

## *Plasmodium falciparum* Histidine-Rich Protein 2 Gene Variation in a Malaria-Endemic Area of Papua New Guinea

Nigani Willie, Peter A. Zimmerman, and Rajeev K. Mehlotra\*

Center for Global Health and Diseases, Case Western Reserve University School of Medicine, Cleveland, Ohio

**Abstract.** Histidine-rich protein 2 of *Plasmodium falciparum* (PfHRP2) forms the basis of many current malaria rapid diagnostic tests (RDTs). It is concerning that there are parasites that lack part or all of the *pfhrp2* gene, and thus do not express the PfHRP2 protein; such parasites are not identifiable by PfHRP2-detecting RDTs. Very limited information is available regarding *pfhrp2* genetic variation in Papua New Guinea (PNG). In the present study, this gene variation was evaluated using 169 samples previously collected from the Wosera area in East Sepik Province of PNG. Molecular diagnosis of these samples showed that 81% were infected, and *P. falciparum* was present in 91% of those infected samples. One hundred and twenty samples were amplified for *pfhrp2* exon-2, from which 12 randomly selected amplicons were sequenced, yielding 18 sequences, all of which were unique. Baker repeat type 2 × type 7 numbers ranged from 0 to 108. Epitope mapping analysis revealed that three major epitopes, DAHHAHHA, AHHAADAHHA, and AHHAADAHH, were present in high prevalence and frequencies. These major epitopes have been shown to be recognized by the monoclonal antibodies 3A4 and PTL-3 (DAHHAHHA), C1-13 (AHHAADAHHA), and S2-5 and C2-3 (AHHAADAHH). This study provides further information on the high genetic variation of *pfhrp2* and its unclear relationship with prediction of RDT detection sensitivity, and identifies major epitopes in this gene from PNG. These results could be relevant and useful to understand the genetic diversity of this gene and the performance of current and future RDTs in this malarious region of the world.

### INTRODUCTION

Histidine-rich protein 2 (HRP2) is a unique protein produced exclusively by *Plasmodium falciparum*, and thus has been used as a biomarker for falciparum malaria infection.<sup>1</sup> In addition, it forms the basis of many current rapid diagnostic tests (RDTs).<sup>2</sup> Over the past 10 years, HRP2-detecting RDTs have become a widely used diagnostic tool for *P. falciparum*, especially in endemic areas that may not have appropriate microscopy capacity available. Several such HRP2-based combination RDTs have been tested in Papua New Guinea (PNG) in various populations: ParaSight-F dipstick in adults<sup>3</sup> and children (0.5–5 years)<sup>4</sup> from the Wosera area (Wosera-Gawi district) in East Sepik Province; ICT (HRP2 and pLDH) in infants (3–27 months) from Mugil (Madang Province) and Maprik (East Sepik Province)<sup>5</sup>; ICT (HRP2 and aldolase) in children (0.5–10 years) from Madang Province<sup>6</sup>; and CareStart™ in pregnant women from Madang Province.<sup>7</sup> In these studies, the sensitivities of the RDTs for *P. falciparum* detection varied from 45.6%<sup>7</sup> to 96%.<sup>6</sup>

A number of host and parasite factors may influence the accuracy and sensitivity of RDTs detecting *P. falciparum*-specific HRP2 (PfHRP2).<sup>8</sup> Most importantly, parasites lacking part or all of the *pfhrp2* gene do not express the PfHRP2 protein and are, therefore, not identifiable by PfHRP2-detecting RDTs. Recent studies have reported *pfhrp2* gene deletions in field isolates of *P. falciparum* from South and Central America, Asia, and Africa, resulting in false-negative test results.<sup>8</sup> Given the emphasis on the World Health Organization test and treat guidelines (<http://www.who.int/malaria/publications/atoz/9789241549127/en/>), *P. falciparum* parasites lacking *pfhrp2* raise concerns that antimalarial treatment

could be withheld from infected patients, and this scenario could potentially undermine malaria elimination efforts.

Studies of the *pfhrp2* gene worldwide show that polymorphism within this gene is extensive, and the correlation between this genetic variation and RDT detection sensitivity remains unclear.<sup>9,10</sup> Initially, testing ParaCheck Pf and ICT Malaria Pf RDTs against 16 cultured lines/isolates from Africa and Asia-Pacific, Baker et al.<sup>10</sup> developed a binary logistic regression model that was able to predict detection sensitivity of these two RDTs based on *pfhrp2* sequence structure. The model predicted an isolate to be detected at ≤ 250 parasites/μL if the number of amino acid repeats type 2 × type 7 was > 43, with an accuracy of 87.5%. Subsequently, using this analysis, 34 PfHRP2-detecting RDTs were tested against 79 global isolates at 200 parasites/μL.<sup>9</sup> In this testing, the regression model did not show a correlation between *pfhrp2* sequence structure and the overall RDT detection rates. Thus, the genetic variation in *pfhrp2* does not appear to affect RDT detection sensitivity at ≥ 200 parasites/μL.<sup>9</sup> Consistent with Baker et al.'s subsequent conclusion, similar results have been reported by Kumar Bharti et al.<sup>11</sup> using *P. falciparum*-positive blood samples collected from 15 sites in eight malaria-endemic states in India and by Willie et al.<sup>12</sup> using samples collected from three health centers in the Ampasimpotsy area in Madagascar.

