Centers for Disease Control and Prevention

Molecular Surveillance for HRP2 and HRP3 Gene Expression in *Plasmodium falciparum* Parasites from South and Central America

Abbreviations

AMI	Amazon Malaria Initiative
CDC	Centers for Disease Control and Prevention
CIDEIM	Centro Internacional de Entrenamiento e Investigaciones Médicas
FIND	Foundation for Innovative New Diagnostics
HRP2	Histidine-rich protein 2 (antigen)
HRP3	Histidine-rich protein 3 (antigen)
MOH	Ministry of Health
MSP2	Merozoite surface protein 2
PAHO	Pan American Health Organization
*Pf*hrp2	*Plasmodium falciparum* histidine-rich protein 2 (gene)
*Pf*hrp3	*Plasmodium falciparum* histidine-rich protein 3 (gene)
RBM	Roll Back Malaria partnership
RDT	Rapid diagnostic test
WHO	World Health Organization

Molecular Surveillance for HRP2 and HRP3 gene expression in *Plasmodium falciparum* parasites from South America and Central America

Progress report: July, 2012

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Acknowledgement

We are thankful for all AMI country partners, ministries of health and country's national institutes involved in the completion of these studies and evaluations presented in this report.

Disclaimer

The findings of this report are expected to be published in the scientific literature. Be mindful in sharing and citing this report.

Objective

The aim of this survey program was to collect and evaluate specimens of *P. falciparum* parasites in malaria-endemic countries of South and Central America for the presence or absence of *P. falciparum* histidine-rich protein-2 gene (*pf*hrp2) and/or *Plasmodium falciparum* histidine-rich protein-3 gene (*pf*hrp3) in order to guide Malaria Control Programs for procurement and implementation of appropriate malaria rapid diagnostic tests for their countries.

Background

Most of the currently available commercial rapid diagnostic tests (RDTs) for malaria use histidine-rich protein 2 (HRP2) as the target for diagnosis of *P. falciparum* parasites. These tests permit the rapid diagnosis of *P. falciparum* malaria making RDTs a component of malaria control measures recommended by the World Health Organization (WHO) and the Roll Back Malaria Partnership (RBM) (1-8). The antibodies used in these kits specifically recognize HRP2 antigen and may also cross-react with another member of the HRP gene family called histidine-rich protein 3 (HRP3). Recent evaluations of RDTs by the US Centers for Disease Control and Prevention (CDC) in collaboration with WHO and Special Programme for Research and Training in Tropical Diseases (TDR) and the Foundation for Innovative New Diagnostics (FIND) determined that a substantial number of malaria parasites in Peru were missing the genes for HRP2 and HRP3, called *pfhrp2* and *pfhrp3*, respectively. In this retrospective study conducted in Peru, 41% of *P. falciparum* parasite samples were missing the HRP2 gene, while 70% were missing the HRP3 one, and 20% of the parasites in this evaluation missing both genes. These gene deletions were reported in detail in a recent publication (9). Because most of the RDTs used to diagnose malaria target HRP2 due to greater sensitivity, deletions of HRP2 and HRP3 genes will negate the ability of the RDTs designed to detect these antigens in parasites in patient blood samples. Countries throughout South America have received Global Fund grants to purchase RDTs to use in malaria control programs. Because of the high proportion of parasites lacking *pfhrp2* and *pfhrp3* in Peru, it was important to conduct a comprehensive molecular surveillance of *P. falciparum* parasites to determine the extent of these gene deletions in South America, which will guide malaria control programs for the procurement and implementation of malaria RDTs. This information is also critical for the use of appropriate diagnostic kits for patient care in this region.

In 2001, eight countries in South America established a surveillance network to address antimalarial drug resistance: the Amazon Network for the Surveillance of Antimalarial Drug Resistance (Red Amazónica para la Vigilancia de la Resistencia a las Drogas Antimaláricas) (RAVREDA). Also in 2001, the United States Agency for International Development (USAID) started to provide financial and technical support to this network via the Amazon Malaria Initiative. A molecular surveillance project was initiated by CDC with additional support from the Amazon Malaria Initiative (AMI), PAHO, and FIND in 2009 to assess the extent of *pfhrp2* and *pfhrp3* deletions in different countries in South America. This report presents the current results and status of genotyping from this ongoing investigation.

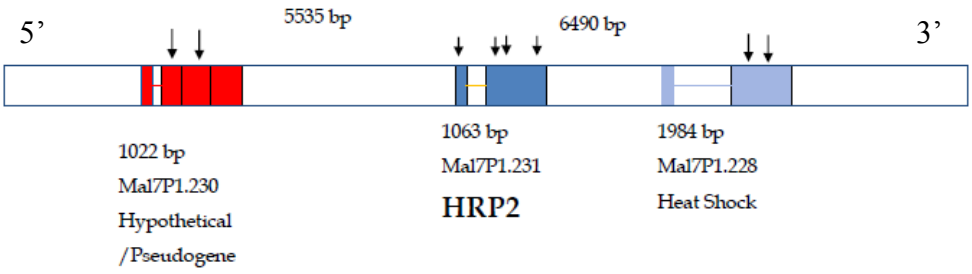
Methods

The protocol recommended collection of specimens of 3 ml of venous blood for the study and save each specimen as three aliquots of plasma and blood pellets; the latter for extracting parasite DNA for genotyping purposes and the former, plasma, for detecting soluble HRP2 protein by ELISA. The proposed plan was to keep two vials of plasma and blood pellets in the host country (for testing as well as a resource for future reference material) and provide one set of plasma and blood pellet to CDC for confirmatory molecular testing and serological confirmation of molecular test results. Unfortunately, some countries did not collect whole blood by venipuncture and decided to collect only filter paper blood spots from finger sticks, a simpler procedure. This led to some important technical challenges, which are discussed in a later part of this report. Finger prick blood (or intravenous blood in some cases) was collected from patients confirmed to have *P. falciparum* infection based on microscopy. Blood spots or an aliquot of venous blood samples was provided to the Malaria Branch laboratory at CDC for genotyping.

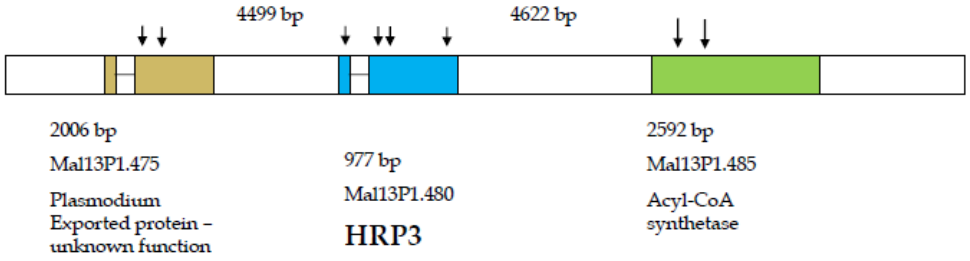
Commercial kits were used for extraction of DNA from blood pellets or blood spots (QIAamp DNA Mini, or QIAamp DNA Blood Mini Kits). *P. falciparum* infection status was further confirmed by species-specific nested PCR, based on amplification of the 18S ribosomal RNA gene [10]. The quality of the DNA for PCR amplification purposes was also evaluated by whether the major surface protein 2 (MSP2) alleles could be amplified. *Pfhrp2* and *pfhrp3* were amplified using primers represented by arrows below in the schematics of figure 1A & 1B and given specifically in Appendix II. For further confirmation and characterization of chromosomal deletions, the following upstream and downstream genes that flank *pfhrp2* or *pfhrp3* were also amplified. The Mal7P1.230 gene is located ~5.5 kb upstream and Mal7P1.228 is located ~6.5kb downstream of *pfhrp2* (Figure 1A). The Mal13P1.475 gene is located ~1.7 kb upstream and Mal13P1.485 is located ~4.4 kb downstream of *pfhrp3* (Figure 1B). In order to verify the specimens had good quality parasite DNA material, they were genotyped with *msp2* markers and only those samples that amplified at least one allele of this gene and an 18sRNA gene were determined to be of good quality (qualified) for further use in the analysis for *pfhrp2* and *pfhrp3* deletion.

