Pf*
Sudan	BDA1	2016	0.16	8,000	+/+	1	2
Sudan	BDA2	2016	0.79	39,500	–/+	1	3
Sudan	BDA3	2016	0.3	15,000	–/+	0	1
Sudan	BDA4	2016	0.02	1,000	–/+	0	1
Sudan	BDA37	2014	<0.01	NA	–/+	0	0
South Sudan	BDD4	2018	1.24	62,000	+/–	1†	3
South Sudan	BDC98	2018	0.5	25,000	–/+	1	1
South Sudan	BDB94	2017	0.5	25,000	–/+	1	1
South Sudan	BDB99	2017	<0.01	NA	–/+	0	1†
Nigeria	BDA24	2015	1.1	55,000	–/+	0	1
Nigeria	BDA92	2012	4	200,000	–/+	1	1
Nigeria	BDA91	2012	2.5	125,000	–/+	1	1
Nigeria	BDB31	2011	0.08	4,000	–/+	0	0
Kenya	BDA42	2014	0.12	6,000	–/+	2	1
Kenya	BDB19	2011	<0.01	NA	+/+	0	3
Ghana	BDA5	2016	<0.01	NA	–/+	1‡	1‡
Tanzania	BDA28	2015	<0.01	NA	–/+	0	1
Zambia	BDA31	2015	0.2	10,000	–/+	0	1
Togo	BDA50	2014	0.27	13,500	–/+	0	1
Peru	BDA52	2014	0.4	20,000	+/–	1	3
Papua New Guinea	BDB6	2012	2.81	140,500	+/+	1‡	2
Papua New Guinea	BDB5	2012	<0.01	NA	+/+	1‡	1
Africa	BDA99	2012	0.46	23,000	+/+	0	3
Africa	BDA90	2012	1.2	60,000	+/+	0	3
Africa	BDA100	2012	<0.01	NA	+/+	0	1
Africa	BDB11	2011	<0.01	NA	–/+	0	0
Africa	BDB46	2010	0.01	500	–/+	0	0

*Gray shading signifies a negative result with both the pan-pLDH and pfHRP2 antigen tests on the RDT. Africa indicates unknown country of origin. ID, identification; NA, not applicable; pan, pan–*Plasmodium* spp.; *Pf*, *P. falciparum*; pfHRP2, *Plasmodium falciparum* histidine-rich protein 2; pLDH, *Plasmodium* lactate dehydrogenase; RDT, rapid diagnostic test.
†0–4 result scored by World Health Organization guidelines.
‡Sample results matched outcomes using CareStart Malaria RDT, whereas parasites were not detected by BinaxNOW RDT (Appendix Figures 2, 3, https://wwwnc.cdc.gov/EID/article/27/2/19-1410-App1.pdf).

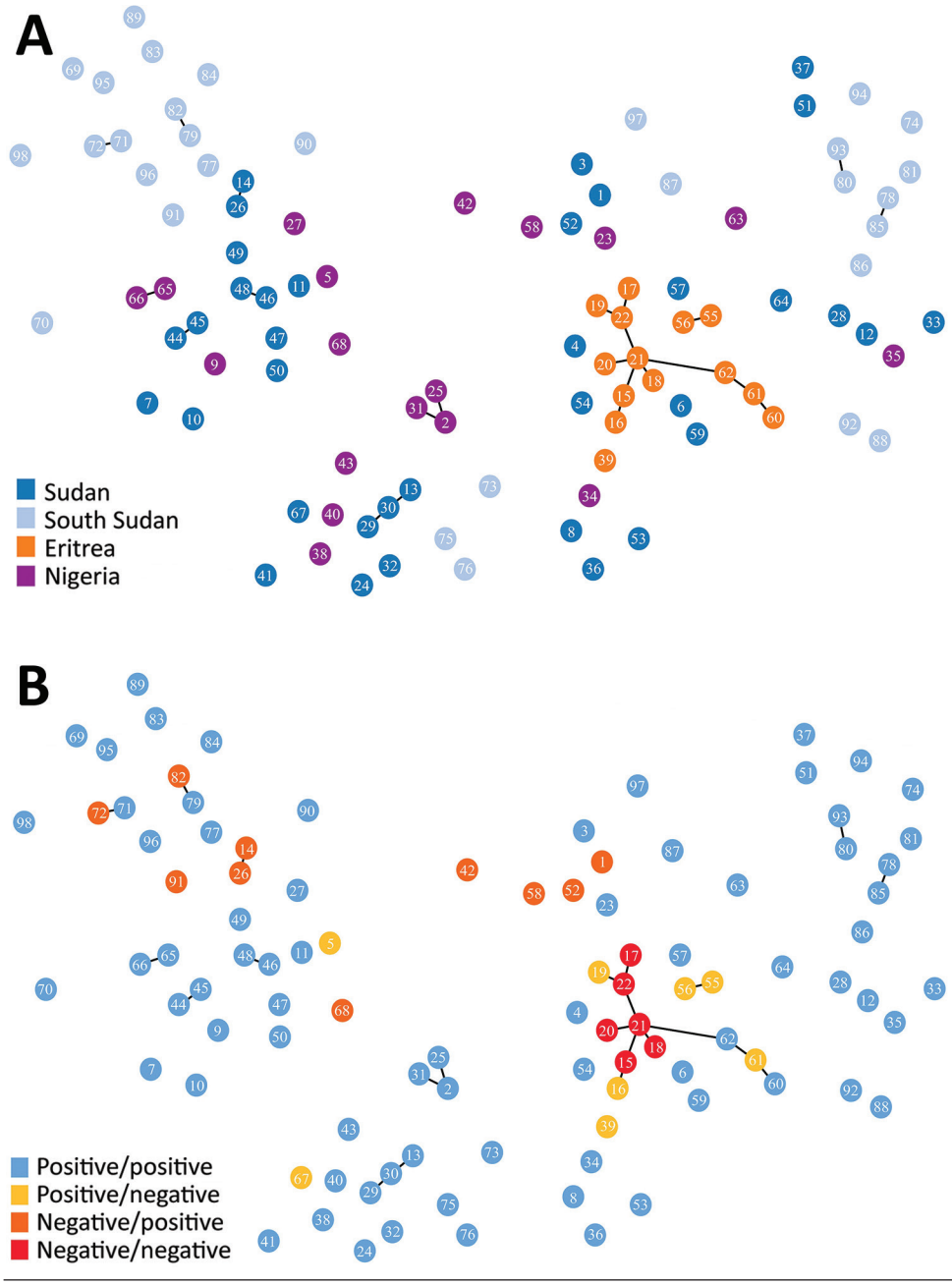

Figure 2. Minimum spanning tree of microsatellite allelic data showing genetic relatedness of *Plasmodium falciparum* populations from Sudan, South Sudan, Nigeria, and Eritrea (A), and *pfhrp2* and *pfhrp3* deletion status of haplotypes (B) (positive: gene present; negative: gene absent). Numbered circles represent specific haplotypes. Plots were generated using PHYLOViZ software (*25*) with a cutoff value of 2 (minimum differences for 2 microsatellite loci) depicted as lines connecting haplotypes and a cutoff value of 3 depicted as haplotype circle arrangements/proximities.

observed between gene-deleted parasites, including between the gene-deleted parasites observed in this study and those analyzed within Eritrea in a previous study (*10*), suggests independent, de novo emergence of parasites with gene deletions. The high level of genetic diversity may reflect the broad geographic and temporal sampling range, as well as the heterogenicity, of natural *P. falciparum* populations in areas with moderate or high transmission intensities.