Previous studies included a very limited number of samples from PNG.<sup>9,10</sup> In one study, nine samples from PNG (three, Bougainville; six, collection area not defined) were included.<sup>10</sup> *Plasmodium falciparum* histidine-rich protein 2 exon-2 sequencing revealed high genetic diversity among these samples (89%, eight unique sequences). In the second study, 17 samples from PNG (collection area not defined) were included, which also showed extensive genetic variation (71%, 12 unique sequences).<sup>9</sup> Although one parasite line (D10) from PNG has been reported to have *pfhrp2* gene deletion,<sup>13</sup> neither of these two studies found any evidence of *pfhrp2* deletion in PNG.<sup>9,10</sup> Finally, the studies that have tested PfHRP2-detecting RDTs did not analyze *pfhrp2* variation.<sup>3–7</sup>

\* Address correspondence to Rajeev K. Mehlotra, Center for Global Health and Diseases, Case Western Reserve University School of Medicine, Biomedical Research Bldg., Room 409A, 2109 Adelbert Rd., Cleveland, OH 44106-4983. E-mail: rkm@case.edu

The primary aim of the present study was to provide further insights into *pfhrp2* variation in PNG. For this aim, 169 samples were randomly selected from a previous malaria epidemiological study conducted in the Wosera area.<sup>14</sup> Using these samples, molecular assays to detect *Plasmodium* spp. infection and analyze *pfhrp2* gene sequences were performed.

## MATERIALS AND METHODS

**Study samples.** Samples previously collected for a malaria epidemiological study conducted in collaboration with the PNG Institute of Medical Research at Case Western Reserve University, Cleveland, Ohio, were used.<sup>14</sup> In this study, there were four serial cross-sectional surveys conducted between August 2001 and June 2003 in 29 villages in the Wosera area. From these surveys, a total of 16,209 blood samples were collected from 8,793 villagers aged < 2 (2.1%) to ≥ 40 (16.7%) years. In all surveys, most participants were asymptomatic. All these blood samples were analyzed for *Plasmodium* blood-stage infections using standard blood smear microscopy and molecular *Plasmodium* species-specific, post-polymerase chain reaction (PCR)/ligase detection reaction-fluorescent microsphere assay (LDR-FMA).<sup>15</sup> It is to be noted that the samples collected in this epidemiological study were before the RDTs were made widely available at the point-of-care in the country, and, therefore, RDT-based diagnosis was not performed. From this study,<sup>14</sup> 169 already extracted genomic DNA samples, which represented 10% of all samples, were randomly selected, which belonged to five of the 29 villages. These were Kumunugum 1 (also called Kuminkum 1), Kumunugum 2 (also called Kuminkum 2), Kumunugum 3 (also called Kuminkum 3), Mikau 1 (also called Miko 1), and Mikau 2 (also called Miko 2).

**Molecular diagnosis of *Plasmodium* spp. infections.** Molecular diagnosis of *Plasmodium* spp. was performed using PCR followed by LDR-FMA. All methods for PCR amplification of small subunit rRNA target sequences and *Plasmodium* species-specific detection by LDR-FMA have been described in detail by McNamara et al.<sup>15</sup> Genomic DNA extracted from *P. falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*-infected blood samples, provided by the Malaria Research and Reference Reagent Resource Center and Dr. W. E. Collins (Centers for Disease Control and Prevention), served as positive controls. DNA was extracted from 50 to 100 μL of each sample using a QIAamp<sup>®</sup> DNA Micro Kit (QIAGEN, Valencia, CA).

**Amplification and sequence analysis of *pfhrp2* gene.** The *pfhrp2* exon-2 (expected size 836 base pairs [bp]) was amplified using the primers and amplification conditions described elsewhere.<sup>12,16</sup> Genomic DNA from the *P. falciparum* strains 3D7 and HB3 were used as positive controls, whereas that from the Dd2 strain, which lacks the *pfhrp2* gene,<sup>17</sup> was used as a negative control in these amplification reactions. Amplicons were purified using a QIAquick<sup>®</sup> PCR Purification Kit (QIAGEN). The nucleotide sequences in all purified amplicons were determined by Sanger sequencing, in both forward and reverse directions, which was performed using the Big-Dye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit protocol from Applied Biosystems (Foster City, CA).

CodonCode Aligner program (v.6.0.2; CodonCore Corporation, Centerville, MA) was used for the alignment and base-calling of the raw sequences. Geneious software (v.10.0.2;

Biomatters Ltd., Auckland, New Zealand) was used for the alignment of sequences, virtual construction of the *pfhrp2* gene, translation into protein sequences, grouping of specific amino acid repeats, identification of insertions/deletions, and comparison with the *pfhrp2* sequences from previous studies. The sequences generated in this study ( $N = 18$ , see Results) were compared with a total of 15 sequences from the two previous studies (GenBank accession numbers AY816241.1–AY816243.1, AY816253.1–AY816258.1<sup>10</sup>, FJ871161.1–FJ871163.1, FJ871241.1, FJ871294.1, and FJ871358.1<sup>9</sup>).

The *pfhrp2* gene sequences were translated into protein sequences and 24 amino acid repeat types (1–24) were classified as described by Baker et al.<sup>9,10</sup> Of these 24 amino acid repeat types (called Baker repeats), 20 (repeats 1–14, 19–24) are present in *pfhrp2*, whereas four (repeats 15–18) are present in the *pfhrp2* paralog gene *pfhrp3*.<sup>9</sup> Because RDT-based diagnosis was not performed on these samples,<sup>14</sup> both scenarios of the predictive model, based on the number of type 2 × type 7 repeats, were used to predict the sensitivity of an RDT detecting PfHRP2: (a) an isolate would be detectable at ≤ 250 parasites/μL if the number of repeat types is > 43<sup>10</sup> and (b) an isolate would be detectable at ≥ 200 parasites/μL regardless of the number of repeat types.<sup>9</sup> In addition, the sequences from this study ( $N = 18$ ) and previous studies ( $N = 15$ ) were analyzed for the identification and distribution of 13 major epitopes, ranging 8–15 amino acids, that are recognized by 11 PfHRP2-specific commercially available monoclonal antibodies (MAbs).<sup>18</sup>

The prevalence and frequency of each amino acid repeat type or epitope were calculated as described.<sup>12</sup>

**Ethics statement.** This study was conducted under the protocols approved by the PNG Medical Research Advisory Committee and University Hospitals of Cleveland Institutional Review Board.

