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# Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 in the China-Myanmar border area

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## Abstract

Deletion of the *Plasmodium falciparum histidine-rich protein 2* (*pfhrp2*) gene may affect the performance of PfHRP2-based rapid diagnostic tests (RDTs). Here we investigated the genetic diversity of the *pfhrp2* gene in clinical parasite isolates collected in recent years from the China-Myanmar border area. Deletion of *pfhrp2* has been identified in 4 out of 97 parasite isolates. Sequencing of the *pfhrp2* exon 2 from 67 isolates revealed a high level of genetic diversity in *pfhrp2*, which is reflected in the presence of many repeat types and their variants, as well as variable copy numbers and different arrangements of these repeats in parasite isolates. In addition, we observed *pfhrp3* deletion in three of the four parasites harboring *pfhrp2* deletion, suggesting of double deletions of both genes in these three isolates. Analysis of two cases, which were *P. falciparum*-positive by microscopy and PCR but failed by two PfHRP2-based RDTs, did not find *pfhrp2* deletion. Further correlational studies of *pfhrp2* polymorphisms with detection sensitivity are needed to identify factors influencing the performance of RDTs in malaria-endemic areas.

### Keywords

malaria; rapid diagnostic tests; *pfhrp2*; deletion; variation

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Authors' contributions

PL and HX carried out the experimental work and data analysis. ZZ, ZY, YC, GY and JS participated in data analysis. PL performed manuscript writing. QF and LC conceived the study and participated in writing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

## 1. Introduction

Malaria remains endemic in 104 countries and estimated 3.4 billion people are at risk of malaria globally (WHO, 2013). Case management and treatment of malaria is highly dependent on accurate early diagnosis. Rapid diagnostic tests (RDTs) for malaria have the potential to improve case management and thereby reduce morbidity and mortality (Maltha et al., 2013; Mouatcho and Goldring, 2013). At least four target antigens are captured by various available RDTs: *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and lactate dehydrogenase (LDH); the pan-plasmodial aldolase and LDH (Abba et al., 2011). PfHRP2 is an abundant antigen produced during the blood stages of *P. falciparum*. It consists of a number of Ala- and His-rich amino acid repeats (Wellems and Howard, 1986) and shares epitopes with another histidine-rich protein, PfHRP3. Thus, antibodies specific for PfHRP2 have been found to also across react with PfHRP3 (Lee et al., 2006).

The performance of malaria RDTs is generally associated with the product quality, storage conditions, parasite or operator factors and parasite/antigen concentrations. One important factor is the variability within the parasite antigens detected by the RDTs, which includes presence or absence of the target epitopes and variations in the number of epitopes in a particular parasite isolate (Lee et al., 2006; Lee et al., 2012; Maltha et al., 2014; Talman et al., 2007). Genetic diversity may be particularly important for PfHRP2-based RDTs, since most of the RDTs are based on this antigen (Mouatcho and Goldring, 2013). Studies to date have documented extensive size variations between parasite strains and a high degree of genetic diversity within this antigen (Baker et al., 2010b; Baker et al., 2005; Deme et al., 2014; Kumar et al., 2012; Lee et al., 2006; Rock et al., 1987). An extreme situation is that certain parasite isolates even harbor a deletion of the *pfhrp2* gene, leading to false negative results in diagnosis with PfHRP2-based RDTs. This phenomenon was first discovered in the Peruvian Amazon in 2010 (Gamboa et al., 2010). Since then, *pfhrp2* deletion-associated poor performance of RDTs has been reported in many malaria-endemic regions (Cheng et al., 2014; Koita et al., 2012; Maltha et al., 2012; Pava et al., 2010; Wurtz et al., 2013). To date, unequivocal evidence for *pfhrp2* and *pfhrp3* gene deletions in *P. falciparum* isolates has been obtained in Peru (Gamboa et al., 2010; Maltha et al., 2012), Brazil (Houze et al., 2011), Senegal (Wurtz et al., 2013), and India (Kumar et al., 2012). Apart from gene deletion, PfHRP2 sequences in different regions showed differences in the number of amino acid repeats and even some rare amino acid variants (Baker et al., 2010b; Baker et al., 2005; Deme et al., 2014; Kumar et al., 2012; Lee et al., 2006). Given the significance of PfHRP2-based RDTs in malaria diagnosis, systematic mapping of PfHRP2 diversity in global malaria endemic regions is highly demanded, especially in areas where poor performance or failure of PfHRP2-based RDTs have been reported (Cheng et al., 2014).