We analyzed microsatellite peaks for the presence of multiple peaks (indicating multiple unique haplotypes within an individual infection). Three samples had evidence of infection by multiple strains with cocirculating strains potentially present at lower density; all other samples had no secondary peaks exceeding our thresholds for calling (this necessary threshold may prevent the detection of minor alleles). Although it detected few multiclonal infections in this sample set, microsatellite analysis was able to detect a very high level of heterogeneity (88 haplotypes/86 samples) within and between countries, demonstrating the quality of the analysis. The low level of multiple clone infections may reflect the source of samples. For instance, Nigeria is a high-transmission country

with a high median multiplicity of infection; however, the Nigerian cohort was composed of travelers or immigrants to Australia who returned home for family events, usually traveling for <2 weeks.

A notable consideration when interpreting results from this study is the opportunity sampling. Using imported *P. falciparum* from travelers provided small sample sizes for most countries of origin and a broad collection timeframe (2010–2018). The sampling timeframe does not capture the true prevalence of *pfhrp2* and *pfhrp3* deletion in contemporary parasite populations or allow us to consider the effects of seasonal profiles (*38*). As a result, the cohort is not representative of cases within endemic regions, which is noteworthy because *pfhrp2/3* deletion needs to be interpreted considering clinical relevance. Deletion proportions between symptomatic and asymptomatic patients within groups were too small and too often status unknown for meaningful analysis. This limitation restricts the conclusions that can be drawn from the screening results, although analyses of imported malaria cases in persons entering Australia has the added benefit of informing local case management.

Because clinicians' notes informed patient data, the specific geographic origins were limited to the country level (and, in 27 cases, were reported only as having origins in Africa). To the best of our knowledge, travelers contracted malaria parasites from their country of origin. However, we cannot exclude the possibility that parasites were contracted from another endemic region. Specimens from South Sudan were obtained primarily from refugees who had reported staying in camps for long periods (3–12 months), including settlements bordering Uganda and Ethiopia. Therefore, parasites may have originated from bordering endemic regions.

Malaria control is complicated in regions bordering other endemic nations by human–vector migration. Border regions are often rural, which may lead to high transmission coupled with inadequate health services (*36*). Similarly, the remoteness, limited resources, and political complexity of border regions often produces suboptimal surveillance responses (*37*). Given the genetic exchange expected between adjacent parasite populations, monitoring of *pfhrp2* and *pfhrp3* for Sudan and South Sudan would ideally be coordinated together with neighboring countries.

In conclusion, analysis of imported *P. falciparum* cases revealed *pfhrp2* and *pfhrp3* deletion from 12 countries, including levels of *pfhrp2*-deleted parasites exceeding 10% from Nigeria, Sudan, and South Sudan, where *pfhrp2*-based malaria RDT failure would constitute a major public health threat. These nations require urgent prevalence surveys and ongoing monitoring for early detection of emergent double deletion parasites.

About the Author

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Article DOI: https://doi.org/10.3201/eid2702.191410

Plasmodium falciparum Histidine-Rich Protein 2 and 3 Gene Deletions in Strains from Nigeria, Sudan, and South Sudan

Appendix

Appendix Table 1. Primers and PCR conditions: PCR conditions for assays investigating presence or absence of *pfhrp2* and *pfhrp3* exon1 and exon2, and assays amplifying 7 neutral microsatellite loci for genetic diversity and genetic relatedness analysis

Target	Primer	Primer sequence (5'-3')	PCR conditions	Reaction mixture (μL per reaction)	Expected product size	Reference
pfhrp2 exon2	*pfhrp2ex2*F	CAA AAG GAC TTA ATT TAA ATA AGA G	94°C/10min [94°C/50s, 55°C/50s, 70°C/1min] × 45 cycles 72°C/10min	Total reaction volume: 25 (Template DNA: 10) + (10× PCR Buffer: 2.5) + (25 mM $MgCl_2$: 2) + (1.25 mM dNTPs: 4) + (Primer *pfhrp2ex2*F @75 μg/mL: 1.5) + (Primer *pfhrp2ex2*R @75 μg/mL: 1.5) + (Amplitaq Gold: 0.25) + (Molecular grade H_2O: 3.25)	600–1000 bp	(*1*)
	*pfhrp2ex2*R	AAT AAA TTT AAT GGC GTA GGC A				
pfhrp2 exon1-2	*2E12F1*	GGT TTC CTT CTC AAA AAA TAA AG	94°C/10min [94°C/50s, 55°C/50s, 60°C/1min] × 45 cycles 68°C/10min	Total reaction volume: 25 (Template DNA: 10) + (10× PCR Buffer: 2.5) + (25 mM $MgCl_2$: 2) + (1.25 mM dNTPs: 4) + (Primer *2E12F1* @75 μg/mL: 1.5) + (Primer *2E12R1* @75 μg/mL: 1.5) + (Amplitaq Gold: 0.25) + (Molecular grade H_2O: 3.25)	36 bp	(*2*)
	2E12R1	TCT ACA TGT GCT TGA GTA GTT TCG				
pfhrp3 exon2	*pfhrp3ex2*F	AAT GCA AAA GGA CTT AAT TC	94°C/10min [94°C/50s, 50°C/50s, 70°C/1min] × 45 cycles 72°C/7min	Total reaction volume: 25 (Template DNA: 10) + (10× PCR Buffer: 2.5) + (25 mM $MgCl_2$: 2) + (1.25 mM dNTPs: 4) + (Primer *pfhrp3ex2*F @75 μg/mL: 1.5) + (Primer *pfhrp3ex2*R @75 μg/mL: 1.5) + (Amplitaq Gold: 0.25) + (Molecular grade H_2O: 3.25)	250–600 bp	(*1*)
	*pfhrp3ex2*R	TGC ATG ATG GGC ATC ACC TG				
pfhrp3 exon1-2	*pfhrp3ex1-2*F	TAT CCG CTG CCG TTT TTG CTT CC	94°C/10min [94°C/50s, 55°C/50s, 70°C/1min] × 45 cycles 72°C/10min	Total reaction volume: 25 (Template DNA: 10) + (10× PCR Buffer: 2.5) + (25 mM $MgCl_2$: 2) + (1.25 mM dNTPs: 4) + (Primer *pfhrp3ex1-2*F @75 μg/mL: 1.5) + (Primer *pfhrp3ex1-2*R @75 μg/mL: 1.5) + (Amplitaq Gold: 0.25) + (Molecular grade H_2O: 3.25)	303 bp	(*2*)
	*pfhrp3ex1-2*R	TGC ATG ATG GGC ATC ACC TG				
pfmsp1	O1 (F)	CACATGAAAGTTATCAAGAACTTGTC	94°C/10min [94°C/25s, 50°C/35s, 68°C1min] × 45 cycles 68°C/10min	Total reaction volume: 25 (Template DNA: 10) + (10× PCR Buffer: 2.5) + (25 mM MgCl2: 2.5) + (2.5 mM dNTPs: 4) + (Primer *pfmsp1*O1 @50 μg/mL: 1) + (Primer *pfmsp1*O2 @50 μg/mL: 1) + (Amplitaq Gold: 0.25) + (Molecular grade H_2O: 3.75)	Variable	(*3*)
	O2 (R)	GTACGTCTAATTCATTTGCACG				
pfmsp2	S3 (F)	GAAGGTAATTAAAACATTGTC		Total reaction volume: 25	Variable	(*3*)

