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Review

Genetic diversity and deletion of *Plasmodium falciparum* histidine-rich protein 2 and 3: a threat to diagnosis of *Plasmodium falciparum* malaria

Running title: Absence of *P. falciparum* histidine-rich protein 2 and 3

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Abstract

Background: PfHRP2-based rapid diagnostic tests (RDTs), based on the recognition of the *P. falciparum* histidine-rich protein 2, are currently the most used tests in malaria detection. Most of the antibodies used in RDTs also detect PfHRP3. However, false-negative results were reported. Significant variation in the *pfhrp2* gene could lead to the expression of a modified protein that would no longer be recognized by the antibodies used in PfHRP2-based RDTs. Additionally, parasites lacking the PfHRP2 do not express the protein and are, therefore, not identifiable.

Aims: This review aims to assess the *pfhrp2* and *pfhrp3* genetic variation or the prevalence of gene deletion in malaria endemic areas and describe its implications on RDT use.

Sources: Publications of interest were identified using PubMed, Google Scholar and Google.

Content: More than 18 types of amino acid repeats were identified from the PfHRP2 sequences. Sequencing analysis revealed high-level genetic variation in the *pfhrp2* and *pfhrp3* genes (above 90% of variation in Madagascar, Nigeria or Senegal) both within and between countries. However, genetic variation of PfHRP2 and PfHRP3 does not seem to be a major cause of false-negative results. The countries which showed the highest proportions of *pfhrp2*-negative parasites were Peru (20 to 100%) and Guyana (41%) in South America, Ghana (36%) and Rwanda (23%) in Africa. High prevalence of *pfhrp2* deletion causes a high rate of false-negatives results.

Implications: Presence of parasites lacking *pfhrp2* gene may pose a major threat to malaria control programs because *P. falciparum* infected patients are not diagnosed and properly treated.

In 2016, approximately 3.2 billion people, which is nearly half of the population of the world, were at risk of contracting malaria [1]. According to the latest World Health Organization (WHO) estimates, there were 216 million cases of malaria in 2016 and 445 000 deaths. Sub-Saharan Africa continues to carry a disproportionately high share of the global malaria burden, with 90% of malaria cases and 90% of malaria deaths, mostly in young children. The WHO recommends diagnostic confirmation of *P. falciparum* in all patients with suspected malaria before treatment with antimalarial drugs. This parasitological confirmation can be done by microscopy or malaria rapid diagnostic tests (RDTs) [2]. The sale of 221 million malaria RDTs across the globe in 2016 is a proof of their utility [1].

Malaria RDTs are flow devices based on the detection of parasite proteins by immunochromatography. Currently, more than 200 different malaria RDTs are commercially available. Most of them are designed to identify *Plasmodium falciparum*-specific proteins and target either the *P. falciparum* histidine-rich protein 2 (PfHRP2) or the *P. falciparum* lactate dehydrogenase (PfLDH). RDTs can detect other pan-malaria (all species) antigens, such as pLDH or pALD (aldolase). PfHRP2-based RDTs are more sensitive for the detection of *P. falciparum* and heat-stable than PfLDH-based RDTs [3,4]. However, most of the antibodies used in RDTs for the detection of PfHRP2 also detect the *P. falciparum* histidine-rich protein 3 (PfHRP3) due to structural homology [5].

False negative RDT results were reported with PfHRP2-based RDTs [6]. Numerous factors are implicated, leading to decreases in the performance of the RDTs. The first factor is operator dependent (errors in performing the test and interpreting the result) and can be reduced by training [7]. Second, poor product design or manufacturing quality may be involved and can be eliminated by using only good quality RDTs that have been verified, tested and qualified by the WHO. In this context, the WHO has created two programmes, the Foundation for Innovative New Diagnostics (FIND) malaria RDT quality assurance