In the remote border regions of Southeast Asian countries, malaria remains prevalent (Cui et al., 2012) and RDTs have been widely used for malaria diagnosis as a case management practice. Recently, we compared two different RDTs in malaria diagnosis at the China-Myanmar border (Yan et al., 2013). From a limited analysis, we identified two *P. falciparum* cases, which were positive by both microscopy and PCR but consistently failed two

PfHRP2-based RDTs. This has prompted us to examine *pfhrp2* genetic diversity in greater detail and determine whether the two false-negative cases were due to *pfhrp2* gene deletions.

## 2. Materials and Methods

### 2.1. Parasite isolates and DNA extraction

A total of 97 *P. falciparum* parasite isolates from the China-Myanmar border and western Thailand were analyzed in this study. Among them, 87 were obtained from malaria patients in the China-Myanmar border area between May 2011 and December 2012, of which 36 were also evaluated by two PfHRP2-based RDTs (Wondfo, China and Tycolpharm Co. Limited, United Kingdom) (Yan et al., 2013). Based on microscopy of Giemsa stained thick smears, parasite densities ranged from 40 to 105,920 parasites/μL of blood, assuming 8000 leukocytes/μL. The remaining ten parasite isolates were collected in Tak Province, western Thailand during mass blood surveys conducted between August 2011 and May 2012 (Li et al., 2014). Parasite DNA was extracted from dried blood spots on Whatman 3 M filter paper using the Qiagen DNA Mini Kit (Qiagen, Valencia, CA, USA). DNA was eluted in 50 μL of distilled water and stored under −20°C.

### 2.2. Amplification of the pfhrp2 and pfhrp3 genes

*P. falciparum* infections in 97 samples were verified by a nested PCR method (Yan et al., 2013). Previously established protocols were used for amplifications of *pfhrp2* and *pfhrp3* (Table S1). Specifically, the A262/A264 primers were used in the primary PCR reaction for *pfhrp2* (Trouvay et al., 2013). For nest PCR, two sets of primers were used to amplify the exon 1/intron 1 part (+29 nt – +155 nt) and the exon 2 (+135 nt – 7 nt after the stop codon) of the *pfhrp2* gene, respectively (Baker et al., 2005; Trouvay et al., 2013). All amplification conditions used were the same as the reference articles. The PCR products of the exon 2 were cloned in the T-vector (Takara Biotechnology, Dalian, China) for sequencing. For amplification of *pfhrp3*, primers targeting exon 2 (+77 nt – +180 nt) were used (Baker et al., 2005; Mariette et al., 2008; Trouvay et al., 2013). Samples were considered *pfhrp2*-negtive or *pfhrp3*-negtive if amplifications were not successful after two attempts.

### 2.3. Flanking genes and genotyping of pfhrp2-negative samples

To determine whether *pfhrp2*-negative samples by PCR were due to lack of parasite DNA in the samples, three polymorphic genes *pfmsp1*, *pfmsp2* and *pfglurp* were amplified using established protocols (Meng et al., 2010). DNA from the reference strains 3D7, 7G8 and HB3 were used as positive controls. Two genes flanking *pfhrp2*, *PF3D7_0831900* and *PF3D7_0831700*, were further detected by the nested-PCR using modified primers and conditions as described earlier (Akinyi et al., 2013).

### 2.4. DNA sequencing and analysis

The PCR products were purified using the Qiagen PCR purification kit (Qiagen, Valencia) and cloned into the pMD18-T vector. Cloned PCR products were sequenced on an ABI 3730XL DNA Analyzer with primers M13-47 (CGCCAGGGTTTTCCCAGTCA CGAC) and RV-M (GAGCGGATAACAATTTCACACAGG) (Takara, Dalian). The nucleotide sequences of *pfhrp2* exon 2 were assembled using DNASTAR (Madison, WI, USA).

Different types of amino acid repeats within exon 2 were identified and given a numeric code (1–18) as described earlier (Baker et al., 2005). These repeat types were aligned to visualize the similarity between parasite isolates and sequence from 3D7 was used as the reference. *Pfhrp2* sequences were deposited in GenBank under accession numbers KP712709 – KP712775.