## RESULTS

**Diagnosis of *Plasmodium* spp. infections.** Polymerase chain reaction diagnosis showed that 81% of all samples (137/169) were infected by *Plasmodium* spp. The LDR-FMA analysis showed that *P. falciparum* was present in 91% (125/137), *P. vivax* in 32% (44/137), *P. malariae* in 23% (31/137), and *P. ovale* in 2% (3/137) of the infected samples. Among those infected samples, 37% (51/137) showed mixed infections, predominantly including *P. falciparum* (36%, 50/137). On the other hand, only 26% of all samples (44/169) were positive by microscopy, all of them for *P. falciparum*, the majority (95%) as single infections. *Plasmodium falciparum* parasitemia ranged from 40 to 39,960 parasites/μL, and 32% (14/44) samples had parasitemia < 200 parasites/μL.

**Amplification of the *pfhrp2* gene.** Amplification of *pfhrp2* exon-2 showed band sizes of 450–900 bp on 1% agarose gels for 120 samples. A comparison between *pfhrp2* PCR and *P. falciparum* detection by LDR-FMA, used as the gold standard, is presented in Table 1. This analysis showed that among the 120 samples that were *pfhrp2* PCR positive, 109 samples were *P. falciparum* positive and 11 samples were *P. falciparum* negative. Thus, these 11 samples were considered false positives. Of these, only one sample was *P. falciparum* positive by microscopy (parasitemia 1,120 parasites/μL), whereas 10 samples were *P. falciparum* negative by microscopy. Thus, these 10 samples, positive for *pfhrp2* but *P. falciparum*

TABLE 1

Comparison of *pfhrp2* polymerase chain reaction and *Plasmodium falciparum* ligase detection reaction–fluorescent microsphere assay

	<i>P. falciparum</i> <sup>+</sup>	<i>P. falciparum</i> <sup>-</sup>	Total
<i>pfhrp2</i> <sup>+</sup>	109	11*	120
<i>pfhrp2</i> <sup>-</sup>	16†	33	49
Total	125	44	169

*pfhrp2* = *P. falciparum* histidine-rich protein 2 gene.

\* 1, microscopy<sup>+</sup>; 10, microscopy<sup>-</sup>.

† 2, microscopy<sup>+</sup>; 14, microscopy<sup>-</sup>.

negative by both LDR-FMA and microscopy, were considered to be “true” false positives. In addition, 16 *P. falciparum*-positive samples did not amplify for the *pfhrp2* gene. Thus, these 16 samples were considered false negatives. Of these, 14 samples were *P. falciparum* negative by microscopy, whereas two samples were *P. falciparum* positive by microscopy (parasitemia 40 and 840 parasites/μL). Thus, these two samples, negative for *pfhrp2* but *P. falciparum* positive by both LDR-FMA and microscopy, were considered to be “true” false negatives.

The overall concordance between *pfhrp2* PCR and *P. falciparum* LDR-FMA molecular assays for this set of samples was 84%. Combining all three diagnostic assays, namely, *pfhrp2* PCR, *P. falciparum* LDR-FMA, and microscopy, the sensitivity and specificity of *pfhrp2* gene detection were 98% and 82%, respectively. Rapid diagnostic test-based diagnosis of malaria was not available for these samples.

**Sequence analysis of the *pfhrp2* gene.** Of the 109 *pfhrp2* amplicons from the samples that were *P. falciparum* positive (Table 1), 12 were randomly selected for sequencing. From these 12 amplicons, a total of 18 exon-2 sequences were generated, ranging 468–858 bp (155–285 amino acids). A comparison among these 18 sequences revealed multiple-strain infections, inferred by the presence of two different sequences, in six of the 12 samples. It also showed that all 18 sequences were unique. Finally, a comparison between these 18 sequences and the 15 previous sequences from PNG showed that no sequence was shared between these two groups. Exon-2 sequences from this analysis were submitted to GenBank (accession numbers MF673786–MF673803).

Of the 20 amino acid repeats present in *pfhrp2* (repeats 1–14, 19–24), 13 repeats in our sequences and 12 repeats in previous PNG sequences were identified (Table 2). Repeat types 1, 2, 6, 7, and 12 were present in almost all sequences (89–100%). Repeat types 3, 5, 8, and 10 were also highly prevalent in all sequences (56–100%). Prevalence of repeat types 4, 13, and 19 was low to moderate in all samples (11–33%). The average frequency of each repeat within our and previous sequences is presented in Figure 1. Repeat types 2 and 7 were the most frequent within our (40%, range = 4–16, mean = 10; 16%, range = 0–9, mean = 4, respectively) and previous (37%, range = 7–15, mean = 11; 19%, range = 3–9, mean = 6, respectively) sequences.