Target	Primer	Primer sequence (5'-3')	PCR conditions	Reaction mixture (μL per reaction)	Expected product size	Reference
	S2 (R)	GAGGGATGTTGCTGCTCCACAG	94°C/10min [94°C/50s, 55°C/60s, 65°C/1min] × 45 cycles 65°C/10min	(Template DNA: 10) + (10× PCR Buffer: 2.5) + (25 mM MgCl2: 2.5) + (2.5 mM dNTPs: 4) + (Primer *pfmsp2*S3 @50 μg/mL: 1) + (Primer *pfmsp2*S2 @50 μg/mL: 1) + (Amplitaq Gold: 0.25) + (Molecular grade H_2O: 3.75)		
pfglurp	G4 (F) G5 (R)	ACATGCAAGTGTTGATCC GATGGTTTGGGAGTAACG	94°C/10min [94°C/25s, 50°C/35s, 68°C/1min] × 45 cycles 68°C/10min	Total reaction volume: 25 (Template DNA: 10) + (10× PCR Buffer: 2.5) + (25 mM MgCl2: 2.5) + (2.5 mM dNTPs: 4) + (Primer *pfglurp*G4 @50μg/mL: 1) + (Primer *pfglurp*G5 @50μg/mL: 1) + (Amplitaq Gold: 0.25) + (Molecular grade H_2O: 3.75)	Variable	(*3*)
PolyA	Round 1: PolyA-F PolyA-R	AAA ATA TAG ACG AAC AGA ATC AGA TAA TTG TTG GTA	94°C/5min [94°C/30s, 42°C/30s, 40°C/30s, 65°C/40s] × 25 cycles 65°C/5min	Total reaction volume: 25 (Template DNA: 2) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.8) + (1.25 mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10 μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 7.7)	131–185 bp (PET dye color: red)	(*4*)
	Round 2: PolyA-2-F PolyA-3IR	AAA ATA TAG ACG AAC AGA ***PET**-GAA ATT ATA ACT CTA CCA	94°C/5min [94°C/30s, 42°C/30s, 65°C/30s] × 25 cycles 65°C/5min	Total reaction volume: 15 (Template DNA: 1) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.5) + (1.25 mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 9)		
TA1	Round 1: TA1-3F TA1-R	CTA CAT GCC TAA TGA GCA TTT TAT CTT CAT CCC CAC	94°C/5min [94°C/30s, 42°C/30s, 40°C/30s, 65°C/40s] × 25 cycles 65°C/5min	Total reaction volume: 25 (Template DNA: 2) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.8) + (1.25 mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 7.7)	162–189 bp (6-FAM dye color: blue)	(*4*)
	Round 2: TA1-2-R TA1-2-R	***6FAM**-CCG TCA TAA GTG CAG AGC TTT TAT CTT CAT CCC CAC	94°C/5min [94°C/30s, 42°C/30s, 65°C/30s] × 25 cycles 65°C/5min	Total reaction volume: 15 (Template DNA: 1) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.5) + (1.25 mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10 μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 9)		
PK2	Round 1: PFPK2-F PFPK2-R	CTT TCA TCG ATA CTA CGA CCT CAG ACT GAA ATG CAT	94°C/5min [94°C/30s, 42°C/30s, 40°C/30s, 65°C/40s] × 25 cycles 65°C/5min	Total reaction volume: 25 (Template DNA: 2) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.8) + (1.25mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 7.7)	162–183 bp (NED dye color: black/yellow)	(*4*)
	Round 2: PK2-2-F PK2-2-R	TAG TAA CGA TGT TGA CAA ***NED**-AAA AAG GAG GAT AAA TAC AT	94°C/5min [94°C/30s, 42°C/30s, 65°C/30s] × 25 cycles 65°C/5min	Total reaction volume: 15 (Template DNA: 1) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.5) + (1.25 mM dNTPs: 0.6) + (Primer F @10μM: 0.6) + (Primer R @10 μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 9)		

Target	Primer	Primer sequence (5'-3')	PCR conditions	Reaction mixture (μL per reaction)	Expected product size	Reference
TA109	Round 1: TA109_3-F	TAG GGA ACA TCA TAA GGA T	94°C/5min [94°C/30s, 42°C/30s, 40°C/30s, 65°C/40s] × 25 cycles 65°C/5min	Total reaction volume: 25 (Template DNA: 2) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.8) + (1.25 mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10 μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 7.7)	163–178 bp (6-FAM dye color: blue)	(*4*)
	TA109_3-R	CCT ATA CCA AAC ATG CTA AA				
	Round 2: TA109-2-F	***FAM**-GGTTAAATCAGGACAACAT	94°C/5min [94°C/30s, 42°C/30s, 65°C/30s] × 25 cycles 65°C/5min	Total reaction volume: 15 (Template DNA: 1) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.5) + (1.25 mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10 μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 9)		
	TA109-2-R	CCT ATA CCA AAC ATG CTA AA				
2490	Round 1: 2490-F	TTC TAA ATA GAT CCA AAG	94°C/5min [94°C/30s, 42°C/30s, 40°C/30s, 65°C/40s] × 25 cycles 65°C/5min	Total reaction volume: 25 (Template DNA: 2) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.8) + (1.25mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10 μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 7.7)	69–83 bp (FAM dye color: blue)	(*4*)
	2490-R	ATG ATG TGC AGA TGA CGA				
	Round 2: 2490-2-F	TTC TAA ATA GAT CCA AAG	94°C/5min [94°C/30s, 42°C/30s, 65°C/30s] × 25 cycles 65°C/5min	Total reaction volume: 15 (Template DNA: 1) + (10× PCR Buffer no Mg: 1.5) + (25 mM $MgCl_2$: 1.5) + (1.25 mM dNTPs: 0.6) + (Primer F @10 μM: 0.6) + (Primer R @10 μM: 0.6) + (FastTaq: 0.25) + (Molecular grade H_2O: 9)		
	2490-2-R	***FAM**-AGA ATT ATT GAA TGC AC				

*Fluorescent labeled primers (boldface type) are light sensitive; exposure to light should be minimized. Microsatellite fluorescent primers were ordered through Applied Biosystems (https://www.thermofisher.com) as Custom 5' Fluorescent Labeled Oligo, 10,000 picomoles.