## 3. Results

### 3.1. Pfhrp2 gene size variation and deletion

Of the 97 *P. falciparum* isolates, amplification of *pfhrp2* exon 1/intron 1 and exon 2 were successful in 93 isolates; 4 samples (M0100441, M0N00199, M0N00529, and M0N00302) failed to amplify any *pfhrp2* fragments. Sixty seven *pfhrp2* PCR fragments were cloned and sequenced. The results showed that the amplified exon 2 fragments ranged from 319 to 902 bp, with most of them (~79%) being 500-900 bp (Fig. 1). Sequencing analysis revealed that 65.7% (44/67) isolates had unique sequences, whereas 23 (34.3%) sequences were present in >1 parasite isolate. It is noteworthy that nine identical sequences were shared among nine parasite isolates collected in a single village, an indication of the same clone in circulation.

For the four parasite isolates that failed to amplify any *pfhrp2* fragments, three (M0100441, M0N00199, and M0N00302) also failed to yield a PCR product for *pfhrp3* exon 2, suggesting possible deletions of both *pfhrp2* and *pfhrp3* genes in these samples. The presence of parasite DNA in these four samples was confirmed by successful amplifications of three polymorphic genes *pfmsp1*, *pfmsp2* and *pfglurp* (data not shown). In addition, two genes (*PF3D7_0831700* and *PF3D7_0831900*) flanking *pfhrp2* were also successfully amplified from these four samples.

### 3.2. Correlation between pfhrp2 gene deletion and RDT results

Thirty-six of the samples from the China-Myanmar border area have been evaluated by two PfHRP2-based RDTs (Yan et al., 2013). Two samples (M0100304 and M0500035) were slide-positive for *P. falciparum* infections but were negative by both RDTs. Based on microscopy, parasite densities of these two samples were estimated at 480 (M0100304) and 120 parasites/μL (M0500035), respectively. *P. falciparum* infections in these two samples were further confirmed by PCR. PCR and sequencing of *pfhrp2* exon 2 from these two samples yielded *pfhrp2* products of 633 and 690 bp, respectively. This result indicated that these two RDT-negative samples were not due to *pfhrp2* gene deletion. Further comparison revealed that M0500035 shared the same *pfhrp2* sequence with another sample M0100418, which was *P. falciparum*-positive by the two RDTs. In addition, the *pfhrp2* exon 2 sequence fromM0100304 was very similar to those from three additional samples. These results suggest that other reasons (low parasitemia, mishandling of the RDTs, etc.) may be accountable for the false-negative results by the two RDTs.

Of the four samples with potential *pfhrp2* deletion, one sample (M0100441) was positive by the two RDTs. However, since RDTs were not performed on the remaining three samples, a direct correlation between *pfhrp2* gene deletion and RDT results could not be firmly established for these samples.

### 3.3. Repeat types of pfhrp2

The PfHRP2 protein from exon 2 is complex and composed of varying numbers of different types of amino acid repeats (Baker et al., 2010b; Baker et al., 2005). In this study, the protein sequences predicted from PfHRP2 exon 2 ranged from 104 to 286 amino acids. Analysis of the 67 sequences identified 33 repeat variants, which for convenience we assigned them to 13 repeat types that have been previously reported (Table 1) (Baker et al., 2010b; Kumar et al., 2013). In most cases, the PfHRP2 sequences harbor many types of these repeat motifs with each motif having variable copies, which give rise to large variation in the size of PCR fragments. Almost all sequences (66/67) begin with 1-4 copies of the Type 1 motif (AHHAHHVAD), whereas only one begins with a slight variation of this repeat motif (AHHAHHVAY). Similarly, 63 sequences end with a Type 12 motif (AHHAAAHHEAATH), four with minor variations of the Type 12 motif (AHHAAAHHEAATQ, AHHAAAHREAATH and AHHAAAHHGAATH). Type 2 (AHHAHHAAD) and 7 (AHHAAD) motifs, which were considered to correlate with the sensitivity of PfHRP2-based RDTs, were the most prominent in our PfHRP2 sequences with 100% and 92.5% prevalence, respectively. In particular, Type 2 motif is present in multiple copies in most isolates. Type 6 motif (AHHATD) was also prevalent in our study region and observed in 95.5% samples. In comparison, Type 3 (AHHAHHAAY), 5 (AHHAHHASD), 8 (AHHAAY) and 10 (AHHAAAHHATD) motifs were relatively abundant and found in more than half of the study populations, occurring at 76.1%, 61.2%, 77.6% and 58.2% , respectively. Type 13 repeat was observed in 4 isolates (6.0%), 3 of which were from Thailand. The type 14 (AHHAHHATD) repeat was present in 6 samples (9.0%), all of which were from China-Myanmar border area. One sequence contained a Type 15 motif (AHHAHHAAN), a motif most often detected in PfHRP3 (Baker et al., 2010a) (Table 1). Type 9 (AAY) and 11 (AHN) repeats were not found in the parasites of this study.