**Prediction of RDT detection sensitivity.** To predict the detection of an isolate by a PfHRP2-detecting RDT, an analysis of Baker repeat type 2 × type 7 numbers and microscopy diagnosis for the 18 sequences are presented in Table 3. The model, which predicts that the number of repeat types > 43 would be needed to detect ≤ 250 parasites/μL (scenario [a]),<sup>10</sup>

TABLE 2

Prevalence of Baker repeat types in *pfhrp2* sequences

Repeat type	Sequence	Prevalence	
		This study (N = 18)	Previous studies (N = 15)
		% (n)	% (n)
1	AHHAHVAD	94 (17)	100 (15)
2	AHHAHHAAD	100 (18)	100 (15)
3	AHHAHHAAY	89 (16)	100 (15)
4	AHH	17 (3)	33 (5)
5	AHHAHHASD	78 (14)	93 (14)
6	AHHAATD	89 (16)	100 (15)
7	AHHAAD	94 (17)	100 (15)
8	AHHAAY	83 (15)	93 (14)
9	AAY	6 (1)	0 (0)
10	AHHAHHATD	56 (10)	93 (14)
11	AHN	0 (0)	0 (0)
12	AHHAHHHEAATH	100 (18)	100 (15)
13	AHHASD	17 (3)	13 (2)
14	AHHAHHATD	0 (0)	0 (0)
15*	AHHAHHAAN	0 (0)	0 (0)
16*	AHHAAN	0 (0)	0 (0)
17*	AHHDG	0 (0)	0 (0)
18*	AHHDD	0 (0)	0 (0)
19	AHHA	11 (2)	13 (2)
20	SHHDD	0 (0)	0 (0)
21	AHHAHHATY	0 (0)	0 (0)
22	AHHAHHAGD	0 (0)	0 (0)
23	ARHAAD	0 (0)	0 (0)
24	AHHTHHAAD	0 (0)	0 (0)

*pfhrp2* = *Plasmodium falciparum* histidine-rich protein 2 gene.

\* Not present in *pfhrp2*, present in *pfhrp3* (Baker et al.<sup>9</sup>).

showed one such sequence (77 repeats, 200 parasites/μL). There were seven sequences (39%) where the number of repeat types was < 43 and the parasite densities were ≤ 250 parasites/μL; five of these sequences were from the samples that had submicroscopic infections (SMI, *pfhrp2*<sup>+</sup> *P. falciparum*<sup>+</sup> microscopy<sup>-</sup>). In addition, three sequences with the number of repeat types > 43 were also from the samples that had SMI. The model, which predicts that ≥ 200 parasites/μL would be detected regardless of the number of repeat types (scenario [b]),<sup>9</sup> showed four such sequences (0–32 repeats, 280–600 parasites/μL). Finally, there were three sequences where the number of repeat types was > 43 and the parasite densities were well over 250 parasites/μL. Among these results, those where the number of repeat types was < 43 and the parasite densities were ≤ 250 parasites/μL, and those with SMI, regardless of the number of repeat types, make any prediction difficult.

**Major epitopes in *pfhrp2* exon-2 targeted by MAbs in RDTs.** Epitope identification analysis on all sequences revealed that eight of the 13 major epitopes were present in majority of the sequences (78–100%) (Table 4). Among these, three major epitopes, DAHHAHHA, AHHAADAHHA, and AHHAADAHH, were present within those sequences at much higher average frequencies (18–26%) than the other five major epitopes (2–8%). These three major epitopes have been shown to be recognized by PfHRP2-specific MAbs 3A4 and PTL-3 (DAHHAHHA), C1-13 (AHHAADAHHA), and S2-5 and C2-3 (AHHAADAHH).<sup>18</sup>

## DISCUSSION

Similar to previous malaria epidemiological studies in PNG, which used both standard blood smear microscopy and PCR-based

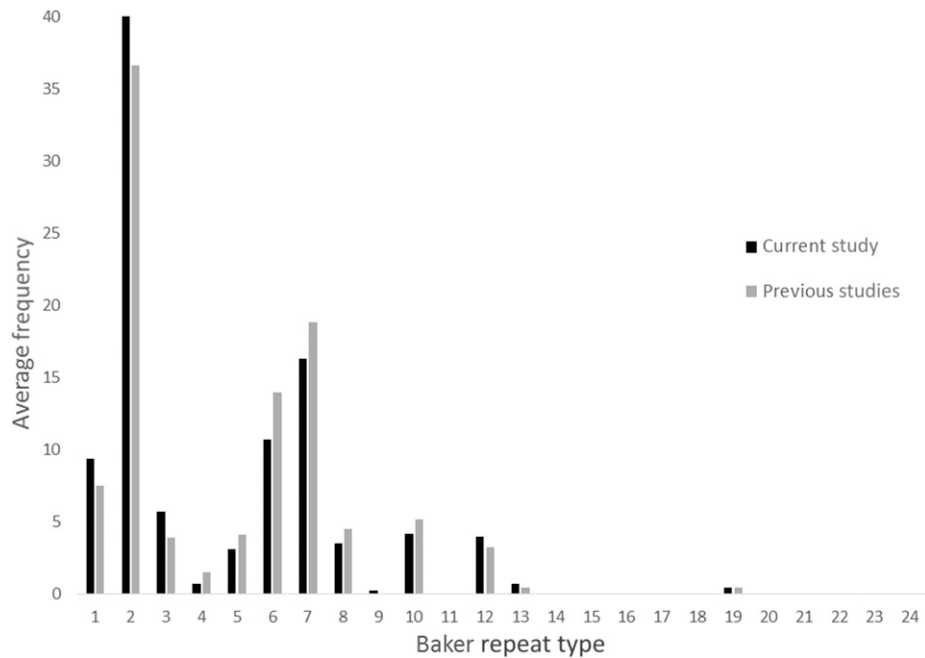


FIGURE 1. Average frequency of amino acid repeat types (Baker repeat types) in the sequences from the current study ( $N = 18$ ) and previous studies ( $N = 15$ ) from Papua New Guinea (PNG).