programme and the WHO-FIND malaria RDT lot testing programme [8]. Another factor in the false negative result is antibody degradation due to poor thermal stability, as well as incorrect transport or storage conditions. Parasite density can also lead to false negative results. Very low parasite densities may not be detected [9-11]. Conversely, a false negative result can occur due to a prozone-like effect in high parasite densities [12,13]. Finally, misdiagnosis can also be due to the genetic variation of the *pfhrp2* gene leading to a modified protein PfHRP2 with a new epitope that is not recognized by RDT antigens or parasites lacking the PfHRP2 protein because of *pfhrp2* gene deletion [9,14,15]. In the absence of the PfHRP2 protein, most of the antibodies used in RDTs for the detection of PfHRP2 also detect the PfHRP3 due to structural homology [5]. However, more *P. falciparum* parasites lacking *pfhrp3* gene were observed across the globe than parasites lacking *pfhrp2* gene [9]. Additionally, most of the parasites lacking *pfhrp2* gene also lost *pfhrp3* gene [9,15].

This review summarizes the publications that reported *pfhrp2* and *pfhrp3* genetic variation or deletion and describe their implications on RDT use and clinical management. Information sources were identified using PubMed, Google Scholar and Google. Searches were performed in these electronic databases using the search term ‘PfHRP2’ or ‘PfHRP3’ alone or in combination with ‘rapid diagnostic test’, ‘RDT’, ‘deletion’, ‘genetic variation’ or ‘polymorphism’. A total of 120 and 73 publications were identified using PubMed using the search term ‘PfHRP2’ alone and in combination with the other terms, respectively. Only 41 publications were interested to *pfhrp2* or *pfhrp3* gene deletion or genetic variation. Two of these 41 publications were in Chinese and excluded.

Genetic variation in the *pfhrp2* and *pfhrp3* genes

The first hypothesis was that the accuracy of PfHRP2-based RDTs can be affected by the *pfhrp2* and *pfhrp3* genetic variation of *P. falciparum* parasites. *Pfhrp2* and *pfhrp3* genes are

located on chromosome 8 and 13, respectively, and both include two exons and one intron. Intron 2 of *pfhrp2* (PF3D7_0831800) and *pfhrp3* (PF3D7_1372200) consists of a repeated amino acid pattern [17]. Theoretically, the presence or absence, as well as the numbers of repeats could affect the binding affinity of the antibodies used in RDTs to the parasites antigen. Fourteen repeated amino acid sequences characterized into 18 types were identified. Type 2 and type 7 were described as potential epitopes targeted by monoclonal antibodies used in PfHRP2-based RDTs [17-19]. There is no published information available on the specific target epitopes of the antibodies used in any RDTs or on the monoclonal antibodies that are directed against PfHRP2. Because of the limited supplies of monoclonal antibodies, it is very likely that most of the PfHRP2-based RDTs use similar antibodies. Additionally, it is likely that either some or all of the epitopes detected by antibodies used in PfHRP2-based RDTs also exist in PfHRP3. The number of AHHAHHAAD (type 2) x AHHAAD (type 7) repeats for PfHRP2 were determined. PfHRP2 sequences were classified into four groups as a function of the number of type 2 x type 7 repeats. Isolates were classified as follows: very sensitive for the detection of PfHRP2 protein (group A) if the number of type 2 x type 7 repeats was ≥ 100 ; sensitive (group B) if the number of type 2 x type 7 repeats ranged from 50 to 100; borderline (group I) if the number of type 2 x type 7 repeats ranged from 43 to 50; and non-sensitive (group C) if the number of type 2 x type 7 repeats was < 43 . The variation in the number and combination of repeats within PfHRP2 affected the sensitivity of PfHRP2-based RDTs [17]. This classification would predict the performance of PfHRP2-based RDTs at low parasite density (below 250 parasites/ μ l). The low number of type 2 x type 7 repeats in group C and potentially in group I changes the conformation of the epitopes enough so that they are no longer recognized by antibodies used in PfHRP2-based RDTs.

Genetic variations in the *pfhrp2* gene have been investigated in the Asia-Pacific region [17,19], India [20-22], Yemen [23], Madagascar [24], Senegal [9,25], Mali [25], Uganda [25], French Guiana [26], Colombia [27] and in the China-Myanmar border area [28].