### 3.4. Variations in repeats

For eight of the repeat types described earlier (Type 1, 2, 4, 5, 6, 7, 10 and 12), there were variants that differed from the original type sequences by a single amino acid (Table 2). For convenience, the typical sequence and its variants were grouped into the same repeat type. For example, we identified 9 variants of the Type 2 repeat, which differed from the original Type 2 sequence AHHAHHAAD by a single amino acid (Table 2). These Type 2 variants were present in the parasite population as minor alleles. For a total of 734 Type 2 repeats identified in PfHRP2 sequences from the 67 parasite isolates, there were only 14 variant Type 2 repeats (1.9%). Altogether, 25 of the 67 isolates contained variant(s) of the eight repeat types; 18 isolates contained only one variant type. Whereas the majority of the mutations did not change the hydrophilic/hydrophobic property of the amino acids, it is not known whether these changes lead to changes in the antigenicity of the protein.

### 3.5. Variations in repeat type organization

We have shown that PfHRP2 in parasites from the Greater Mekong Subregion displayed a high level of genetic diversity with 52 different *pfhrp2* sequences identified from 67 *P. falciparum* isolates. We have attempted to assign them into groups based the recognition of more or less conserved arrangement of the repeat types within a sequence (Table 3, Fig. S1).

Generally, Type 2 motifs always followed the Type 1 motif. In the middle of the sequences, repeat types were often arranged as 3-5-7-8-2-7-6. Before the ending Type 12, there was either a Type 2, Type 6 or Type 10 repeat in most cases. According to the similarities in domain organization, the 67 PfHRP2 sequences were divided into four groups: sequences in Group 1, 2, and 3 ended with repeat types 6-2-12, 6-12, and 10-12, respectively, while the remaining isolates were classified into Group 4. Detailed organization of repeats is illustrated in Fig. S1.

## 4. Discussion

This study, as the first report of *pfhrp2* genetic diversity from the China-Myanmar border area, was motivated from an earlier finding of false-negative cases of two PfHRP2-based RDTs (Yan et al., 2013). In search of a potential cause, we identified 4 out of 87 (4.6%) isolates in Myanmar as potentially having *pfhrp2* gene deletions. In addition, three of these four isolates may also harbor *pfhrp3* deletions. To date, *hrp2* deletion has been unequivocally identified in several malaria endemic regions of the world, though the proportions of parasites carrying the deletion varied greatly. In Peru, *P. falciparum* isolates lacking *pfhrp2* had reached a staggering 41% (Gamboa et al., 2010), whereas in Senegal the *pfhrp2* deletion rate was still low (2.4%) (Wurtz et al., 2013). The *pfhrp2* deletion rate in our study population is close to the rate (4.2%) reported in India (Kumar et al., 2013).

As found in other studies, sequence variations do not seem to accurately foretell the performance of the RDTs. PfHRP3 also shares antigenic epitopes with PfHRP2 and thus plays a role in the performance of PfHRP2-based RDTs (Baker et al., 2005). In our study, the two false-negative *P. falciparum* cases by PfHRP2-based RDTs contained 633 and 690 bp of *pfhrp2* exon 2 sequences, respectively. Moreover, one even shared the same *pfhrp2* sequence with another sample detected by the PfHRP2-based RDTs. Such a discrepancy could be resulted from relatively low parasite densities of the two samples, or errors made during the RDT testing (e.g., insufficient blood transferred to the RDT, wrong buffer volume or wrong reading time). Conversely, one parasite isolate failed to amplify any *pfhrp2* gene fragments but was detected as *P. falciparum* positive by the RDTs. We do not know the reason for this puzzling result, which could be due to sequence variations in the primer regions of *pfhrp2* or methodological problem that led to amplification failure. A more thorough comparison between the RDTs and other diagnostic methods (microscopy and PCR) is needed to resolve these discrepancies.