Figure 1. Genomic positioning of *pfhrp2, pfhr3,* and flanking genes.

A. *Pfhrp2* and flanking genes



B. *Pfhrp3* and flanking genes


4499 bp
4622 bp
2006 bp
Mal13P1.475
Plasmodium
Exported protein –
unknown function
977 bp
Mal13P1.480
HRP3
2592 bp
Mal13P1.485
Acyl-CoA
synthetase

Results

Bolivia

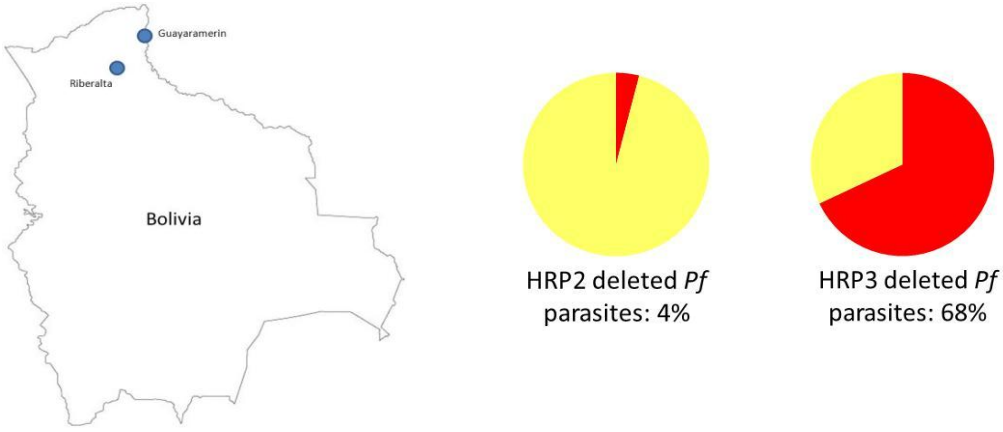
Total samples planned for collection: 100
Total samples provided: 27

Blood specimen type:

- Filter paper blood spot: provided
- Blood pellet: provided
- Blood plasma/serum: provided

Sites of collection: Riberalta and Guayaramerin

Figure 2. Collection sites and results in Bolivia.



Sample collection, training and challenges

Dr. A. Macedo de Oliveira and Dr. V. Udhayakumar visited Bolivia in 2010 for a week to work with Dr. Añez to provide training for sample collection in Riberalta and Guayaramerin (Figure 2). Dr. Jorge Cuba, Head, Malaria Section in the Public Health Laboratory of Riberalta provided assistance and guidance for this project. Ms. Gladys Nakaq, Laboratory Technician (Riberalta Public Health Laboratory) and Ms. Rosling Ocampo Zambrano (Guayaramerin Health Centre) received training for sample collection. Local health centers were selected for intravenous blood collection and filter paper blood spot collection. The targeted sample size was 100 for both sites combined in Bolivia. After one year, only 27 samples with *P. falciparum* mono infection were collected and provided to CDC for testing. A total of 27 filter paper samples were initially provided to the CDC laboratory on 6/8/11. Subsequently, 27 blood pellets and plasma samples corresponding to the filter paper blood samples were provided to the CDC laboratory on 7/5/11. The shipment of blood pellets and plasma did not maintain a cold chain adequately and these samples had thawed (instead of being frozen) when they arrived at CDC laboratory. We also could not identify a strong partner from the Ministry of Health in Bolivia to receive laboratory training for molecular surveillance and this is a major limitation to transfer the appropriate technology for conducting local molecular surveillance in the future.

Samples rejected from analysis:

N	Sample #	18S	MSP2	230	*pfhrp2*	228	485	*pfhrp*3	475
1	BR16	-	-	-	-	+	-	-	-
2	BR27	-	-	-	-	-	-	-	-

Reasons for rejection

These samples were rejected from the final analysis because there was no amplification of either the 18S or one or both alleles of the MSP2 gene. Only the samples that were successfully amplified for these two genes were included in the final analysis, since this confirm the presence of *P. falciparum* in the samples.

Summary of findings

We tested all of the samples, both filter paper blood spots and blood pellets, using the established CDC laboratory procedures and both sets gave identical results. Two samples were rejected from the final analysis since they did not meet the inclusion criteria as noted above. Among the remaining 25 samples, one of the samples was found to have deleted the HRP2 gene (Figure 2, Table 1). Seventeen samples were found to have deletions of the HRP3 gene. Nine samples showed a deletion of Mal7P1.230, which is a pseudogene located near the upstream of HRP2 gene. There were 12 samples showing deletion of the Mal13P1.475 gene and 14 samples showing deletion of Mal13P1.485 gene, genes that flank *pfhrp3* (Table 1).

Table 1. Summary results showing deletions of *pfhrp2*, *pfhrp3* and their neighboring genes in Bolivia.

Country	Collection site	Genes*					
		230	***pfhrp2***	228	475	*pfhrp*3	485
Bolivia	Riberalta and Guayaramerin	9/25 (36.0%)	**1/25 (4.0%)**	0/25 (0.0%)	12/25 (48.0%)	**17/25 (68.0%)**	14/25 (56.0%)

*Data show number (and percentage) of parasites with genetic deletions out of total samples tested

Pending activities and further actions needed to complete the project in Bolivia

The occurrence of a 4% level of *pfhrp2* deletion in a limited number of samples (1 out of 25) indicates there is a need for testing additional samples to get a better estimate of prevalence of this deletion in Bolivia. In addition, to confirm these findings, another aliquot of plasma samples should be shipped to CDC under appropriate cold conditions as the previous shipment was delivered without sufficient cold chain (this could have compromised the integrity of specimens). The new plasma samples will be used to determine the levels of HRP2 proteins and to confirm the molecular results of *pfhrp2* status.

Bolivia should determine whether they would like to conduct further systematic surveillance to more accurately determine the prevalence of *pfhrp2*-deleted *P. falciparum* parasites in different regions as the current sample size of 25 is too small. Given that Bolivia did not receive any technical training for molecular testing, it is important they make use of next

round of molecular testing (if AMI/PAHO decides to support it) to train country staff. It is also relevant to point out here that CDC can support training of a Bolivian scientist (if MOH wants to identify one) for molecular testing of *pfhrp2* and *pfhrp3* deletions.