The PfHRP2 protein conformation was highly diverse in parasite isolates from the same area [9,28] or the same country [20]. Significant differences were seen depending on the geographic localization. First, the average length of the *pfhrp2* amplicons was shorter in the Asia-Pacific region than what was reported in Mozambique and Tanzania [29] but longer than in Vanuatu [19]. Sequencing analysis revealed high-level genetic variation in *pfhrp2*: 34 different sequences (87% of genetic variation) were detected in Tanzania [19], 74 (93%) in Nigeria [19], 120 (98%) in Senegal [9] or 221 (97%) in Madagascar [24]. Then, there was a significant difference between the number of repeats for type 5 and type 6 in the isolates from south, centre and north Madagascar [24]. Type 4 was rarely observed in Dakar (16% in 2009-2012) and was mainly identified in isolates from Pikine (a suburban area of Dakar approximately 15 km from Dakar), Thies (70 km from Dakar) or Velingara (565 km from Dakar in southern Senegal) (96.3%) [9,25]. Sequencing analysis revealed high-level genetic diversity in the *pfhrp2* and *pfhrp3* genes both within and between countries.

Some studies showed that type 2 and type 7 were predictive of the sensitivity of PfHRP2-based RDT detection at low parasite densities [9,17,24]. A PfHRP2-based RDT false negative sample was reported in a soldier in Uganda, certainly due to the low number of type 2 × type 7 repeats (40 repeats) [14]. According to the predictive Baker's model, this isolate belonged to the group C of non-sensitive parasites to PfHRP2-based RDTs, confirming that the numbers of repeats could affect the binding affinity of the antibodies used in RDTs to the parasites antigen and so the performance of the PfHRP2-based RDTs.

However, in other studies, no correlation was observed between variations in the size of repeat types 2 and 7 in *Pfhrp2*, nor between any combination of repeat motifs, and

performance of the PfHRP2-based RDTs at low parasitemia [19,21,28]. The genetic variation of PfHRP2 does not seem to be a major cause of false negative results.

Deletion of *pfhrp2* and *pfhrp3* genes

The presence or absence of *pfhrp2* and *pfhrp3* genes can be characterized by amplifying the full length of exon 2 and a region across exon 1 and exon 2 of both genes. Similarly, genes flanking *pfhrp2* (PF3D7_0831900 and PF3D7_0831700) and *pfhrp3* (PF3D7_1372100 and PF3D7_1372400) genes can be amplified to characterize the extent of the chromosomal deletions in the parasites lacking the *pfhrp2* and *pfhrp3* genes [9,15,17,26,27].

The results of the studies that have reported *P. falciparum* parasites lacking *pfhrp2* and/or *pfhrp3* are summarized in Table 1. The prevalence of *P. falciparum* parasites lacking *pfhrp2* and/or *pfhrp3* per country are presented in Figure 1 and Figure 2, respectively.

South America was the first region to report the occurrence of false negative PfHRP2-based RDTs, due to parasites lacking *pfhrp2* gene [27,30-38]. The first publication on *P. falciparum* parasites lacking *pfhrp2* reported data from Peru in 2010 [37]. This retrospective study showed that 41% and 70% of the samples collected from different areas of the Amazon region of Peru near Iquitos between 2003 and 2007 lacked the *pfhrp2* and *pfhrp3* genes, respectively. The proportion of parasites lacking *pfhrp2* increased from 20.7% in 1998-2001 to 40.6% in 2003-2005 in the Amazon region of Peru [35]. During a *P. falciparum* outbreak on the northern Pacific coast of Peru in 2010-2012, 100% of the 54 analysed samples lacked the *pfhrp2* gene [36]. Analysis in South America revealed high-level differences in the deletion of the *pfhrp2* gene both within and between countries. In 2010, 41% of parasites in Guyana and 15% in Suriname in 2009-2011 lacked the *pfhrp2* gene [33] while very low proportions (ranging from 0 to 4%) were found in Honduras from 2008-2009 [34], in French Guiana in 2009 [26], in Ecuador from 2012-2013 [32] or in Bolivia in 2010 [30]. The

situation can be very different within a country. From 2008-2010, 53.6% of the parasites lacked the *pfhrp2* gene in southeast Colombia (Amazonas) versus 0% in the northwest (Antioquia) [27]. In 2012, 31.6% of the *P. falciparum* parasites collected in Acre, located on the western edge of the Amazon region of Brazil and bordering Peru, lacked the *pfhrp2* gene while none of the samples collected were deleted in Para, which is located in the eastern end of the Brazilian Amazon basin in northern Brazil [30]. In 2011, a French traveller imported malaria from Brazil and presented a false-negative PfHRP2-based RDT with a parasite density of 3.2%, resulting from combined deletions of *pfhrp2* and *pfhrp3* [15].