Analysis of *pfhrp2* genetic diversity also revealed striking geographical differences, which might also be linked to the variability of RDT performance. First, the average length of our *pfhrp2* amplicons was 687 bp, which was shorter than what was reported in Mozambique and Tanzania (Ramutton et al., 2012), but longer than in Vanuatu (Baker et al., 2010b). Our parasite population lacked repeat types 9 and 11. Whereas Type 11 repeat was absent in Senegal (Wurtz et al., 2013), it was detected in all the sequenced isolates from India (Kumar et al., 2013). In addition, Type 4 repeat was present less frequently in <40% of our samples. As Type 4 and 7 were thought to be the targets of RDTs (Deme et al., 2014), such a difference at the population level could potentially result in varied performance of RDTs.

Furthermore, we have detected multiple minor variants of eight typical repeat sequences. It is unknown whether they also affect the sensitivity of the RDTs.

In summary, sequencing analysis revealed deletion as well as high-level genetic diversity of the *pfhrp2* gene in the parasite population from the China-Myanmar border. Genetic diversity is reflected in the presence of different types of repeats, as well as variations in sequence, copy number, and arrangement of these repeats. In addition, we also detected deletion of *pfhrp3* gene in some parasite samples. Future studies should be directed to determine what antigen epitopes are detected by the *pfhrp2* antibodies used in the RDTs in order to improve the detection sensitivity. Discovery of new antigens suitable for RDT development should also be encouraged.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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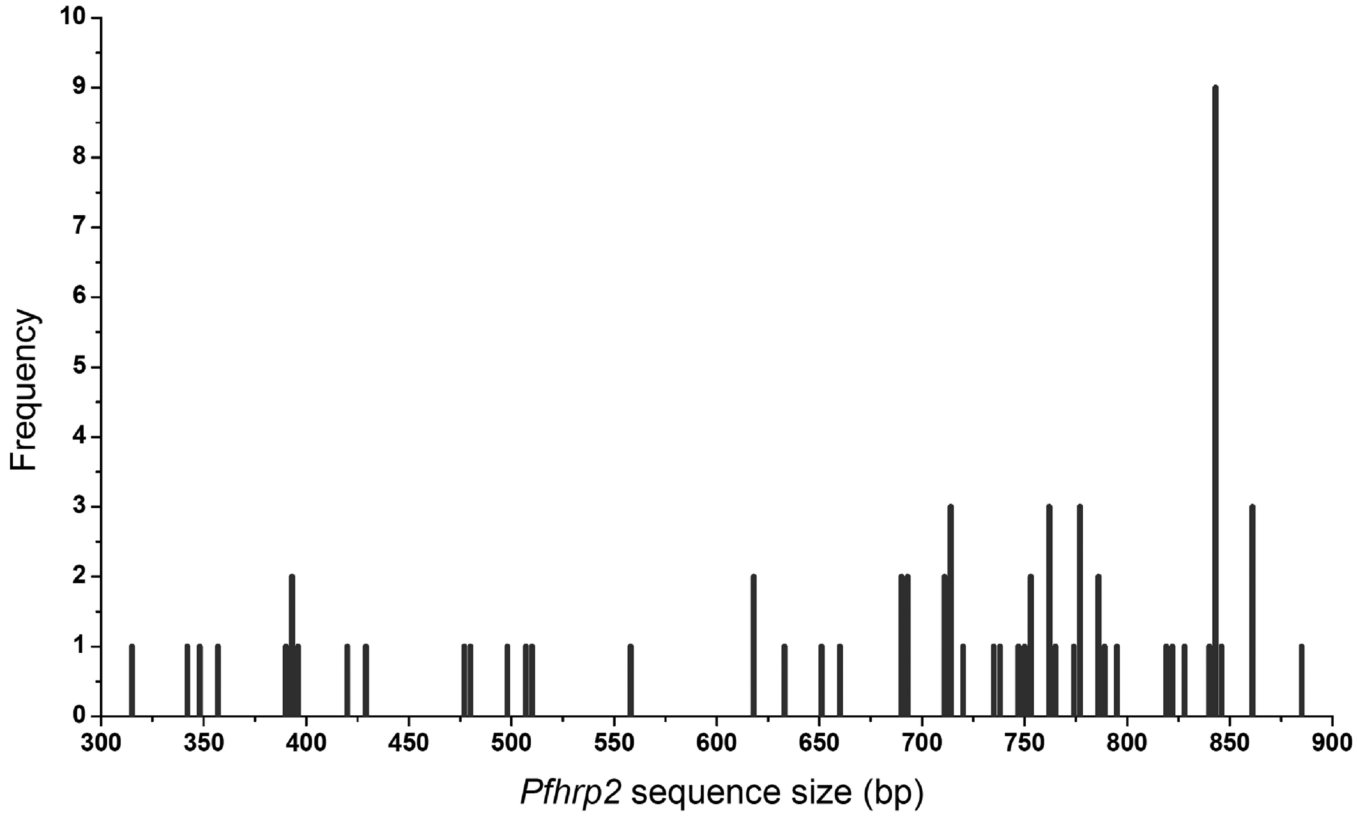