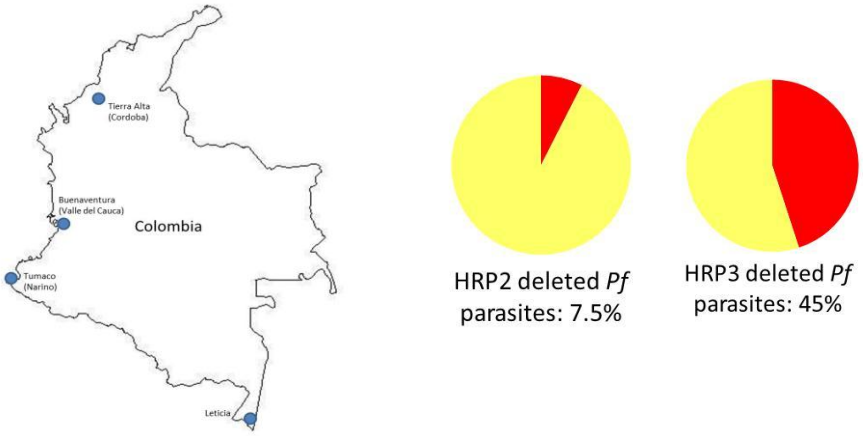
Colombia
Total samples planned for collection: 100
Total samples provided: 53
Blood specimen type:

- Filter paper blood spot: provided
- Blood plasma/serum: not collected
- Blood pellet: not collected

Sites of collection:

- Tierra Alta (Cordoba) (10 samples)
- Tumaco (Narino) (22 samples)
- Buenaventura (Valle del Cauca) (21 samples)

Figure 3. Collection sites and results in Colombia.



Sample collection, training and challenges

FIND provided funding and training for the collection of samples in Colombia. Dr. Claribel Murillo (Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM), Cali, Colombia served as key contact for sample collection. Dr. Murillo also received training in CDC (Feb 14, 2010 to March 27, 2010) in the methodology of molecular testing for *pfhrp2*/*pfhrp3* genetic deletions. Colombia did not collect venous blood as stated in the protocol and decided to collect filter paper blood spots per their association with FIND. This became a limitation in conducting detailed molecular analysis. Further serological confirmation of the molecular data could not be done due to lack of plasma. We received only about 50% of the sample numbers (100 targeted) aimed for Colombia.

Samples rejected from analysis:

N	Sample #	18S	MSP2	230	*pfhrp2*	228	485	*Pfhrp3*	475
1	TA02	+	-	+	+	+	-	-	-
2	TA04	+	-	-	+	-	-	-	-
3	TA06	+	-	-	-	-	-	-	-
4	TA07	+	-	-	-	+	-	-	+
5	BV17	+	-	-	-	-	-	-	-
6	BV20	-	-	-	-	-	-	-	-
7	BV21	-	+	-	-	+	-	-	+
8	BV24	-	-	-	-	+		-	+
9	BV25	-	-	-	-	-	-	+	-
10	BV26	-	+	-	+	+	+	-	+
11	BV27	-	-	-	-	-	-	-	-
12	BV32	-	-	-	+	-	-	-	-
13	BV35	-	+	-	-	-	-	-	-

Reasons for rejection

These samples were rejected from the final analysis because there was no amplification of the 18S and/or the MSP2 gene. Only the samples that were successfully amplified for these two genes were included in the final analysis, since this confirm the presence of *P. falciparum* in the samples.

Summary of analysis

A total of 53 samples collected from 2009–2010 were provided to the CDC malaria laboratory on 09/30/2010. Only 40 samples yielded good quality DNA that met the criteria for further *pfhrp2* testing. The results are reported for these 40 samples in Figure 3 and Table 2. In summary, three samples (7.5%) showed *pfhrp2* deletion and 18 samples (45.0%) showed *pfhrp3* deletion (Figure 3, Table 2). There were 21 samples with Mal7P1.230 deletion, 1 sample with Mal7P1.228 deletion, 17 samples with Mal13P1.475 gene deletion and 13 samples with Mal13P1.485 gene deletion (Table 2). A separate analysis of a small number of retrospectively collected samples conducted during the training session at CDC with Ms. Murillo suggests that levels of *pfhrp2* and *pfhrp3* deletions comparable to that present in Peru may be found around Leticia, in Amazonas province of Colombia, which was not one of the sites looked at prospectively.

Table 2. Summary results showing deletions of *pfhrp2*, *pfhrp3* and their neighboring genes in Colombia.

Country	Collection sites	Genes*					
		230	***pfhrp2***	228	475	***Pfhrp3***	485
Colombia	Tierra Alta (Cordoba)	3/6	**2/6**	1/6	6/6	**6/6**	6/6
	Tumaco (Narion)	11/22	**0/22**	0/22	6/22	**7/22**	5/22
	Buenaventura (Valle del Cauca)	7/12	**1/12**	0/12	5/12	**5/12**	2/12
	Total	21/40 (52.5%)	**3/40 (7.5%)**	1/40 (2.5%)	17/40 (42.5%)	**18/40 (45.0%)**	13/40 (32.5%)

*Data show number (and percentage) of parasites with genetic deletions out of total samples tested

Pending activities and further actions necessary to complete the project in Colombia

The data suggest the presence of some parasites with *pfhrp2* deletions on the Pacific and Caribbean coasts of Colombia, particularly the latter region. However, without additional samples from some of the surveyed areas (Tierra Alta) and other different parts of the country, it is difficult to give a fair estimate of prevalence of *pfhrp2*-deleted parasites throughout Colombia. Most of the samples have been collected only from the Pacific coastal region and a few samples from Tierra Alta on the Caribbean coast, which had 33% (2/6) deletions of *pfhrp2*. Future attempts to collect adequate numbers of samples from Tierra Alta and, as well, other interior sites in the country need to be encouraged. As noted above, an analysis of a limited set of retrospective samples indicated a high rate of *pfhrp2* and *pfhrp3* deletions in the Amazonas province of Colombia. Colombia needs to consider further systematic surveillance approaches to get a fuller understanding of the prevalence of HRP2 and HRP3 gene deletions in *P. falciparum* parasites in the country. In a possible future round sample collection for this surveillance, Colombia can make use of that opportunity to collect samples more widely on a geographical basis with an adequate sample size and respecting collection guidance (plasma and pellet) to estimate prevalence of gene deletions accurately.

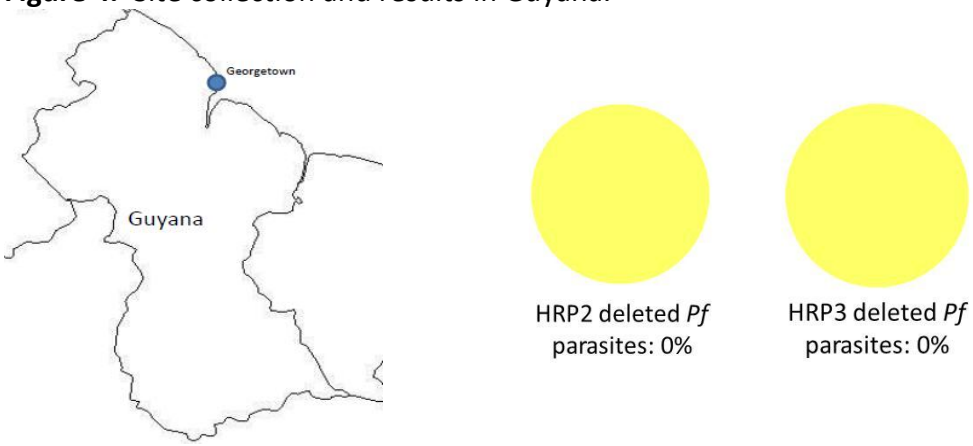
Guyana

Total samples planned for collection: 100
Total samples provided: 100
Blood specimen type

- Filter paper blood spot: provided
- Blood plasma/serum: collected and provided in August 2012
- Blood pellet: collected and provided in August 2012

Sites of collection: Georgetown (All from a single clinic)

Figure 4. Site collection and results in Guyana.