Similar evidence of the emergence of false negative PfHRP2-based RDTs, due to parasites lacking *pfhrp2* gene was followed more recently in Africa [9,39-45]. In Africa, and more particularly in the Democratic Republic of the Congo in 2013-2014, the prevalence of parasites lacking the *pfhrp2* gene differed within the country with a high prevalence of *pfhrp2* gene deletions observed in Sankuru, Nord-Kivu, Sud-Kivu and Kinshasa (from 14.8 to 21.9%) compared to other regions in the Democratic Republic of the Congo, such as Bas-Uele, Haut-Katanga, Kasai or Equateur (from 0.2 to 2.8%) [39]. These data show the difficulty to monitor the emergence and the spread parasites lacking the *pfhrp2* gene. The global proportion of parasites lacking the *pfhrp2* gene was 6.4% in the Democratic Republic of the Congo. High proportions of *pfhrp2* gene deletion were reported in 2015 in some areas of Ghana (36%) and Rwanda (23%) [41,45]. A retrospective study in Mali showed that 2% of *P. falciparum* parasites lacked the *pfhrp2* gene in 1996 [43]. Isolates lacking the *pfhrp2* gene were identified in Senegal from 2009-2011 [9], in Eritrea from 2013-2014 [40], in Kenya in 2014 [42] and in Mozambique [44].

Concerning Asia, a low proportion of parasites lacking the *pfhrp2* gene were reported in India [21,22,46] and at the border between China, Myanmar and Thailand [28]. A PfHRP2-

based RDT negative sample with *pfhrp2* gene-deleted parasites was reported in Bangladesh in 2017 [16].

Implications for clinical management

P. falciparum parasites lacking *pfhrp2* and *pfhrp3* genes are an important source of false-negative cases using PfHRP2-based RDTs and, more particularly, in samples with low parasite densities. Parasites that lack the *pfhrp2* gene cannot express the PfHRP2 protein and therefore cannot be detected by PfHRP2-based RDTs. High proportions of *pfhrp2*-deleted isolates were reported in some areas of South America (Peru, Brazil, Colombia) and in Africa (Ghana, Rwanda, Democratic Republic of Congo). Those countries are major endemic regions of *P. falciparum* malaria, and therefore, the clinical consequences must be considered. Emergence of parasites lacking *pfhrp2* gene poses a major threat to malaria control programs because *P. falciparum* infected patients are not diagnosed and properly treated. High proportions of *pfhrp2*-deleted parasites will lead to a high rate of false-negative PfHRP2-based RDTs, particularly in areas with low malaria transmission and will lead to a high proportion of *P. falciparum* infected patients who will not be diagnosed and properly treated, especially in Africa where the diagnosis is mainly based on RDTs (177 million of RDTs used in sub-Saharan Africa in 2016). The common hypothesis is that parasites lacking *pfhrp2* gene will emerge in a low-transmission area and spread then to a high-transmission area. First, in low-transmission area, each infection is more likely to result in a higher parasite load because of the lower levels of clinical immunity. Second, because immunity is less developed in low transmission areas, parasites lacking *pfhrp2* gene are more likely to survive the host immune response and subsequently to be spread. Third, individuals tend to be infected concurrently by fewer genetically distinct parasites, so parasites lacking *pfhrp2* gene face less competition within the host and an increased probability of transmission success. We can expect a

selection and a spread of the *pfhrp2* deletion in sub-Saharan Africa, and more particularly in Ghana, Rwanda and Democratic Republic of Congo, where high proportions of *pfhrp2*-deleted isolates have been reported and where malaria diagnosis is mainly based on RDTs. False negative RDT results due to parasites lacking *pfhrp2* and *pfhrp3* genes also have implications for non-endemic areas. Untreated, approximately 10% of uncomplicated imported malaria will progress to severe malaria [47].