**Fig. 1.**
Sizes and frequencies of *pfhrp2* PCR fragments from different parasite isolates.

## Table 1

PfHRP2 repeat types and frequencies in two Southeast Asian sites*

| Types | Repeat sequences | Frequency in parasite isolates [n (%)] | | |
|---|---|---|---|---|
| | | Total (n=67) | China-Myanmar border (n=62) | Thailand (n=5) |
| 1 | AHHAHHVAD | 67 (100%) | 62 (100%) | 5 (100%) |
| 2 | AHHAHHAAD | 67 (100%) | 62 (100%) | 5 (100%) |
| 3 | AHHAHHAAY | 51 (76.1%) | 49 (79.0%) | 2 (40.0%) |
| 4 | AHH | 25 (37.3%) | 21 (33.9%) | 4 (80.0%) |
| 5 | AHHAHHASD | 41 (61.2%) | 40 (64.5%) | 1 (20.0%) |
| 6 | AHHATD | 64 (95.5%) | 59 (95.2%) | 5 (100%) |
| 7 | AHHAAD | 62 (92.5%) | 57 (91.9%) | 5 (100%) |
| 8 | AHHAAY | 52 (77.6%) | 48 (77.4%) | 4 (80.0%) |
| 9 | AAY | 0 | 0 | 0 |
| 10 | AHHAAAHHATD | 39 (58.2%) | 35 (56.5%) | 4 (80.0%) |
| 11 | AHN | 0 | 0 | 0 |
| 12 | AHHAAAHHEAATH | 67 (100%) | 62 (100%) | 5 (100%) |
| 13 | AHHASD | 4 (6.0%) | 1 (1.6%) | 3 (60.0%) |
| 14 | AHHAHHATD | 6 (9.0%) | 6 (9.7%) | 0 |
| 15 | AHHAHHAAN | 1 (1.5%) | 1 (1.6%) | 0 |

* Designation of repeat types was based on an earlier publication

**Table 2**

Original repeat types and their variants identified in the study parasite populations

| Type | Original type sequence | Variants * | # isolates with variant§ | # (%) of variant¶ |
|---|---|---|---|---|
| 1 | AHHAHHVAD | AHHAHHVA**Y** | 1 | 1/191 (0.5%) |
| 2 | AHHAHHAAD | AHHA**Y**HAAD | 3 | 14/734(1.9%) |
| | | AHHAH**Y**AAD | 2 | |
| | | A**Y**HAHHAAD | 1 | |
| | | AHHAH**R**AAD | 1 | |
| | | AHHA**R**HAAD | 1 | |
| | | AHHAHH**T**AD | 1 | |
| | | AHHAH**T**AAD | 1 | |
| | | AHHAHHA**V**D | 1 | |
| | | AHHAHHAA**G** | 3 | |
| 4 | AHH | **T**HH | 1 | 5/39 (12.8%) |
| | | A**R**H | 1 | |
| | | AH**R** | 1 | |
| | | A**Q**H | 1 | |
| | | AH**Y** | 1 | |
| 5 | AHHAHHASD | AHHA**Q**HASD | 1 | 1/44 (2.3%) |
| 6 | AHHATD | AH**L**ATD | 1 | 1/185 (0.5%) |
| 7 | AHHAAD | A**R**HAAD | 1 | 5/270 (1.9%) |
| | | AH**R**AAD | 2 | |
| | | AHHA**P**D | 1 | |
| | | AHHAA**E** | 1 | |
| 10 | AHHAAAHHATD | AH**Y**AAAHHATD | 1 | 1/59 (1.7%) |
| 12 | AHHAAAHHEAATH | AHHAAAH**R**EAATH | 1 | 4/67 (6.0%) |
| | | AHHAAAHH**G**AATH | 1 | |
| | | AHHAAAHHEAAT**Q** | 2 | |

*Variants differing from the original type sequences by a single amino acid (highlighted in bold and underlined) are grouped into the same repeat type.