Sample collection, training and challenges

Dr. Nicholas Ceron and Mr. Krishnalal took an active role in organizing the sample collection efficiently. Dr. Udhayakumar visited Guyana in 2010 (March 22–26, 2010) to provide technical support and training for sample collection. During this visit the PAHO office and the Ministry of Health actively engaged in facilitating training and logistics for sample collection. The Health Minister, Honorable Dr. Ramasamy, took a personal interest in facilitating the project and involved the Guyana National Reference Laboratory in Georgetown to make use of laboratory infrastructure for processing samples and storage. Dr. Colin Roach and Mr. Chandrabose from this laboratory provided support for sample processing and storage. The samples were collected from the malaria clinic in Georgetown where most of the malaria patients from coastal and interior Guyana go for treatment (Figure 4). Guyana is the only country that achieved targeted sample size in a very short time and collected venous blood and filter paper blood spots as proposed. Guyana provided filter paper samples to CDC for molecular testing and it could not initially ship blood pellets and plasma to CDC for additional testing due to logistical issues. The blood pellet and plasma specimens were finally sent to CDC in August 2012. Unfortunately the shipment failed to maintain cold chain and the samples were in room temperature when delivered.

Samples rejected from analysis:

N	Sample #	18S	MSP2	230	*pfhrp2*	228	485	*Pfhrp3*	475
1	032	-	-	-	+	+	-	-	-
2	076	-	-	-	-	+	+	-	-
3	092	-	-	-	+	+	+	+	+

Reasons for rejection

These samples were rejected from the final analysis because there was no amplification of either 18S or either of the two MSP2 gene alleles. Only the samples that were successfully amplified for these two genes were included in the final analysis, since this confirm the presence of *P. falciparum* in the samples.

Summary of findings

A total of 100 samples as blood spots on filter paper were provided to the CDC laboratory (in March 2011) for this project. We tested all of the samples using the established CDC laboratory procedures. Three samples were rejected from the final analysis since they did not meet the inclusion criteria as noted above. Among the remaining 97 samples, none of the samples were found to have HRP2 or HRP3 gene deletions (Figure 4, Table 3). There were 40 samples showing deletion of Mal7P1.230, which is a pseudogene located near the upstream of HRP2 gene. One sample was found to have deleted gene Mal7P1.228 (gene located downstream of *pfhrp2*) (Table 3). In summary, we have not found any sample with genetic deletion for *pfhrp2* or *pfhrp3* from this batch of samples analyzed.

Table 3. Summary results data showing deletions of *pfhrp2*, *pfhrp3* and their neighboring genes in Guyana.

Country	Collection site	Genes*					
		230	***pfhrp2***	228	475	***Pfhrp3***	485
Guyana	Georgetown	40/97 (41.2%)	**0/97 (0.0%)**	1/97 (1.0%)	0/97 (0.0%)	**0/97 (0%)**	0/97 (0.0%)

*Data show number (and percentage) of parasites with genetic deletions out of total samples tested

Pending activities and further actions needed to complete the project in Guyana

Molecular testing for HRP2 and HRP3 genes has been completed. Serological confirmation of molecular test results can be completed when Guyana provides additional plasma samples to CDC.

Peru

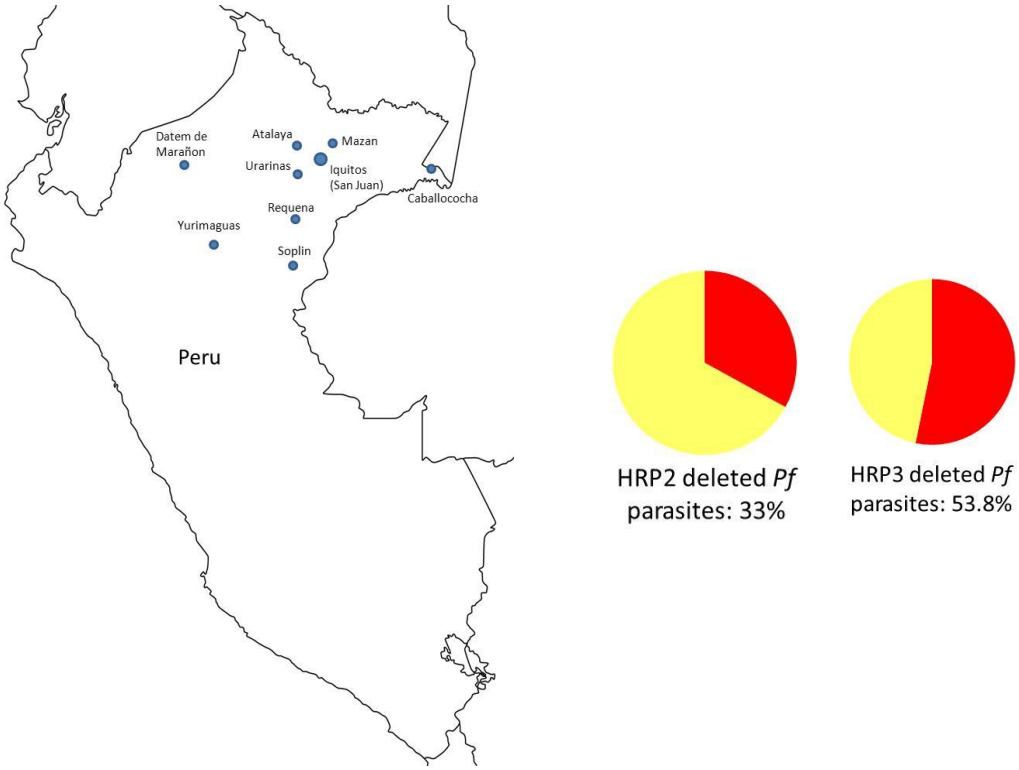
Total samples planned for collection: 100
Total samples provided: 94
Blood specimen type:

- Filter paper blood spot: provided
- Blood plasma/serum: not collected
- Blood pellet: not collected

Sites collected (all within the Loreto region)

- Atalaya (PA)
- Caballococha (PC)
- Datem Marañon (PD)
- Mazan (PM)
- San Juan (PSJ)
- Soplin (PS)
- Requena (PR)
- Yurimaguas (PY)
- Urarinas (PU)

Figure 5. Collection sites and results in Peru.



Sample collection, training and challenges

Dr. Dionicia Gamboa (Universidad Peruana Cayetano Heredia, Lima, Peru) and her staff in collaboration with FIND collected samples for the current study. Samples for this active surveillance were collected from 2009 through 2010 (Figure 5). These samples were only collected as filter paper blood spots. The lack of venous blood samples introduced a challenge for conducting molecular characterization of the expression of HRP2 genes. In the absence of plasma samples, it was not possible to confirm the molecular test results with serological tests. Ms. Katherine Jessica Torres-Fajardo, a graduate student from Dr. Gamboa's laboratory, received training in CDC (7/20/09 to 9/18/09) for molecular testing of *pfhrp2* and *pfhrp3* deletions. Collaboration with Dr. Gamboa moved very smoothly.