Appendix Table 2. Patient data and results summary for the study of *Plasmodium falciparum* histidine-rich protein 2 and 3 gene deletions

Continent	Country	Specimen voucher	Year of patient's birth	Sex	Parasitemia % RBC	Parasitemia Parasites/μL	Further Notes*	Year of collection	*pfhrp2* exon 1–2	*pfhrp2* exon 2	*pfhrp3* exon 1–2	*pfhrp3* exon 2	*pfmsp1/pfmsp2 /pfglurp*
Africa	Unknown	BDA55	1986	M	NA	NA	NA	2014	pos	pos	pos	pos	NA
		BDA57	1970	M	1.4	70,000	NA	2014	neg†	neg	pos	pos	pos/pos/pos
		BDA64	1989	M	<0.01	<500	NA	2014	pos	pos	pos	pos	NA
		BDA66	1961	M	1	50,000	NA	2013	pos	pos	pos	pos	NA
		BDA81	1948	M	0.9	45,000	NA	2013	pos	pos	pos	pos	NA
		BDA88	1978	M	0.9	45,000	NA	2012	pos	pos	pos	pos	NA
		BDA89	1974	M	<0.01	<500	NA	2012	pos	pos	pos	pos	NA
		BDA90	1945	F	1.2	60,000	NA	2012	pos	pos	pos	pos	NA
		BDA99	1964	M	0.46	23,000	NA	2012	pos	pos	pos	pos	NA
		BDA100	1974	M	<0.01	<500	NA	2012	pos	pos	pos	pos	NA
		BDB1	1955	M	2	100,000	NA	2012	neg	neg	pos	pos	pos/pos/pos
		BDB2	1958	M	11	550,000	NA	2012	pos	pos	pos	pos	NA
		BDB4	1964	M	NA	NA	NA	2012	pos	pos	pos	pos	NA
		BDB9	1992	M	1.1	55,000	NA	2012	neg	neg	pos	pos	pos/pos/pos
		BDB10	1977	M	<0.01	<500	NA	2012	pos	pos	pos	pos	NA
		BDB11	1948	F	<0.01	<500	NA	2011	neg	neg	pos	pos	pos/pos/pos
		BDB17	2004	M	NA	NA	NA	2011	pos	pos	pos	pos	NA
		BDB20	1981	F	2.4	120,000	NA	2011	pos	pos	pos	pos	NA
		BDB35	1959	M	<0.01	<500	NA	2011	pos	pos	pos	pos	NA
		BDB38	2006	M	0.3	15,000	NA	2010	pos	pos	pos	pos	NA
		BDB42	1990	F	<0.01	<500	NA	2010	pos	pos	pos	pos	NA
		BDB45	1965	M	0.04	2,000	NA	2010	pos	pos	pos	pos	NA
		BDB46	1980	M	0.01	500	NA	2010	pos	pos	pos	pos	NA
		BDB84	1993	M	2.2	110,000	NA	2017	pos	pos	pos	pos	NA
		BDB86	1976	M	<0.01	<500	NA	2017	pos	pos	pos	pos	NA
		BDB88	2008	F	0.4	20,000	NA	2017	pos	pos	pos	pos	NA
		BDB91	1982	M	NA	NA	NA	2017	pos	pos	pos	pos	NA
	Cameroon	BDA12	1957	M	4.13	206,500	P, R	2015	pos	pos	pos	pos	NA
		BDA13	1957	M	3.2	160,000	P, R	2015	pos	pos	pos	pos	NA
		BDA38	1974	M	<0.01	<500	NA	2014	pos	pos	pos	pos	NA
	Gambia	BDB33	1992	F	3.4	170,000	NA	NA	pos	pos	pos	pos	NA
		BDB36	1992	F	1.9	95,000	NA	2011	pos	pos	pos	pos	NA
		BDB39	2000	NA	0.15	7,500	NA	NA	pos	pos	pos	pos	NA
		BDB40	1949	NA	<0.01	<500	NA	NA	pos	pos	pos	pos	NA
		BDB41	1910	NA	0.03	1,500	NA	NA	pos	pos	pos	pos	NA
	Ghana	BDA85	1967	M	3.7	185,000	PNC	2012	pos	pos	pos	pos	NA
		BDA5	1967	F	<0.01	<500	P	2016	neg	neg	pos	pos	pos/pos/pos
		BDA45	2001	F	0.14	7,000	NA	2014	pos	pos	pos	pos	NA
		BDA46	1973	F	0.64	32,000	PNC	2014	pos	pos	pos	pos	NA
		BDA67	1983	M	0.12	6,000	PNC	2013	pos	pos	pos	pos	NA
		BDA70	1975	M	1.1	55,000	CM	2013	pos	pos	pos	pos	NA
		BDA96	1955	M	1.1	55,000	NA	20 12	pos	pos	pos	pos	NA
		BDA98	1955	M	1	50,000	NA	2012	pos	pos	pos	pos	NA
		BDB24	1972	M	<0.01	<500	NA	2011	pos	pos	pos	pos	NA
		BDB26	1966	M	4.27	213,500	NA	2011	pos	pos	pos	pos	NA