diagnosis,<sup>19,20</sup> we noticed a large difference in the overall prevalence of *Plasmodium* infections determined by using these methods (26% vs. 81%, respectively). These results reflect the well-known low concordance between microscopy and LDR-FMA diagnostic methods and are also consistent with the malaria infection characteristics of the Wosera area reported previously.<sup>14,19,21</sup>

The overall concordance between *pfhrp2* PCR and *P. falciparum* post-PCR/LDR-FMA was 84%, suggesting variation in amplification of two different target sequences in this set of samples. In our recent study conducted on samples

from Madagascar, the overall concordance between these two molecular assays was 97%.<sup>12</sup> The Wosera samples were collected and processed in 2001–2003, 15 or more years ago, and have been used for a number of molecular investigations since then. Thus, their storage and usage over the years, likely leading to some level of degradation, may be a factor to consider for the lower concordance. Regarding the 10 “true” false-positive samples (*pfhrp2*<sup>+</sup> *P. falciparum*<sup>-</sup> microscopy<sup>-</sup>), there could be other possible reasons, such as very low-level infections or, less likely, amplification of the *pfhrp2* paralog gene *pfhrp3*, which has a sequence homology of more than 75% in the tandem repeat region to *pfhrp2*.<sup>18,22</sup> None of these 10 samples were sequenced. There were two “true” false-negative samples (*pfhrp2*<sup>-</sup> *P. falciparum*<sup>+</sup> microscopy<sup>+</sup>). As RDTs were not performed on these samples,<sup>14</sup> *pfhrp2* deletion status of these two samples could not be determined (see Limitations).

The *pfhrp2* exon-2 sequence sizes (468–858 bp, 155–285 amino acids) were similar to those reported previously from PNG and globally.<sup>9,10</sup> All 18 sequences (100%) were found to be unique. This observation is similar to previous observations from PNG, where 89%<sup>10</sup> and 71%<sup>9</sup> *pfhrp2* sequence diversity was reported. In a global analysis, the *pfhrp2* sequence diversity was found to be higher in countries with high transmission intensity.<sup>9</sup> A strong correlation between malaria transmission intensity and *pfhrp2* diversity may be expected, as malaria infections in high-transmission settings, including those in PNG, often involve multiple-strain infections.<sup>23</sup> In the present study, using Sanger sequencing, two different sequences were identified in six of the 12 samples, implying multiple-strain infections. Sanger sequencing has limited ability to detect multiple parasite types in a mixed infection and can miss a significant proportion of minority variants. It is possible that the use of next-generation sequencing technology, which allows high-resolution analyses of a heterogeneous

TABLE 3

Baker repeat types and *Plasmodium falciparum* infection diagnosis

Sample*	Repeat type number (2 × 7)	LDR-FMA	Microscopy	Parasites/μL	RDT detection prediction
DFA4927	32 (16 × 2)	+	+	280	Yes†
DFA4929	80 (10 × 8)	+	-	-	SMI
DFA4934	77 (11 × 7)	+	+	200	Yes‡
DFA4938	22 (11 × 2)	+	-	-	SMI
DFA4940A	66 (11 × 6)	+	-	-	SMI
DFA4940B	42 (6 × 7)	+	-	-	SMI
DFA4942	55 (11 × 5)	+	-	-	SMI
DFA5008A	108 (12 × 9)	+	+	8,440	Yes
DFA5008B	60 (12 × 5)	+	+	8,440	Yes
DFB0007A	24 (8 × 3)	+	-	-	SMI
DFB0007B	21 (7 × 3)	+	-	-	SMI
DFB0013A	39 (13 × 3)	+	+	40	?
DFB0013B	11 (11 × 1)	+	+	40	?
DFB0014A	22 (11 × 2)	+	+	360	Yes†
DFB0014B	16 (4 × 4)	+	+	360	Yes†
DFB0031A	0 (7 × 0)	+	+	600	Yes†
DFB0013B	48 (8 × 6)	+	+	600	Yes
DFB0051	30 (15 × 2)	+	-	-	SMI

LDR-FMA = ligase detection reaction-fluorescent microsphere assay; RDT = rapid diagnostic test; SMI = submicroscopic infections.

\* GenBank accession numbers MF673786–MF673803.

† Scenario (b) (Baker et al.<sup>9</sup>).

‡ Scenario (a) (Baker et al.<sup>10</sup>).

TABLE 4  
Distribution of major epitopes in *pfhrp2* sequences

Major epitope	This study (N = 18)		Previous studies (N = 15)		MAb*
	Prevalence	Average frequency	Prevalence	Average frequency	
	% (n)	%	% (n)	%	
DAHHAHHA	100 (18)	18	100 (15)	18	3A4, PTL-3
DAHHAADAHH	94 (17)	8	100 (15)	7	2G12-1C12
DAHVVADAHH	0 (0)	0	0 (0)	0	2G12-1C12
YAHHAHHA	94 (17)	4	100 (15)	4	1E1-A9, PTL-3
DAHHAHHV	89 (16)	5	100 (15)	3	1E1-A9
HATDAHHAAD	61 (11)	2	67 (10)	3	A6-4
HATDAHHAHA	67 (12)	2	100 (15)	2	A6-4
AHHAADAHHA	100 (18)	26	100 (15)	26	C1-13
DAHHAADAHHA	94 (17)	8	100 (15)	7	N7
AHHAADAHH	100 (18)	26	100 (15)	26	S2-5, C2-3
AHHAADAHH	78 (14)	2	100 (15)	2	S2-5
TDAHHAADAHHAADA	50 (9)	1	53 (8)	1	TC-10
AAYAHHAHHAAY	0 (0)	0	0 (0)	0	Genway

MAb = monoclonal antibody; *pfhrp2* = *Plasmodium falciparum* histidine-rich protein 2 gene.