Summary of findings

Ninety-four samples collected from different sites in the Amazon basin in Peru for the period of 2009–2010 were given to CDC on 07/13/2010. The samples were tested by established CDC procedures and the results are presented in Table 4 for 93 specimens as one sample did not meet the inclusion criteria for DNA integrity. A total of 31 samples showed HRP2 gene deletions (33.3%) and 50 samples (53.8%) showed HRP3 gene deletions (Figure 5, Table 4). There were 73 samples with Mal7P1.230 deletion, 17 samples with Mal7P1.228 deletion, 36 samples with Mal13P1.475 deletion and 18 samples with Mal13P1.485 deletion (Table 4). Thus far, currently, the highest levels of *pfhrp2* deletion have been identified only in the Amazon River basin in Peru. The levels of *Pfhrp2* and *Pfhrp3* deletions in this prospective survey are similar to those found in the retrospective studies that have been published recently (9). It should be noted that the proportion of parasites with *pfhrp2* or *pfhrp3* deletions are quite variable from locality to locality. For instance the

parasite population in the community of Atalaya was 100% *pfhrp2* positive and 100% *pfhrp2* negative in the community of Requena. However, overall this region of Peru shows a high level of *pfhrp2/ pfhrp3* deletions.

Table 4. Summary results showing deletions of *pfhrp2*, *pfhrp3*, and their neighboring genes in Peru.

Country	Collection sites	Genes*					
		230	***pfhrp2***	228	475	***Pfhrp3***	485
Peru	Perú Atalaya (PA)	16/19	**0/19**	2/19	1/19	**3/19**	1/19
	Perú Caballococha (PC)	1/1	**1/1**	0/1	1/1	**1/1**	0/1
	Perú Datem Marañon (PD)	2/2	**2/2**	2/2	0/2	**2/2**	0/2
	Perú Mazan (PM)	11/11	**3/11**	5/11	2/11	**7/11**	3/11
	Perú San Juan (PSJ)	1/1	**1/1**	0/1	1/1	**1/1**	1/1
	Perú Requena (PR)	9/11	**11/11**	1/11	11/11	**11/11**	0/11
	Perú Soplin (PS)	16/31	**8/31**	1/31	15/31	**17/31**	8/31
	Perú Urarinas (PU)	5/5	**3/5**	4/5	4/5	**4/5**	3/5
	Perú Yurimaguas (PY)	12/12	**2/12**	2/12	1/12	**4/12**	2/12
	Total	73/93 (78.5%)	**31/93 (33.3%)**	17/93 (18.3%)	36/93 (38.7%)	**50/93 (53.8%)**	18/93 (19.4%)

*Data show number (and percentage) of parasites with genetic deletions out of total samples tested

Pending activities and further actions needed to complete the project

Dr. Gamboa's laboratory performed independent testing of the same samples for *pfhrp2* and *pfhrp3* deletion. We need to compare the results from the Peru and CDC laboratories for any inconsistencies and resolve if there are differences.

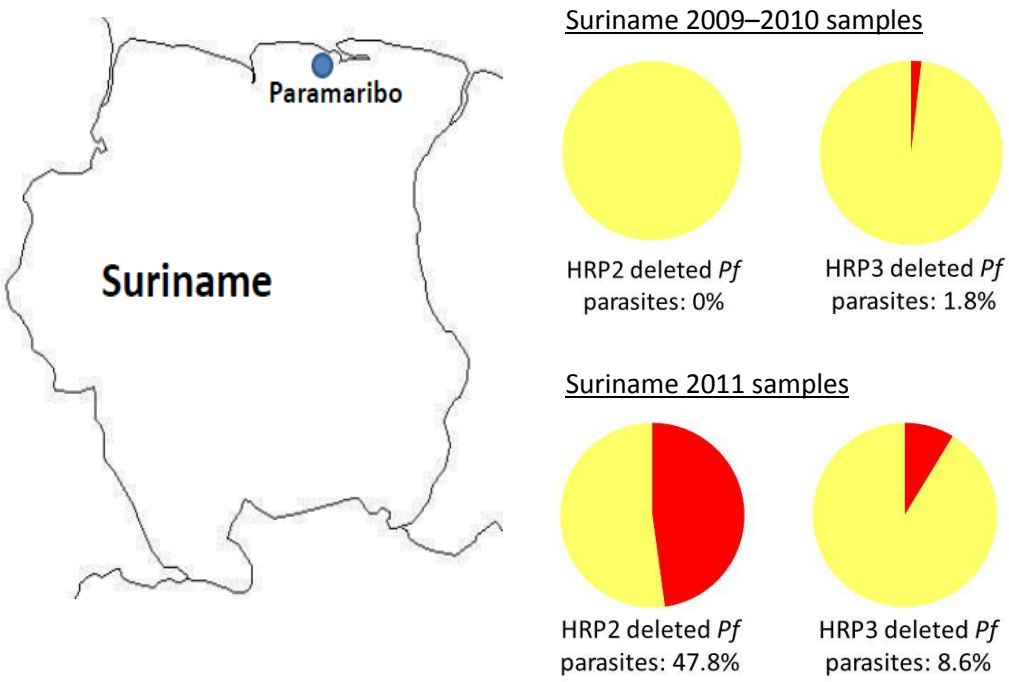
In summary, a substantial number of samples from multiple sites in the Amazon River basin in Peru have been tested. The data indicate widespread distribution of *pfhrp2*- and *pfhrp3*-deleted parasites in this endemic region of Peru. Although it was not known whether *pfhrp2*-deleted parasites were currently present in the coastal region due to lack of sample collection at any sites on the coast (as *P. falciparum* transmission is extremely low currently), testing of less than a dozen samples from a 2001 collection found no *pfhrp2* deletions. However, it has become evident from a very recent investigation of malaria reemergence in Tumbes on the Northern coast of Peru that all the 18 parasite isolates recently collected and analyzed showed *pfhrp2* deletion. Genetic evidence indicates the introduced parasites were probably originally from around Requena in Loreto province. It is well established now that *pfhrp2* deletion is high in Peru. Therefore, HRP2-based diagnostic tests are not recommended for use in Peru for diagnosis of *P. falciparum* malaria.

Suriname
Total samples planned for collection: 100
Total samples provided: 103
Blood specimen type

- Filter paper blood spot: provided
- Blood plasma/serum: not collected
- Blood pellet: not collected

Site of collection: Paramaribo

Figure 6. Collection site and results in Suriname.



Sample collection, training and challenges

Suriname chose to use initially retrospective samples available in their parasite bank collection for this investigation. Samples were preserved as filter paper blood spots and they were made available to this investigation. We had significant technical issues with some of the samples for conducting genotyping. Suriname provided samples in three different batches. It would have been better if all the samples were given in a single batch for logistical reasons related to efficient testing. Ms. Mergiory Y Bracho Garrido from Dr. Malti Adhein's laboratory (Anton de Kom Universiteit van Suriname, Paramaribo, Suriname) received training for molecular testing of *pfhrp2* and *pfhrp3* deletion by participating in the workshop conducted in Brazil. She has gone on to establish a capability for testing for *pfhrp2* and *pfhrp3* deletion in Suriname. Ms. Bracho Garrido has tested the same samples in parallel for comparison with CDC test results.