Continent	Country	Specimen voucher	Year of patient's birth	Sex	Parasitemia % RBC	Parasitemia Parasites/μL	Further Notes*	Year of collection	*pfhrp2* exon 1–2	*pfhrp2* exon 2	*pfhrp3* exon 1–2	*pfhrp3* exon 2	*pfmsp1/pfmsp2 /pfglurp*
		BDB29	1967	F	0.1	5,000	P	2011	pos	pos	pos	pos	NA
		BDB37	1979	M	<0.01	<500	NA	2010	pos	pos	pos	pos	NA
		BDB51	1967	F	<0.01	<500	P	2016	pos	pos	pos	pos	NA
		BDB52	1967	F	6.7	335,000	NA	2016	pos	pos	pos	pos	NA
		BDB65	1980	M	0.3	15,000	PNC	2016	pos	pos	pos	pos	NA
		BDB80	1958	M	0.7	35,000	NA	2017	pos	pos	pos	pos	NA
		BDB100	1959	M	0.16	8,000	NA	2017	pos	pos	pos	pos	NA
	Ivory Coast	BDA75	1973	M	0.12	6,000	PNC	2014	pos	pos	pos	pos	NA
		BDA76	1987	F	0.1	5,000	CC	2014	pos	pos	pos	pos	NA
	Kenya	BDA42	1986	M	1.4	70,000	NA	2014	neg	neg	pos	pos	pos/pos/pos
		BDA58	1986	F	<0.01	<500	NA	2014	pos	pos	pos	pos	NA
		BDA61	1986	M	<0.01	<500	NA	2014	pos	pos	pos	pos	NA
		BDA62	2000	M	0.4	20,000	NA	2013	pos	pos	pos	pos	NA
		BDA63	1994	M	<0.01	<500	NA	2013	pos	pos	pos	pos	NA
		BDA71	1985	M	0.4	20,000	NA	2013	pos	pos	pos	pos	NA
		BDA72	2012	M	7.6	380,000	NA	2013	pos	pos	pos	pos	NA
		BDA73	1937	M	2.3	115,000	NA	2011	pos	pos	pos	pos	NA
		BDA74	1969	NA	NA	NA	NA	NA	pos	pos	pos	pos	NA
		BDB18	1969	NA	NA	NA	NA	NA	pos	pos	pos	pos	NA
		BDB19	1977	F	0.27	13,500	NA	2011	pos	pos	pos	pos	NA
		BDB21	1996	F	0.85	42,500	NA	2017	pos	pos	pos	pos	NA
		BDB30	1996	F	0.09	4,500	NA	2017	pos	pos	pos	pos	NA
		BDB98	1959	M	1	50,000	PNC	2014	pos	pos	pos	pos	NA
		BDC1	1974	M	<0.01	<500	PNC	2013	pos	pos	pos	pos	NA
		BDA59	1986	F	0.02	1,000	NA	2018	pos	pos	pos	pos	NA
		BDA83	1968	F	30.1	1,505,000	PNC	2016	pos	pos	pos	pos	NA
		BDC3	2003	F	1.5	75,000	NA	2016	pos	pos	pos	pos	NA
	Madagascar	BDA7	1984	F	<0.01	<500	NA	2017	pos	pos	pos	pos	NA
	Malawi	BDA6	1988	M	0.08	4,000	NA	2017	pos	pos	pos	pos	NA
		BDB77	2003	F	<0.01	<500	NA	2016	pos	pos	pos	pos	NA
		BDB79	NA	M	0.04	2,000	R	2010	pos	pos	pos	pos	NA
		BDA8	NA	F	1.5	75,000	R	2010	pos	pos	pos	pos	NA
		BDA9	NA	M	<0.01	<500	R	2010	pos	pos	pos	pos	NA
		BDA10	NA	F	<0.01	<500	R	2010	pos	pos	pos	pos	NA
	Mali	BDA14	1980	M	NA	NA	NA	2015	pos	pos	pos	pos	NA
		BDA18	1977	M	1.29	64,500	NA	2015	neg	neg	pos	pos	pos/pos/pos
		BDB62	1971	M	0.29	14,500	NA	2016	pos	pos	pos	pos	NA
	Nigeria	BDA11	1972	M	0.05	2,500	NA	2015	pos	pos	pos	pos	NA
		BDA15	1977	M	0.2	10,000	NA	2015	pos	pos	pos	pos	NA
		BDA17	1968	M	0.15	7,500	NA	2015	pos	pos	pos	pos	NA
		BDA24	1989	M	1.1	55,000	NA	2015	neg	neg	pos	pos	pos/pos/pos
		BDA25	1989	M	0.36	18,000	NA	2015	pos	pos	pos	pos	NA
		BDA29	1966	M	1.1	55,000	NA	2015	pos	pos	pos	pos	NA
		BDA44	1977	M	1.01	50,500	NA	2014	pos	pos	pos	pos	NA
		BDA48	1981	F	<0.01	<500	NA	2014	pos	pos	pos	pos	NA
		BDA60	1977	M	0.61	30,500	P	2014	pos	pos	pos	pos	NA
		BDA77	1982	M	<0.01	<500	NA	2013	pos	pos	pos	pos	NA

Continent	Country	Specimen voucher	Year of patient's birth	Sex	Parasitemia % RBC	Parasitemia Parasites/μL	Further Notes*	Year of collection	*pfhrp2* exon 1–2	*pfhrp2* exon 2	*pfhrp3* exon 1–2	*pfhrp3* exon 2	*pfmsp1/pfmsp2 /pfglurp*
		BDA78	1976	M	NA	NA	NA	2013	pos	pos	pos	pos	NA
		BDA79	1976	M	1	50,000	NA	2013	pos	pos	pos	pos	NA
		BDA80	1976	M	<0.01	<500	NA	2013	pos	pos	pos	pos	NA
		BDA86	1968	M	<0.01	<500	P	2012	pos	pos	pos	pos	NA
		BDA87	1972	M	1.1	55,000	NA	2012	pos	pos	pos	pos	NA
		BDA91	1981	M	2.5	125,000	NA	2012	neg	neg	pos	pos	pos/pos/pos
		BDA92	1979	M	4	200,000	NA	2012	neg	neg	pos	pos	pos/pos/pos
		BDA93	1979	M	0.1	5,000	NA	2012	pos	pos	pos	pos	NA
		BDA94	1988	M	<0.01	<500	NA	2012	pos	pos	pos	pos	NA
		BDB7	1992	M	<0.01	<500	NA	2012	pos	pos	pos	pos	NA
		BDB8	1992	M	<0.01	<500	NA	2012	pos	pos	pos	pos	NA
		BDB16	1987	M	0.2	10,000	NA	2011	pos	pos	pos	pos	NA
		BDB22	1932	M	<0.01	<500	NA	2011	pos	pos	pos	pos	NA
		BDB23	1932	M	0.3	15,000	NA	2011	pos	pos	pos	pos	NA
		BDB25	1939	M	NA	NA	TF	2011	pos	pos	pos	pos	NA
		BDB31	1982	M	0.08	4,000	CC	2011	neg	neg	pos	pos	pos/pos/pos
		BDB44	1970	M	<0.01	<500	NA	2010	pos	pos	pos	pos	NA
		BDB58	1987	M	0.1	5,000	NA	2016	pos	pos	pos	pos	NA
		BDB72	1976	M	<0.01	<500	NA	2016	pos	pos	pos	pos	NA
		BDA65	1956	M	1	50,000	NA	2013	pos	pos	pos	pos	NA
	Sierra Leone	BDA43	1991	F	12.25	612,500	NA	2014	pos	pos	pos	pos	NA
		BDA49	1972	M	<0.01	<500	R	2014	pos	pos	pos	pos	NA
		BDA56	1992	M	0.1	5,000	NA	2014	pos	pos	pos	pos	NA
		BDA84	1966	F	0.2	10,000	NA	2013	pos	pos	pos	pos	NA
		BDB13	1994	NA	0.06	3,000	PNC	NA	pos	pos	pos	pos	NA
		BDB66	1969	M	<0.01	<500	NA	2016	pos	pos	pos	pos	NA
		BDB78	1971	M	<0.01	<500	NA	2017	pos	pos	pos	pos	NA
		BDB81	1982	F	0.33	16,500	NA	2017	pos	pos	pos	pos	NA
		BDB83	1972	M	0.15	7,500	NA	2017	pos	pos	pos	pos	NA
		BDB85	1978	M	<0.01	<500	NA	2017	pos	pos	pos	pos	NA
		BDB87	1989	F	0.38	19,000	NA	2017	pos	pos	pos	pos	NA
		BDB96	1979	M	0.05	2,500	NA	2017	pos	pos	pos	pos	NA
		BDC4	1986	M	0.5	25,000	NA	2018	pos	pos	pos	pos	NA
	South Africa	BDA40	1957	F	NA	NA	P	2014	pos	pos	pos	pos	NA
		BDB82	1959	F	<0.01	<500	NA	2017	pos	pos	pos	pos	NA
	South Sudan	BDA68	1993	M	0.4	20,000	NA	2013	pos	pos	pos	pos	NA
		BDA69	1993	M	1.5	75,000	NA	2013	pos	pos	pos	pos	NA
		BDA97	1945	F	0.03	1,500	NA	2012	pos	pos	pos	pos	NA
		BDB60	1970	M	0.15	7,500	NA	2016	pos	pos	pos	pos	NA
		BDB63	1983	M	<0.01	<500	NA	2016	pos	pos	pos	pos	NA
		BDB90	2000	F	<0.01	<500	R	2017	pos	pos	pos	pos	NA
		BDB94	1988	M	0.5	25,000	NA	2017	neg	neg	pos	pos	pos/pos/pos
		BDB95	1942	M	1.3	65,000	NA	2017	pos	pos	pos	pos	NA
		BDB97	1972	M	1.3	65,000	NA	2017	pos	pos	pos	pos	NA
		BDB99	1983	M	<0.01	<500	NA	2017	neg	neg	pos	pos	pos/pos/pos
		BDC99	2017	F	0.13	6,500	R	2018	pos	pos	pos	pos	NA
		BDD01	1988	M	0.26	13,000	R	2018	pos	pos	pos	pos	NA