\* Lee et al.<sup>18</sup>

mixture of the parasites within the host,<sup>24,25</sup> may have enabled more comprehensive detection of *pfhrp2* sequences present in those samples. Although comparative analysis of the sequences showed that no two sequences were the same between our study and previous studies, the prevalence and frequency of each of the 12 Baker repeat types, common between these two groups, were comparable. This was also true for the repeat types 2 and 7, which were used in a model to predict RDT detection sensitivity.<sup>9,10</sup>

Detection sensitivity for different RDTs tested in different populations in PNG was as low as 45.6%<sup>7</sup> to as high as 96%.<sup>6</sup> Although the correlation between RDT performance and *pfhrp2* variation remains unclear,<sup>9-12</sup> the studies that have tested RDTs in PNG did not analyze *pfhrp2* variation.<sup>3-7</sup> All 18 sequences in the present study were generated from 12 samples that were *P. falciparum* positive by LDR-FMA. Thirty-nine percent (7/18) of these sequences had the number of type 2 × type 7 repeats < 43, ranging from 0 (7 × 0, respectively) to 42 (6 × 7, respectively). The parasitemia in these samples was from 40 parasites/μL to submicroscopic. This suggests that under scenario (a), these samples would not be detected, that is, they would be considered nonsensitive to RDT detection.<sup>10,16</sup> In addition, there were samples where the sequences had > 43 repeats, but those samples had SMI. It is important to recognize that commercial malaria RDTs were approved for detecting symptomatic malaria cases and not for detecting submicroscopic cases. Therefore, it is difficult to predict whether all these samples would be detected or not by an RDT. Given the field sample-based observations made by Kumar Bharti et al.<sup>11</sup> and Willie et al.,<sup>12</sup> verification of these RDT detection predictions made on the basis of the repeat numbers is required, particularly for the samples that had very low (< 50 parasites/μL) or submicroscopic parasitemia levels.

Depending on the RDT used and study setting and design, RDT may<sup>26,27</sup> or may not<sup>28,29</sup> perform better than microscopy in detecting SMI. Such infections, if undetectable by the current generation of RDTs, may compromise the current focus on malaria elimination in PNG.<sup>30</sup> Recently, funding agencies, manufacturers, and researchers have been working toward developing ultra-sensitive RDTs (uRDT) with limits of detection similar to those nucleic acid amplification-based methods. As a step in this direction, uRDT (Alere™ Malaria

Ag P.f (05FK140), Standard Diagnostics/Alere, San Diego, CA) has been commercialized with claims of an analytical sensitivity approximately 10 times the detection limit of the best conventional RDTs. Under laboratory conditions, using different *P. falciparum* culture strains, uRDT showed a greater than 10-fold improvement over the SD Bioline Malaria Ag P.f RDT (05FK50); *pfhrp2*-positive strains ITG and HB3 were detected down to 49 parasites/μL by the SD Bioline Malaria Ag P.f RDT, whereas the same strains were detected down to 3.13 parasites/μL by uRDT.<sup>31</sup> It may be valuable to test whether the use of such an RDT can improve detection of very low level infections/SMI in PNG.

Investigations of the effect of *pfhrp2* sequence variation on the binding of MAbs to PfHRP2<sup>1</sup> have led to the characterization of 13 major epitopes recognized by 11 such antibodies (Table 4).<sup>18,22</sup> The information regarding PfHRP2-specific MAbs being used in RDTs is generally not disclosed by the manufacturers. Epitope identification in all PNG sequences showed that epitopes DAHHAHHA, AHHAADAHHA, and AHHAADAHH were present in high prevalence and frequencies. Epitopes AHHAADAHHA and DAHHAHHA are recognized by MAbs C1-13 and PTL-3, respectively, both of which have shown the best potential (based on the prevalence and frequency of epitopes together with the heat durability profile) for use in an RDT.<sup>18</sup> These epitope identification results should be helpful for studies comparing performances of different RDTs in PNG and for those evaluating new antibodies for the development of improved RDTs.<sup>32</sup>

## LIMITATIONS

It is acknowledged that no RDT data were available pertaining to the samples analyzed in the present study. Based on recent field sample-based studies, where no direct relationship between *pfhrp2* repeat types and the ability to detect low-density infections was observed,<sup>11,12</sup> the detection predictions made in the present study should be verified with RDTs. This verification is also important given the finding that there was no correlation between *pfhrp2* sequence length or repeat type and PfHRP2 plasma concentration in African children.<sup>33</sup> In the present study, two samples, positive by *P. falciparum* LDR-FMA and microscopy, were *pfhrp2* PCR

negative (“true” false negatives in Table 1). In the absence of RDT data, *pfhrp2* deletion status of these two samples could not be predicted. In addition, attempts to amplify the *pfhrp2* paralog gene *pfhrp3* and the *pfhrp2* flanking genes PF3D7\_0831900 and PF3D7\_0831700, which provide confirmatory evidence of *pfhrp2* deletion,<sup>8</sup> could not be made because of exhaustion of the samples. HRP3 has a sequence homology of more than 75% in the tandem repeat region to HRP2 and is recognized by most anti-HRP2 MAbs including C1-13.<sup>18,22</sup> Although HRP3 is less abundant than HRP2,<sup>34</sup> in situations of low-level parasitemia or when parasites have *pfhrp2* deletions, it could enhance the sensitivity of the RDTs.<sup>31,35,36</sup> Finally, the samples in the present study were collected from a single area in PNG, before the RDTs were made widely available at the point-of-care and the selection pressure on the parasite population may have been exerted. Using more recent samples from multiple areas in PNG, further studies should evaluate the current status of *pfhrp2* variation together with performance of the PfHRP2-detecting RDTs.