By modelling the prevalence of parasites lacking PfHRP2 and the treatment coverage estimates, Watson et al could predict the spread of the *pfhrp2* deletion in sub-Saharan Africa and regions in which surveillance should be prioritized [48]. The model showed that low malaria prevalence and high rates of people treated by antimalarial drugs resulted in the greatest selection of parasites lacking the *pfhrp2* gene. The selection pressure favouring parasites lacking the *pfhrp2* gene was predicted to be weaker in the presence of the *pfhrp3* gene. Using all these data, Watson et al created a map of 160 locations in Africa most at risk of rapid selection of parasites lacking the *pfhrp2* gene, and more particularly in Angola, Cameroon, Gabon, Kenya, Mali, Mauritania, Republic of Central Africa, Senegal Sudan or Tanzania. These maps could be used by public health authorities to determine where they should more closely monitor the emergence and spread of parasites lacking the *pfhrp2* gene.

Additionally, a recent study using an agent-based stochastic simulation model of *P. falciparum* transmission predicted that the use of PfHRP2-based RDTs in populations where *P. falciparum* parasites that lacked the *pfhrp2* gene are introduced was sufficient to select parasites with the *pfhrp2* gene deletion. These parasites were therefore undetectable by PfHRP2-based RDTs [49]. Parasites lacking the *pfhrp2* gene were transmitted in more than 80% of cases compared with 30% of parasites with a normal *pfhrp2* gene when detection of *P. falciparum* depended solely on PfHRP2-based RDTs. The only use of PfHRP2-based RDTs leads to select individuals with parasites lacking *pfhrp2* gene as parasites reservoir.

Additionally, misdiagnosis due to the non-detection of parasites lacking the *pfhrp2* gene leads to delayed treatment resulting in a higher load of parasites lacking the *pfhrp2* gene and a longer period for mosquitoes feeding, leading a better transmission of parasites with this deletion. In Eritrea, in March 2016, 80.8% (21/26) and 92.3% (24/26) of the patients infected by *P. falciparum* at Ghindae Hospital were infected with parasites lacking the *pfhrp2* and *pfhrp3* genes, respectively [50]. All the samples were confirmed by microscopy and PCR. The spread of *pfhrp2*-negative parasites was probably due to selection by use of HRP2-based RDTs. The conditions were ideal in Eritrea for selection of parasites lacking the *pfhrp2* gene: very good compliance with RDT before treatment since 2006, low malaria prevalence and high treatment adherence. The introduction and spread of parasites lacking the PfHRP2 protein will increase the mutant parasite prevalence, transmission, morbidity, and ultimately malaria mortality.

Due to the potential impact at the clinical, public health and economic levels, recommendations are being developed to monitor the presence of *pfhrp2* gene deletions in *P. falciparum* [51]. The WHO has formulated guidelines [52]. PfHRP2 deletion must be investigated when a patient sample gives negative results by at least two quality-assured HRP2-based RDTs; this sample must be assessed microscopically for *P. falciparum* by two qualified microscopists. When parasites lacking the *pfhrp2* gene have been confirmed, surveillance of febrile patients and other people around the index case must be carried out to determine the prevalence of the *pfhrp2* gene deletion in *P. falciparum* in the specific region and country. In countries where the prevalence of *pfhrp2*-deleted parasites is low, many isolates are required to properly assess the proportion of parasites lacking the PfHRP2 protein.

There are still many regions where the evaluation of the presence of *pfhrp2* gene deletions in *P. falciparum* has not been yet investigated.

It is imperative to use three-band RDTs that incorporate both pfHRP2 and pLDH antigens over a two-band RDT (HRP2 alone) to minimize misdiagnosis [53] or to abandon the use of PfHRP2-based RDTs.