Continent	Country	Specimen voucher	Year of patient's birth	Sex	Parasitemia % RBC	Parasitemia Parasites/μL	Further Notes*	Year of collection	*pfhrp2* exon 1–2	*pfhrp2* exon 2	*pfhrp3* exon 1–2	*pfhrp3* exon 2	*pfmsp1/pfmsp2 /pfglurp*
		BDD02	1975	M	0.05	2,500	R, CC	2018	pos	pos	pos	pos	NA
		BDD03	1980	M	0.5	25,000	R, CC	2018	pos	pos	pos	pos	NA
		BDD04	1991	M	1.24	62,000	R	2018	pos	pos	neg	neg	pos/pos/pos
		BDD05	1985	M	0.03	1,500	R	2018	pos	pos	pos	pos	NA
		BDC98	1964	F	0.50	25,000	R	2018	neg	neg	pos	pos	pos/pos/pos
	Sudan	BDA2	1996	M	0.79	39,500	P, TF	2016	neg	neg	pos	pos	pos/pos/pos
		BDA3	1995	M	0.3	15,000	P	2016	neg	neg	pos	pos	pos/pos/pos
		BDA4	1983	M	0.02	1,000	NA	2016	neg	neg	pos	pos	pos/pos/pos
		BDA16	1983	M	1.7	85,000	P	2015	pos	pos	pos	pos	NA
		BDA19	1975	F	0.2	10,000	P	2015	pos	pos	pos	pos	NA
		BDA21	1978	M	<0.01	<500	NA	2015	pos	pos	pos	pos	NA
		BDA22	1978	M	0.2	10,000	NA	2015	pos	pos	pos	pos	NA
		BDA23	1978	M	1.2	60,000	NA	2015	pos	pos	pos	pos	NA
		BDA26	1978	F	<0.01	<500	NA	2015	pos	pos	pos	pos	NA
		BDA27	1971	M	0.02	1,000	PNC	2015	pos	pos	pos	pos	NA
		BDA33	2007	F	1.29	64,500	NA	2015	pos	pos	pos	pos	NA
		BDA34	2007	F	0.6	30,000	NA	2015	pos	pos	pos	pos	NA
		BDA37	1946	M	<0.01	<500	NA	2014	neg	neg	pos	pos	pos/pos/pos
		BDA39	1946	F	NA	NA	TF	2014	pos	pos	pos	pos	NA
		BDA41	1970	M	0.06	3,000	NA	2014	pos	pos	pos	pos	NA
		BDA51	1976	F	0.67	33,500	NA	2014	pos	pos	pos	pos	NA
		BDA53	1979	M	0.09	4,500	NA	2014	pos	pos	pos	pos	NA
		BDA95	1957	M	3	150,000	NA	2012	pos	pos	pos	pos	NA
		BDB14	1977	M	0.5	25,000	PNC	2011	pos	pos	pos	pos	NA
		BDB15	2006	M	0.8	40,000	NA	2011	pos	pos	pos	pos	NA
		BDB27	1955	NA	NA	7,000	NA	NA	pos	pos	pos	pos	NA
		BDB28	1975	M	2	100,000	P	2011	pos	pos	pos	pos	NA
		BDB50	1974	M	0.63	31,500	NA	2010	pos	pos	pos	pos	NA
		BDB53	1975	M	3.7	185,000	PNC	2016	pos	pos	pos	pos	NA
		BDB54	1975	M	0.63	31,500	NA	2016	pos	pos	pos	pos	NA
		BDB55	1996	M	NA	NA	NA	NA	pos	pos	pos	pos	NA
		BDB56	1976	M	0.07	3,500	NA	2016	pos	pos	pos	pos	NA
		BDB57	1976	M	0.5	25,000	NA	2016	pos	pos	pos	pos	NA
		BDB64	1961	M	0.6	30,000	CC	2016	pos	pos	pos	pos	NA
		BDB67	1984	M	0.8	40,000	NA	2016	pos	pos	pos	pos	NA
		BDB68	1988	M	0.02	1,000	NA	2016	pos	pos	pos	pos	NA
		BDB70	1992	F	<0.01	<500	NA	2016	pos	pos	pos	pos	NA
		BDB71	1947	M	<0.01	<500	NA	2016	pos	pos	pos	pos	NA
		BDB73	2011	M	1.3	65,000	NA	2016	pos	pos	pos	pos	NA
		BDB74	1998	F	<0.01	<500	NA	2016	pos	pos	pos	pos	NA
		BDB75	2010	F	0.5	25,000	NA	2016	pos	pos	pos	pos	NA
		BDB76	1977	M	0.1	5,000	NA	2016	pos	pos	pos	pos	NA
		BDB89	2001	M	<0.01	<500	NA	2017	pos	pos	pos	pos	NA
		BDC5	2001	F	2	100,000	NA	2018	pos	pos	neg	neg	pos/pos/pos
	Sumatra	BDA36	1965	M	1.7	85,000	NA	2014	pos	pos	pos	pos	NA
		BDC2	1968	M	0.1	5,000	NA	2017	pos	pos	neg	neg	pos/pos/pos
	Tanzania	BDA28	1968	M	<0.01	<500	CC	2015	neg	neg	pos	pos	pos/pos/pos