In conclusion, the present study provides in-depth information regarding *pfhrp2* genetic variation in a malaria-endemic area of PNG, which could be a valuable starting point for testing and interpreting the results of RDTs. As heavy reliance is placed on RDTs for point-of-care malaria diagnosis in PNG,<sup>5-7</sup> it is essential from a public health perspective to perform routine monitoring, where the use of RDT is combined with the analysis of *pfhrp2* variation. Such monitoring may help alert the authorities to potential diagnostic failure risks and the need to explore complimentary/alternative diagnostic approaches in this malarious region of the world.

Received February 15, 2018. Accepted for publication May 24, 2018.

Published online July 2, 2018.

Acknowledgments: We thank all study participants and field technicians for their participation and support, and are grateful to Rosalind Howes for her valuable time and critical evaluation of this manuscript.

Financial support: N. W. was supported by a training grant from Fogarty International Center, National Institutes of Health (D43TW007377) to P. A. Z.

Authors' address: Nigani Willie, Peter A. Zimmerman, and Rajeev K. Mehlotra, Center for Global Health and Diseases, Case Western Reserve University School of Medicine, Cleveland, OH, E-mails: nxw144@case.edu, paz@case.edu, and rkm@case.edu.

## REFERENCES

- Parra ME, Evans CB, Taylor DW, 1991. Identification of *Plasmodium falciparum* histidine-rich protein 2 in the plasma of humans with malaria. *J Clin Microbiol* 29: 1629–1634.
- Moody A, 2002. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev* 15: 66–78.
- Genton B, Hii J, Paget S, Alpers MP, 1996. Rapid manual diagnosis of *Plasmodium falciparum* malaria using ParaSight-F dipsticks applied to human blood and urine. *J Travel Med* 3: 172–173.
- Genton B, Paget S, Beck HP, Gibson N, Alpers MP, Hii J, 1998. Diagnosis of *Plasmodium falciparum* infection using ParaSight®-F test in blood and urine of Papua New Guinean children. *Southeast Asian J Trop Med Public Health* 29: 35–40.
- Senn N, Rarau P, Manong D, Salib M, Siba P, Robinson LJ, Reeder J, Rogerson S, Mueller I, Genton B, 2012. Rapid diagnostic test-based management of malaria: an effectiveness study in Papua New Guinean infants with *Plasmodium falciparum* and *Plasmodium vivax* malaria. *Clin Infect Dis* 54: 644–651.
- Manning L, Laman M, Rosanas-Urgell A, Turlach B, Aipit S, Bona C, Warrell J, Siba P, Mueller I, Davis TM, 2012. Rapid antigen detection tests for malaria diagnosis in severely ill Papua New Guinean children: a comparative study using Bayesian latent class models. *PLoS One* 7: e48701.
- Umbers AJ et al., 2015. Accuracy of an HRP-2/panLDH rapid diagnostic test to detect peripheral and placental *Plasmodium falciparum* infection in Papua New Guinean women with anaemia or suspected malaria. *Malar J* 14: 412.
- Cheng Q, Gatton ML, Barnwell J, Chiodini P, McCarthy J, Bell D, Cunningham J, 2014. *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. *Malar J* 13: 283.
- Baker J et al., 2010. Global sequence variation in the histidine-rich proteins 2 and 3 of *Plasmodium falciparum*: implications for the performance of malaria rapid diagnostic tests. *Malar J* 9: 129.
- Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, Bell D, Cheng Q, 2005. 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* 192: 870–877.
- Kumar Bharti P, Singh Chandel H, Krishna S, Nema S, Ahmad A, Udhayakumar V, Singh N, 2017. Sequence variation in *Plasmodium falciparum* histidine rich proteins 2 and 3 in Indian isolates: implications for malaria rapid diagnostic test performance. *Sci Rep* 7: 1308.
- Willie N, Mehlotra RK, Howes RE, Rakotomanga TA, Ramboarina S, Ratsimbaoa AC, Zimmerman PA, 2018. Insights into the performance of SD Bioline Malaria Ag P.f/Pan rapid diagnostic test and *Plasmodium falciparum* histidine-rich protein 2 gene variation in Madagascar. *Am J Trop Med Hyg* 98: 1683–1691.
- Kemp DJ, Thompson JK, Walliker D, Corcoran LM, 1987. Molecular karyotype of *Plasmodium falciparum*: conserved linkage groups and expendable histidine-rich protein genes. *Proc Natl Acad Sci USA* 84: 7672–7676.
- Kasehagen LJ et al., 2006. Changing patterns of *Plasmodium* blood-stage infections in the Wosera region of Papua New Guinea monitored by light microscopy and high throughput PCR diagnosis. *Am J Trop Med Hyg* 75: 588–596.
- McNamara DT, Kasehagen LJ, Grimberg BT, Cole-Tobian J, Collins WE, Zimmerman PA, 2006. Diagnosing infection levels of four human malaria parasite species by a polymerase chain reaction/ligase detection reaction fluorescent microsphere-based assay. *Am J Trop Med Hyg* 74: 413–421.
- Mariette N, Barnadas C, Bouchier C, Tichit M, Menard D, 2008. Country-wide assessment of the genetic polymorphism in *Plasmodium falciparum* and *Plasmodium vivax* antigens detected with rapid diagnostic tests for malaria. *Malar J* 7: 219.
- Walker-Jonah A, Dolan SA, Gwadz RW, Panton LJ, Wellem TE, 1992. An RFLP map of the *Plasmodium falciparum* genome, recombination rates and favored linkage groups in a genetic cross. *Mol Biochem Parasitol* 51: 313–320.
- Lee N, Gatton ML, Pelecanos A, Bubb M, Gonzalez I, Bell D, Cheng Q, McCarthy JS, 2012. Identification of optimal epitopes for *Plasmodium falciparum* rapid diagnostic tests that target histidine-rich proteins 2 and 3. *J Clin Microbiol* 50: 1397–1405.
- Mehlotra RK, Kasehagen LJ, Baisor M, Lorry K, Kazura JW, Bockarie MJ, Zimmerman PA, 2002. Malaria infections are randomly distributed in diverse holoendemic areas of Papua New Guinea. *Am J Trop Med Hyg* 67: 555–562.
- Mehlotra RK, Lorry K, Kastens W, Miller SM, Alpers MP, Bockarie M, Kazura JW, Zimmerman PA, 2000. Random distribution of mixed species malaria infections in Papua New Guinea. *Am J Trop Med Hyg* 62: 225–231.
- Genton B, al-Yaman F, Beck HP, Hii J, Mellor S, Narara A, Gibson N, Smith T, Alpers MP, 1995. The epidemiology of malaria in the Wosera area, East Sepik Province, Papua New Guinea, in preparation for vaccine trials. I. Malarimetric indices and immunity. *Ann Trop Med Parasitol* 89: 359–376.
- Lee N, Baker J, Andrews KT, Gatton ML, Bell D, Cheng Q, McCarthy J, 2006. Effect of sequence variation in *Plasmodium falciparum* histidine-rich protein 2 on binding of specific monoclonal antibodies: implications for rapid diagnostic tests for malaria. *J Clin Microbiol* 44: 2773–2778.