List of samples rejected from analysis:

Suriname shipment 1

N	Sample #	18S	MSP2	230	*pfhrp2*	228	485	*Pfhrp3*	475
1	M10-0127	-	-	+	-	+	-	-	-
2	M10-0286	+	-	-	+	+	+	+	+
3	M10-0373	-	-	-	+	+	-	-	-
4	M10-0867	+	-	+	+	+	+	+	+
5	M10-1055	-	-	+	-	+	+	+	+

Suriname shipment 2

N	Sample #	18S	MSP2	230	*pfhrp2*	228	485	*pfhrp3*	475
1	M9-0029	-	+	-	+	+	+	+	+
2	M9-0201	-	+	-	+	+	+	-	+
3	M9-0272	-	+	-	+	+	+	+	+
4	M9-0374	-	-	-	-	+	+	-	+
5	M9-0816	+	-	+	+	+	+	+	+
6	M9-0825	-	-	-	-	+	+	-	+
7	M9-1049	+	-	-	-	-	+	-	+
8	M9-1187	-	-	-	+	+	+	-	+
9	M9-1204	-	-	-	-	-	+	-	+
10	M9-1255	+	-	+	+	+	+	+	+
11	M9-1300	-	-	-	-	-	+	+	+

Suriname Shipment 3

N	Sample #	18S	MSP2	230	*pfhrp2*	228	485	*pfhrp3*	475
1	M10-0147	-	-	-	-	-	NA	-	-
2	M11-0161	-	-	-	-	-	-	-	+
3	M10-0176	-	-	-	-	-	NA	-	-
4	M10-0886	-	-	-	+	-	NA	+	-
5	M10-1124	-	-	+	-	-	NA	-	+
6	M11-0192	-	-	-	-	-	-	-	-
7	M11-0207	-	-	-	-	-	-	-	-
8	M11-0370	-	-	-	-	-	-	-	-
9	M11-0379	-	-	-	-	-	-	-	-

Reasons for rejection

These samples were rejected from the final analysis because there was no amplification of the 18S and/or one or both of the MSP2 gene alleles. Only the samples that were successfully amplified for these two genes were included in the final analysis, since this is the molecular marker of *P. falciparum* presence in the sample.

Summary of findings

A total of 53 samples were provided initially to the CDC laboratory in February 2011 (collected in 2010) and May 2011 (collected in 2009) for this project. Shipment 1 had 23 samples and shipment 2 had 30 samples. We tested all of the samples using the established CDC laboratory procedures. Sixteen samples (5 from Shipment 1, 11 from Shipment 2) were rejected from the final analysis as they did not meet the inclusion criteria. Among the remaining 37 samples, none of the samples were found to have *pfhrp2* deletions. One sample was found to have a *pfhrp3* deletion (Figure 6, Table 5). There were 12 samples showing deletion of Mal7P1.230. None of the samples showed evidence for deletion of the Mal7P1.228 gene, the Mal13P1.475 gene, or the Mal13P1.485 gene (Table 5). A third batch of samples (50) was sent from Suriname to CDC in February 2012 and these samples were recently analyzed for deletions of *pfhrp2* and *pfhrp3*. Eighteen of these samples meeting our inclusion criteria were collected in 2010 and none were found to have deleted *pfhrp2* or *pfhrp3* (Figure 6 for 2009-2010, which includes a total of 55 samples). One specimen had deleted the Mal7P1.228 gene. The other 23 specimens meeting inclusion criteria were collected in 2011. Eleven of these 23 samples (47.82%) collected in 2011 were found to have deleted all or a portion (eight samples with partial deletion and three samples with complete deletion) of the gene for HRP2. Two of these 2011 specimens also deleted the HRP3 gene. Two samples failed to amplify Mal7P1.228, five failed to amplify Mal7P1.230, and one did not amplify Mal7P1.485.

Table 5. Summary results showing deletions of *pfhrp2*, *pfhrp3,* and their neighboring genes in Suriname.

Country	Collection site	Genes*					
		230	***pfhrp2***	228	475	***Pfhrp3***	485
Suriname	Paramaribo	17/78 (21.8%)	**11/78 (14.10%)**	3/78 (3.9%)	0/78 (00.0%)	**3/78 (3.84%)**	1/78 (1.3%)

*Data show number (and percentage) of parasites with genetic deletions out of total samples tested

Pending activities and further actions needed to complete the project in Suriname

Some samples yielded poor or conflicting results because they were collected on Whatman #1 filter paper. This has led to multiple testing to confirm the results, which has contributed to a delay in completing the testing on these samples. A number of these specimens (25) as such were excluded because of poor DNA quality. We have provided a summary of CDC test results for the first two batches of samples to Suriname. We have provided a separate summary for the third set of samples, which because of a significant number of samples with *pfhrp2* deletions may have implications for a need of renewed surveillance using good quality blood samples. We have not received the individual test results from Suriname to compare with the test results obtained in CDC.

Brazil

Total samples planned for collection: 400 (100 in each of four different states)
Total samples provided to CDC: none, collection is still active

Blood specimen type

- Blood plasma/serum
- Blood pellet (extracted DNA)

Sites of collection

- Para
- Rondonia
- Acre
- Amazonas

Sample collection, training and challenges

Sample collection started in Brazil only recently, but it is anticipated that sample collection for three sites will be completed between August and October, 2012. The slow start in collections of specimens was in part the result of a number of delays (over two years) in obtaining institutional review board (IRB) clearances from the local and national levels with several requests for clarifications and modifications being made by the various IRB committees. Clearance for collection has yet to be obtained from the Amazonas IRB although clearance at the national level has been granted. Molecular testing at CDC will be initiated when the blood specimens are processed and DNA extracted at the Instituto Evandro Chagas (IEC). These extracted blood samples along with an aliquot of plasma are to then to be transferred to CDC.

In order to provide training for conducting molecular testing for detecting *pfhrp2* and *pfhrp3* deletions in *P. falciparum* parasite populations, a training workshop was conducted at the Instituto Evandro Chagas, Belem, Brazil from August 30th to September 10, 2010. In this workshop ten participants (eight from Brazil, one from Guyana and one from Suriname) attended. This training was provided by Dr. John Barnwell and Dr. V. Udhayakumar with Ms. Amanda Poe acting as technical assistant. The purpose of this training was to establish local capacity for testing in Brazil, Guyana and Suriname. List of attendees to the training:

- Marinete M. Povoa, senior reserach scientist, IEC, Belem, Brazil
- Giselle M.R. Viana, scientist, IEC, Belem, Brazil
- Danielle R.L. Barbosa, student, IEC, Belem, Brazil
- Yonne Cheuhan, scientist, Fundacao de Medicina Tropical de Amazonas, Manaus, Brazil
- Marilia L.O. Guedes, student, FIMCA, Porto Velho, Brazil
- Eponina C.M. Melo, staff, Laboratorio Central de Cruzeiro do Sul, Cruzeiro do Sul, Acre, Brazil
- Joana D.N. Costa, staff, Centro de Pesquisa em Medicina Tropical de Rondonia, Porto Velho, Brazil
- Maristela G.D. Cunha, professor, Federal University of Para, Belem, Brazil
- Javin Chandrabose, senior medical technologist, National Public Health Reference Laboratory, Georgetown, Guyana
- Mergiory Labadie-Bracho, scientist, Institute for Biomedical Sciences, Paramaribo, Suriname

Summary of current collections and findings

The current plan for the analysis of specimens from the collection sites in Brazil is to send all aliquots of blood pellets and plasma to IEC in Belem, Para. There one aliquot set (blood and plasma) of each specimen will be archived at -80^{o}C. The other two aliquots of a blood sample will be extracted for DNA; one to be analyzed at IEC and the other transported to Atlanta to be analyzed at CDC. To date 85 blood specimens have been collected in Acre, another 62 in Rondonia, 42 from Itaituba in Western Para state, and at least 33 from Goianesia do Para in Eastern Para state near Tucurui. Extraction and analysis has begun at IEC on a portion of the blood samples. Initial results obtained at CDC on 14 specimens collected in Eastern Para state found no deletions of *pfhrp2* but all 14 (100%) of the isolates appear to have deleted *pfhrp3*. As noted above, it is anticipated that collections will be completed between August and October, 2012 except for Amazonas state due to a lack of clearance from the local IRB.