Further studies are needed to identify and develop new protein targets for RDTs. The *P. falciparum* insulin-degrading enzyme homologue (PfIDE_h) could be a suitable candidate to replace PfHRP2 by using immuno-polymerase chain reaction detection [54]. This assay could detect less than one parasite/ μ l. It would be desirable to develop immunochromatographic flow device for detection of this new protein in practical field diagnostic. Detection of HRP2 at sub-picogram levels was effective using an antigen capture-based immunoassay through a bead-based system [55]. This bead-based immunoassay could be developed for other antigens, such as PflDH.

Transparency declaration

The authors declare that they have no competing interests.

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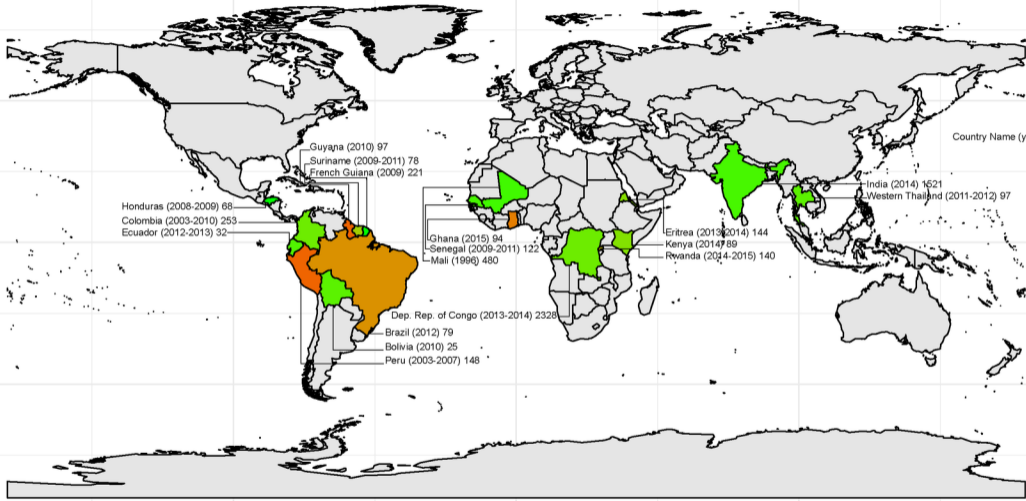
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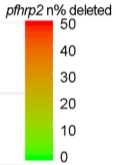
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Figure 1. Geographical distribution of the prevalence of *P. falciparum* parasites lacking the *pfhrp2* gene.

Figure 2. Geographical distribution of the prevalence of *P. falciparum* parasites lacking the *pfhrp3* gene.



Country Name (year of study) number of isolates



Guyana (2010) 97
Suriname (2009-2011) 78
French Guiana (2009) 221

Honduras (2008-2009) 68
Colombia (2003-2010) 253
Ecuador (2012-2013) 32

Ghana (2015) 94
Senegal (2009-2011) 122
Mali (1996) 480

Dep. Rep. of Congo (2013-2014) 2328
Brazil (2012) 79
Bolivia (2010) 25
Peru (2003-2007) 148