23. Fola AA, Harrison GLA, Hazairin MH, Barnadas C, Hetzel MW, Iga J, Siba PM, Mueller I, Bary AE, 2017. Higher complexity of infection and genetic diversity of *Plasmodium vivax* than *Plasmodium falciparum* across all malaria transmission zones of Papua New Guinea. *Am J Trop Med Hyg* 96: 630–641.
24. Lalremruata A et al., 2017. Species and genotype diversity of *Plasmodium* in malaria patients from Gabon analysed by next generation sequencing. *Malar J* 16: 398.
25. Levitt B, Obala A, Langdon S, Corcoran D, O'Meara WP, Taylor SM, 2017. Overlap extension barcoding for the next generation sequencing and genotyping of *Plasmodium falciparum* in individual patients in western Kenya. *Sci Rep* 7: 41108.
26. Golassa L, Enweji N, Erko B, Aseffa A, Swedberg G, 2013. Detection of a substantial number of sub-microscopic *Plasmodium falciparum* infections by polymerase chain reaction: a potential threat to malaria control and diagnosis in Ethiopia. *Malar J* 12: 352.
27. Harris I et al., 2010. A large proportion of asymptomatic *Plasmodium* infections with low and sub-microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: challenges for malaria diagnostics in an elimination setting. *Malar J* 9: 254.
28. Tripura R et al., 2017. Submicroscopic *Plasmodium* prevalence in relation to malaria incidence in 20 villages in western Cambodia. *Malar J* 16: 56.
29. Zaw MT et al., 2017. Asymptomatic and sub-microscopic malaria infection in Kayah State, eastern Myanmar. *Malar J* 16: 138.
30. Rosewell A, Makita L, Muscatello D, John LN, Bieb S, Hutton R, Ramamurthy S, Shearman P, 2017. Health information system strengthening and malaria elimination in Papua New Guinea. *Malar J* 16: 278.
31. Das S, Peck RB, Barney R, Jang IK, Kahn M, Zhu M, Domingo GJ, 2018. Performance of an ultra-sensitive *Plasmodium falciparum* HRP2-based rapid diagnostic test with recombinant HRP2, culture parasites, and archived whole blood samples. *Malar J* 17: 118.
32. Leow CH, Jones M, Cheng Q, Mahler S, McCarthy J, 2014. Production and characterization of specific monoclonal antibodies binding the *Plasmodium falciparum* diagnostic biomarker, histidine-rich protein 2. *Malar J* 13: 277.
33. Ramutton T et al., 2012. Sequence variation does not confound the measurement of plasma PfHRP2 concentration in African children presenting with severe malaria. *Malar J* 11: 276.
34. Baker J, Gattton ML, Peters J, Ho MF, McCarthy JS, Cheng Q, 2011. Transcription and expression of *Plasmodium falciparum* histidine-rich proteins in different stages and strains: implications for rapid diagnostic tests. *PLoS One* 6: e22593.
35. Beshir KB, Sepulveda N, Bharmal J, Robinson A, Mwanguzi J, Busula AO, de Boer JG, Sutherland C, Cunningham J, Hopkins H, 2017. *Plasmodium falciparum* parasites with histidine-rich protein 2 (*pfhrp2*) and *pfhrp3* gene deletions in two endemic regions of Kenya. *Sci Rep* 7: 14718.
36. Gamboa D et al., 2010. A large proportion of *P. falciparum* isolates in the Amazon region of Peru lack *pfhrp2* and *pfhrp3*: implications for malaria rapid diagnostic tests. *PLoS One* 5: e8091.