Continent	Country	Specimen voucher	Year of patient's birth	Sex	Parasitemia % RBC	Parasitemia Parasites/μL	Further Notes*	Year of collection	*pfhrp2* exon 1–2	*pfhrp2* exon 2	*pfhrp3* exon 1–2	*pfhrp3* exon 2	*pfmsp1/pfmsp2 /pfglurp*
		BDA47	1971	M	<0.01	<500	NA	2014	pos	pos	pos	pos	NA
		BDB61	1990	M	<0.01	<500	NA	2016	pos	pos	pos	pos	NA
		BDA82	1985	M	0.9	45,000	PNC	2013	pos	pos	pos	pos	NA
	Togo	BDA50	1983	M	0.27	13,500	NA	2014	neg	neg	pos	pos	pos/pos/pos
	Uganda	BDA54	1984	M	1.07	53,500	NA	2014	pos	pos	pos	pos	NA
		BDB47	1980	M	1.2	60,000	NA	2010	pos	pos	pos	pos	NA
	Zambia	BDA31	1958	M	0.2	10,000	NA	2015	neg	neg	pos	pos	pos/pos/pos
		BDB3	1960	M	<0.01	<500	NA	2012	pos	pos	pos	pos	NA
		BDB32	1956	F	0.04	2,000	NA	2011	pos	pos	pos	pos	NA
		BDB59	1939	F	8	400,000	NA	2016	pos	pos	pos	pos	NA
		BDB92	1943	M	0.6	30,000	P	2017	pos	pos	pos	pos	NA
	Zimbabwe	BDA1	1975	F	0.16	80,00	NA	2016	pos	pos	pos	pos	NA
Asia	Cambodia	BDC6	1971	F	3.3	165,000	TF	2018	pos	pos	pos	pos	NA
	India	BDB48	1940	M	<0.01	<500	NA	2010	pos	pos	pos	pos	NA
		BDB49	1940	M	0.1	5,000	NA	2010	pos	pos	pos	pos	NA
		BDB69	2011	F	3	150,000	NA	2016	pos	pos	pos	pos	NA
	Indonesia	BDB43	1958	M	2	100,000	NA	2010	pos	pos	pos	pos	NA
	Papua New Guinea	BDA30	1958	M	<0.01	<500	P, TF	2015	pos	pos	pos	pos	NA
		BDA35	1986	F	0.3	15,000	NA	2015	pos	pos	pos	pos	NA
		BDB5	1996	M	<0.01	<500	NA	2012	pos	pos	pos	pos	NA
		BDB12	1973	M	0.7	35,000	NA	2011	pos	pos	pos	pos	NA
		BDB34	1991	NA	0.8	40,000	NA	NA	pos	pos	pos	pos	NA
	Thailand	BDA20	1981	M	0.19	9,500	NA	2015	pos	pos	pos	pos	NA
South America	Peru	BDA52	1986	F	0.4	20,000	CC	2014	pos	pos	neg	neg	pos/pos/pos

* CC, reported concurrent condition; NA, not applicable; neg, negative; P, prophylaxis compliant; PNC, prophylaxis noncompliant; pos, positive; R, known refugee status; TF, previous treatment failure.
†Green shading indicates negative results for the target from both assays.

References

<jrn>1. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, et al. Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. J Infect Dis. 2005;192:870–7. PubMed https://doi.org/10.1086/432010</jrn>

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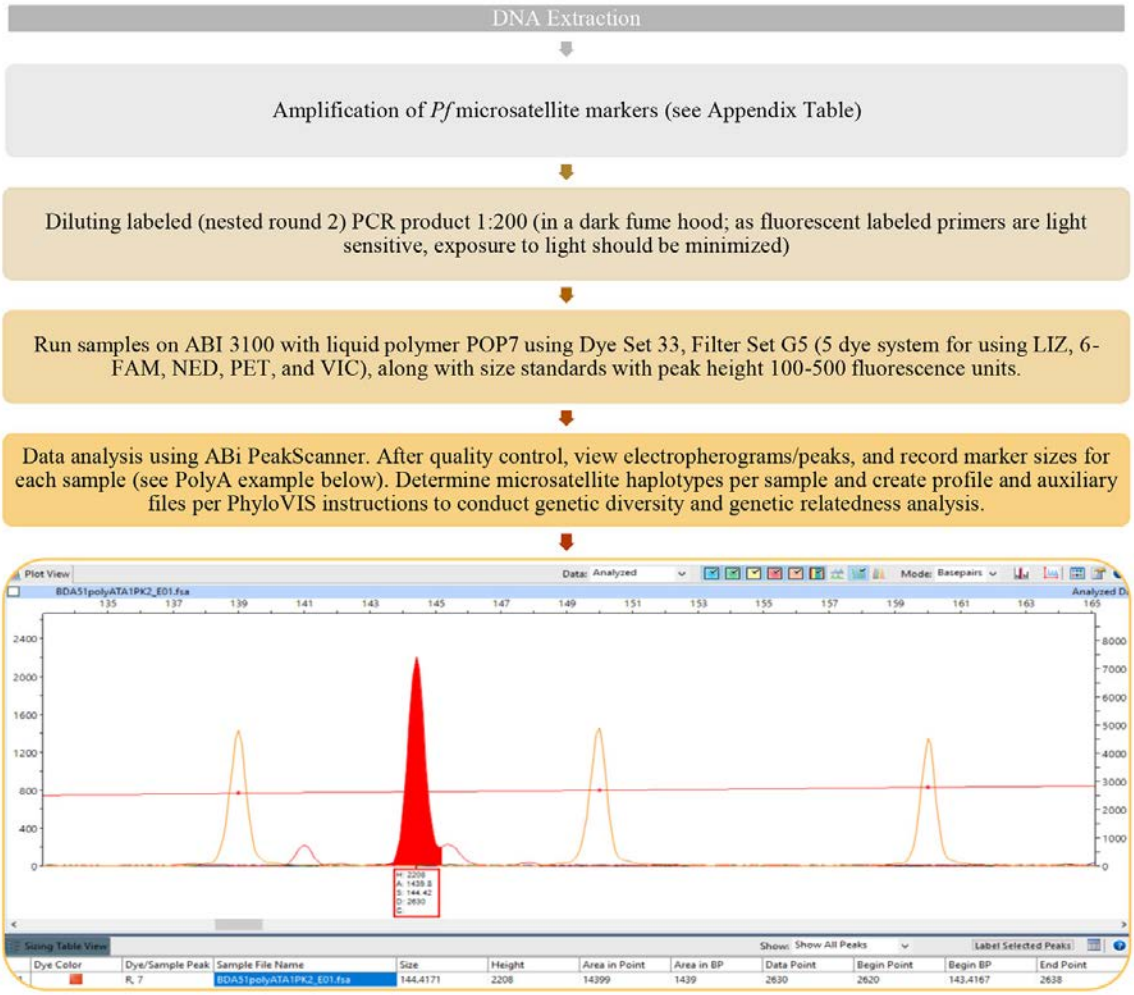
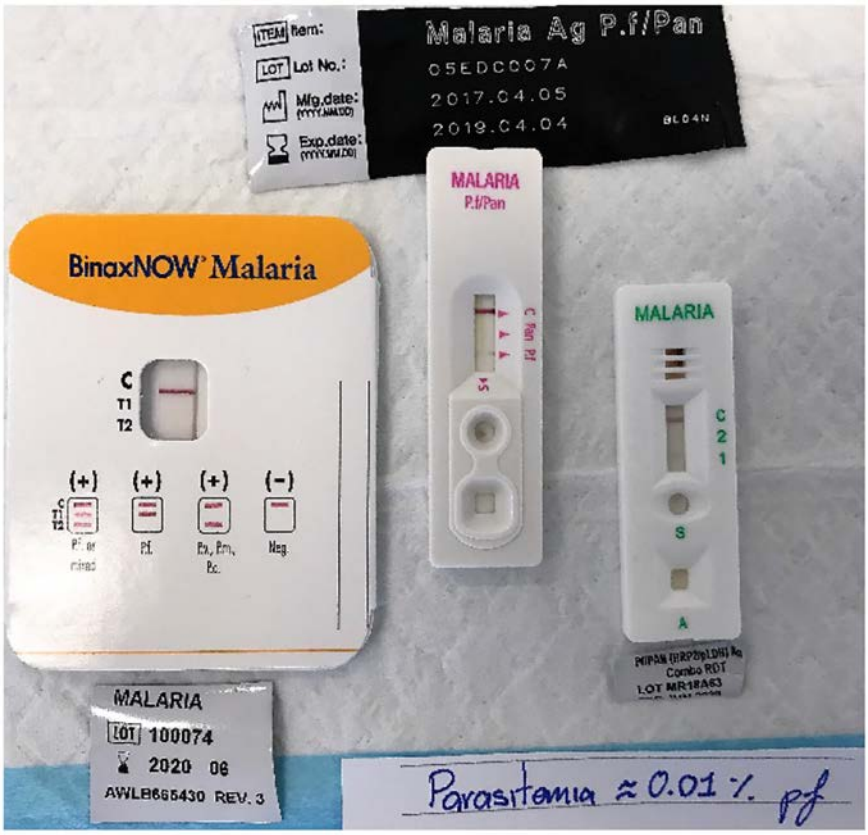
<jrn>3. Cheng Q, Gatton ML, Barnwell J, Chiodini P, McCarthy J, Bell D, et al. *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. Malar J. 2014;13:283. https://doi.org/10.1186/1475-2875-13-283</jrn>