Retrospective survey using samples collected from Honduras

Total samples available for testing : 68

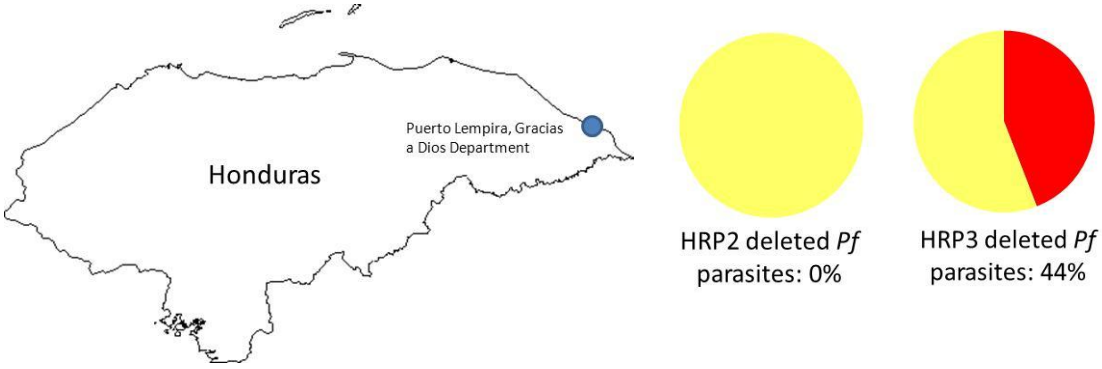
Blood specimen type:

- Filter paper blood spot: provided
- Blood pellet: not collected
- Blood plasma/serum: not collected

Site of collection

- Puerto Lempira, Gracias a Dios Province

Figure 7. Collection site and results in Honduras.



Sample collection, training and challenges

There were 68 samples available from a chloroquine efficacy study conducted from September 2008 through September 2009. These samples were brought to CDC for testing molecular markers of drug resistance and the left over samples were utilized for this study as requested by the Honduras. Dr. Gustavo Fontecha and Ms. Meisy Mendoza-Montoya (National Malaria Laboratory, Ministry of Health, Honduras) received training in CDC (1/20/2011 to 2/22/2011) for molecular testing of *pfhrp2* and *pfhrp3* deletions (along with

molecular testing of chloroquine resistance markers). This collaboration was well executed and the country partners are attempting to improve the local laboratory capacity for conducting molecular surveillance for *pfhrp2* deletion.

Summary of findings

We tested 68 samples using the established CDC laboratory procedures. No samples were rejected from the final analysis. Among these samples, none was found to have deleted the HRP2 gene (Table 6). Thirty samples were found to have deletions of the HRP3 gene (Table 6). No samples showed a deletion of Mal7P1.230, which is a pseudogene located near the upstream of HRP2 gene, or a deletion of Mal7P1.228, a heatshock gene located near the downstream of HRP2 gene. There were 32 samples showing deletion of the Mal13P1.475 gene and 13 samples showing deletion of Mal13 P1.485 gene, genes that flank *pfhrp3* (Table 6).

Table 6. Summary results showing deletions of *pfhrp2*, *pfhrp3,* and their neighboring genes in Honduras.

Country	Collection site	Genes*					
		230	***pfhrp2***	228	475	***Pfhrp3***	485
Honduras	Puerto Limpera, Gracias a Dios Department	0/68 (0%)	**0/68 (0%)**	0/68 (0%)	32/68 (47.1%)	**30/68 (44.1%)**	13/68 (19.1%)

*Data show number (and percentage) of parasites with genetic deletions out of total samples tested

Final reporting and publication

As soon as collection and analysis of molecular testing results are completed in all participating countries a final report will be prepared and submitted to participating countries and other AMI partners, especially PAHO. The various results will also be prepared as manuscripts and submitted for publication in one or several scientific journals.

Summary

The absence of the HRP2 gene in a large proportion of *P. falciparum* parasites in the Amazon River watershed of Peru has been previously reported (9). This mutation results in no HRP2 antigen being produced and *P. falciparum* parasites in the blood of patients are not detected by malaria rapid diagnostic tests that are designed to detect HRP2. HRP2-based RDTs are the most sensitive, most stable and most commonly used RDTs for the diagnosis of *P. falciparum*. Further initial prospective surveys for deletion of the HRP2 and HRP3 genes in populations of *P. falciparum* have been or are about to be completed in Peru, Colombia, Bolivia, Brazil, Guyana, Suriname, and other countries taking part in the Amazon Malaria Initiative.

In Peru the survey served to confirm the levels and extent of *pfhrp2*-and also *pfhrp3*-negative parasites. A subsequent event and study has shown that these parasites can jump to a new distant focus of transmission and set up an outbreak of parasite infections resistant to diagnosis. The level of *pfhrp2*-negative parasites in Colombia overall was about 7.5%,

however, on the Pacific coast this was only 3.0% (1/34), but in Tierra Alta on the Caribbean coast it was nearly 33% (2/6) with *pfhrp3* deletions from 32% to 100%. An additional retrospective investigation indicated the levels of *pfhrp2*-negative parasites around Leticia, Colombia were comparable to those in Peru. Other participating countries have shown low levels of *pfhrp2* deletions in the parasite populations ranging from 0% in Guyana and Honduras to 4% and 7.7% in Bolivia and Suriname, respectively. At this time, the level and extent of *pfhrp2*-negative *P. falciparum* in Brazil remains unknown, but it is expected that by October/November, 2012 a much clearer idea of *pfhrp2* deletions in that country will be available.

Pfhrp2 and *pfhrp3* deletions appear to be widespread but patchy with levels of *pfhrp2*-negative parasites generally low outside of the Amazon River basin of Peru and the adjoining region of Colombia where HRP2 gene deletions in the *P. falciparum* populations remain high (>25%). However, examples such as the outbreak of *pfhrp2*-negative parasites on the coast of Peru and the increase in *pfhrp2*-negative cases in Suriname in 2011 illustrate the need for continued vigilance.

Recommendations

1. Surveillance for *P. falciparum* populations with HRP2 gene deletions should continue with surveys conducted at least every three years to be able to assess any continuing or new trends in diagnostic resistant parasites.

2. The number of sites examined should adequately represent and cover the different zones of *P. falciparum* transmission in a country. Additionally, the number of samples collected in any given survey site should be large enough to adequately detect and assess trends in the presence and level *pfhrp2*-negative parasites where rates are less than 10% (around 100 samples).