Eritrea (2013-2014) 144
Kenya (2014) 89
Rwanda (2014-2015) 140

India (2014) 1621
Western Thailand (2011-2012) 97

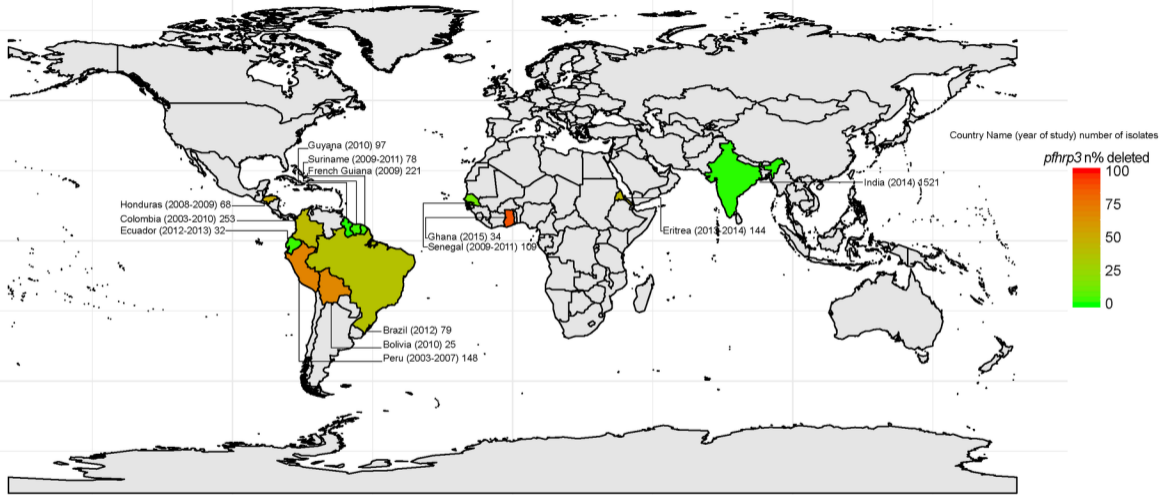


Table 1. Prevalence of the *Plasmodium falciparum* histine-rich protein 2 (*phhrp2*) and *pfhrp3* genes in *P. falciparum* isolates in the world

Country	Region	Year	Isolates with <i>pfhrp2</i> deletion			Isolates with <i>pfhrp3</i> deletion			Reference
			Total no	Deletion no	Deletion %	Total no	Deletion no	Deletion %	
South America									
Bolivia	Beni	2010	25	1	4	25	17	68	[30]
Brazil	Acre	2012	79	25	31,6	79	30	38	[30]
Brazil	Rondonia	2010-2011	60	2	3,3	60	11	18,3	[30]
Brazil	Para	2011-2012	59	0	0	59	30	50,9	[30]
Colombia	Antioquia, Amazonas, Guaviare, Choco, Narino	2003-2010	253	15	5,9	253	106	41,9	[27]
Colombia	Cordoba (22%), Narino (39%), Valle del Cauca (39%)	2008-2009	100	18	18	100	52	52	[31]
Colombia	Antioquia, Amazonas, Guaviare, Choco, Narino	2011-2012	112	0	0	112	51	45,5	[27]
Ecuador	Esmaraldas	2012-2013	32	1	3,1	32	1	3,1	[32]
French Guiana	Cayenne (59%), Saul (19%), St Laurent du Maroni (12%)	2009	221	0	0	221	10	4,5	[26]
Guyana	Cuyuni-Mazaruni (55%), Potao-Siparuni (34%)	2010	97	40	41	97	1	1	[33]

Honduras	Puerto Lempira	2008-2009	68	0	0	68	34	50	[34]
Peru	Loreto	1998-2001	92	19	20,7				[35]
Peru	Loreto	1999-2000	82	63	77				[36]
Peru	Loreto (Iquitos)	2003-2005	96	39	40,6				[35]
Peru	Peruvian Amazon (Iquitos, 77%)	2003-2007	148	61	41	148	103	70	[37]
Peru	Peruvian Amazon	2010-2011	74	19	25,7	78	34	43,6	[38]
Peru	Tumbes	2010-2012	54	54	100				[36]
Suriname	Sipaliwini (46%), Brokopondo (19%)	2009-2011	78	12	15	78	3	4	[33]

Africa

Democratic Republic of Congo	national	2013-2014	2328	149	6,4				[39]
Eritrea	Gash Barka	2013-2014	144	14	9,7	144	14	43	[40]
Ghana	Accra	2015	94	34	36	34	30	88,2	[41]
Kenya	Mbita, Kilifi	2014	89	8	9				[42]
Mali	Sirakoro, Bancoumana, Donéguébougou	1996	480	10	2				[43]
Mozambique	national	2010-2016	69	1	1.4	69	0	0	[44]
Rwanda	Busogo, Rikara, Kibiziri	2014-2015	140	32	23				[45]
Senegal	Dakar	2009-2011	122	3	2,4	109	14	12,8	[9]

Asia

India	Chhattisgarh (Centrl India)	2010	48	2	4,2	48	2	4,2	[46]
India	national	2014	1392	1	0,01	1392	2	0,14	[21]
India	national	2014	1521	36	2,4	1521	27	1,7	[22]
China-Myanmar	China-Myanmar border, western Thailand	2011-2012	97	4	4,1				[28]