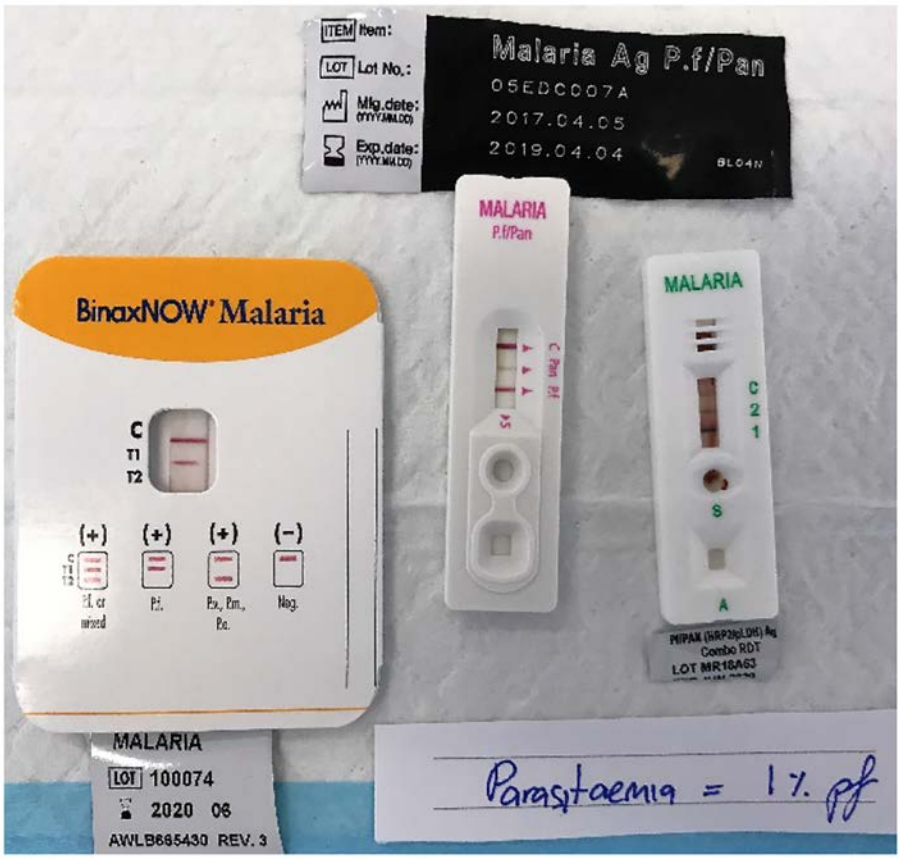
<jrn>4. Berhane A, Anderson K, Mihreteab S, Gresty K, Rogier E, Mohamed S, et al. Major threat to malaria control programs by *Plasmodium falciparum* lacking histidine-rich protein 2, Eritrea. Emerg Infect Dis. 2018;24:462–70. https://doi/org/10.3201/eid2403.171723</jrn>



Appendix Figure 1. Workflow for *P. falciparum* microsatellite analysis.



Appendix Figure 2. Results for sample BDB99 (originating from South Sudan, 2017, <0.01% parasitemia, *pfhrp2* negative/*pfhrp3* positive) using BinaxNOW, CareStart, and BioLine HRP2-based RDTs. CareStart and SD BioLine show no pan *spp.* band and a faint (1) *pf* band. BinaxNOW shows a negative pan *spp.* and *pf* outcome. Testing was conducted at Westmead Institute for Medical Research, Westmead, Australia, on January 4, 2019.



Appendix Figure 3. Results for sample BDD4 (originating from South Sudan, 2018, 1.24% parasitemia, *pfhrp2* positive/*pfhrp* negative) using BinaxNOW, CareStart, and BioLine HRP2-based RDTs. CareStart and SD BioLine show a faint (1) pan *spp.* band and a strong (4) *pf* band. BinaxNOW shows a negative pan *spp.* outcome and a moderate (2) *pf* band. Testing was conducted at Westmead Institute for Medical Research, Westmead, Australia, on January 4, 2019.