§Number of parasites carrying the variant repeat type (from a total of 67 parasite isolates).

¶Indicates the number (%) of the variant type repeat within the total number of the corresponding type repeat identified in the 67 parasite isolates.

**Table 3**

Organization of hrp2 exon 2 repeat types

| Group[#] | N | PfHRP2 amino acid sequence | | | |
|---|---|---|---|---|---|
| | | N-terminal | Repeat types [§] | | C-terminal |
| 1a | 9 | LLHETQAHVDD[*] | **1111** 22222222222 | **357827** **6**27 6 7 6 777 | **6 2 12** | CLRH |
| 1b | 3 | LLHETQAHVDD | **1** 2222223222222 | 8 **57827** **6**2 | 10 **6 2 12** | CLRH |
| 1c | 4 | LLHETQAHVDD | **111** 22324222222 | **357827** **6** 6 7 777 | **6 2 12** | CLRH |
| 1d | 2 | LLHETQAHVDD | **11**(11) 22222211222 | **357827** 7 7 6 77 | **6 2 12** | CLRH |
| 1e | 2 | LLHETQAHVDD | **111**(1) 222 | (7) **6** (7)6 777(7) | **6 2 12** | CLRH |
| 2a | 3 | LLHETQAHVDD | **11** 223222222 | **357827** **6**2622626 14 26262 | **6** **12** | CLRH |
| 2b | 7 | LLHETQAHVDD | **1**(111) 2223242(4)2222222 | **57827** **6**2 (7 6 77)62 | 10 **6** **12** | CLRH |
| 2c | 3 | LLHETQAHVDD | **1**(1111) 22(222)322(22) | **(827)** **6**2 (7 6 77)62 | 10 **6** **12** | CLRH |
| 3a | 6 | LLHETQAHVDD | **11**(11) 2232(3/67777)2222222 | **357827**(7) **6** 7(77) 6(7/2/5)62(22)(1010) | **10** **12** | CLRH |
| 3b | 2 | LLHETQAHVDD | **11** 22326222222 | **357 2** **3** 5 2 7 10 | **10** **12** | CLRH |
| 3c | 3 | LLHETQAHVDD | **1111**(1) 22(2)32422 22(15) 222(2) | **57827** **6**2 (4)/(67 6777 62) | **10** **12** | CLRH |
| 3d | 5 | LLHETQAHVDD | **11**(1) 2(22)(324)222222(222) | **827**(772)**6**2(62)62 67 1010 | **10** **12** | CLRH |
| 3e | 4 | LLHETQAHVDD | **1**(11) (22)424 22(4)2222222(77) 13 **7827** **6**(2) (77677626777)64 1010 | **10** **12** | CLRH |
| 3f | 7 | LLHETQAHVDD | **1**(111) 2(22)(324 22222222) (7 **6**2 7776227) (1010) | **10** **12** | CLRH |
| 4a | 2 | LLHETQAHVDD | **1**(1) 2(22)(32422 4)/(6776)2222(22) **357827**(827 **6**2 777) | **12** | CLRH |
| 4b | 2 | LLHETQAHVDD | **111** 2 77 32 677772777 222222 **3** 2**8** | **12** | CLRH |
| 4c | 3 | LLHETQAHVDD | **111**(11) 22(22)(3242) **(5)** (**6**) **14** | **12** | CLRH |

[#]Pfhrp2 sequences with a similar organization of the repeat types were grouped into 4 groups, with each starting with the same number.

[*]Identical to 3D7 sequence.

[§]Details of the arrangement of the repeat types are shown in Fig. S1. The conservative repeat types are highlighted in bold. The repeat types marked in bracket indicated that some parasite isolates carried these repeat types. The repeat types inserted in Type 2 repeats are underlined.