3. Specimens should be collected in the form of blood with separation of the samples into blood pellets for DNA extraction and molecular analysis and plasma for detection of HRP2 by ELISA to provide cross-checking the results from molecular analysis. Assays should be performed under established protocols and with appropriate standards and quality control measures.

4. When rates of *pfhrp2* negativity of 10% or greater are detected in a parasite population in an area under surveillance the use of HRP2-based RDTs should be discouraged and discontinued. Alternative RDTs detecting *P. falciparum*–specific pLDH or a pan pLDH should be selected from lists of recommended products selected by the Global Fund or WHO based upon the WHO/TDR/FIND/CDC program for the evaluation malaria RDTs. It is extremely important to mention that, in areas of low and very low malaria transmission, lower thresholds for deletion should be considered, as all possible efforts to minimize false-negative results should be made. In addition, despite RDT implementation, public health authorities should continue efforts to maintain and improve microscopy capacity in South and Central American countries, as this continues to be the cornerstone for malaria diagnosis for routine case management.

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Appendix I: Summary of samples with genetic deletion in HRP2, HRP3 and their flanking genes in South America and Central America

Genes tested	Countries					
	Bolivia (%)	Colombia (%)	Guyana (%)	Peru (%)	Suriname (%)	Honduras (%)
230	9 (36%)	21 (52.2%)	40 (41.2%)	73 (78.5%)	17 (21.8%)	0 (0%)
Pfhrp2	**1 (4%)**	**3 (7.5%)**	**0 (0%)**	**31 (33.3%)**	**11 (14.1%)**	**0 (0%)**
228	0 (0%)	1 (2.5%)	1 (1.0%)	17 (18.3%)	3 (3.8%)	0 (0%)
475	12 (48%)	17 (42.5%)	0 (0%)	36 (38.7%)	0 (0%)	32 (47.5%)
Pfhrp3	**17 (68%)**	**18 (45 %)**	**0 (0%)**	**50 (53.8%)**	**3 (3.84%)**	**30 (44.1%)**
485	14 (56%)	13 (32.5%)	0 (0%)	18 (19.4%)	1 (1.3%)	13 (19.1%)
Total samples	25	40	97	93	78	68

Appendix II: Primers used for amplification of targeted genes

The following primers were used for the amplification of the indicated genes

1) 18S ribosomal DNA primers (for the confirmation of presence of *Plasmodium falciparum* in the specimens)

   1st reaction (genus specific)

   AL3765: 5’ CCT GTT GTT GCC TTA AAC TTC 3’

   AL3766: 5’ TTA AAA TTG TTG CAG TTA AAA CG 3’

   2nd reaction (species specific, *P. falciparum*)

   AL3767: 5’ TTA AAC TGG TTT GGG AAA ACC AA ATA TAT T 3’

   AL3768: 5’ ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC 3’

2) MSP2 primers (multiplex)

   1st reaction

   M1-OF: 5’ CTA GAA GCT TTAGAA GAT GCA GTA TTG 3’
   M1-OR: 5’ CTT AAA TAG TAT TCT AAT TCA AGT GGA TCA 3’
   M2-OF: 5’ ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA 3’
   M2-OR: 5’ CTT TGT TAC CAT CGG TAC ATT CTT 3’
   G-OF: 5’ TGA ATT TGA AGA TGT TCA CAC TGA AC 3’
   G-OR: 5’ GTG GAA TTG CTT TTT CTT CAA CAC TAA ’

   2nd reaction

   M1-KF: 5’ AAA TGA AGA AGA AAT TAC TAC AAA AGG TGC 3’
   M1-KR: 5’ GCT TGC ATC AGC TGG AGG GCT TGC ACC AGA 3’
   M1-MF: 5’ AAA TGA AGG AAC AAG TGG AAC AGC TGT TAC 3’
   M1-MR: 5’ ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC 3’
   M1-RF: 5’ TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG 3’
   M1-RR: 5’ CAA GTA ATT TTG AAC TCT ATG TTT TAA ATC AGC GTA 3’
   M2-FCF: 5’ AAT ACT AAG AGT GTA GGT GCA TAT GCT TCC A 3’
   M2-FCR: 5’ TTT TAT TTG GTG CAT TGC CAG AAC TTG AAC 3’
   M2-ICF: 5’ AGA AGT ARG GCA GAA AGT AAG CCT TCT ACT 3’
   M2-ICR: 5’ GAT TGT AAT TCG GGG GAT TCA GTT TGT TCG 3’

3) MSP2 primers (nested)

   1st reaction

   MSP2F1: GAA GGT AAT TAA AAC ATT GTC

   MSP2R1(2): GAT GTT GCT GCT CCA CAG

   2nd reaction

   MSP2F2: GAG TAT AAG GAG AAG TAT G

   MSP2R2: CTA GAA CCA TGA ATA TGT CC

4) HRP2 exon 1-2

   1st reaction

   HRP2E12F1: 5' GGT TTC CTT CTC AAA AAA TAA AG 3'

   HRP2E12R1: 5' TCT ACA TGT GCT TGA GTT TCG 3'

   2nd reaction

   HRP2E12F: 5' GTA TTA TCC GCT GCC GTT TTT GCC 3'

   HRP2E12R: 5' CTA CAC AAG TTA TTA TTA AAT GCG GAA 3'

5) Mal7P1.230 (5 prime gene to HRP2; a pseudo gene)

1st reaction

230F1: 5’ GAT ATC ATT AGA AAA CAA GAG CTT AG 3’

230R: 5' TAT CCA ATC CTT CCT TTG CAA CAC C 3'

2nd reaction

230F: 5' TAT GAA CGC AAT TTA AGT GAG GCA G 3'

230R:5' TAT CCA ATC CTT CCT TTG CAA CAC C 3'

6) Mal7P1.228 (3 prime to HRP2 gene; a heat shock protein)

1st reaction

228F: 5' AGA CAA GCT ACC AAA GAT GCA GGT G 3'

228R: 5' TAA ATG TGT ATC TCC TGA GGT AGC 3'

2nd reaction

228F1: 5' CCA TTG CTG GTT TAA ATG TTT TAA G 3'

228R: 5' TAA ATG TGT ATC TCC TGA GGT AGC 3'

7) HRP3 exon 1-2

1st reaction

HRP3E12F1: 5' GGT TTC CTT CTC AAA AAA TAA AA 3'

HRP3E12R1: 5' CCT GCA TGT GCT TGA CTT TA 3'

2nd reaction

HRP3E12F: 5' ATA TTA TC GCT GCC GTT TTT GCT 3'

HRP3E12R: 5' CTA AAC AAG TTA TTG TTA AAT TCG GAG 3'

8) Mal13PMal13PMal13P1.475 (5 prime to HRP3 gene; an exported protein of unknown function)

1st reaction

475F: 5' TTC ATG AGT AGA TGT CCT AGG AG 3'

475R: 5' TCG TAC AAT TCA TCA TAC TCA CC 3'

2nd reaction

475F: 5' TTC ATG AGT AGA TGT CCT AGG AG 3'

475R1: 5' GGA TGT TTC GAC ATT TTC GTC G 3'

9) Mal13PMal13PMal13P1.485 (3 prime to HRP3 gene; coding for Acyl-coA synthetase)

1st reaction

485F: 5' TTG AGT GCA ATG ATG AGT GGA G 3'

485R: 5' AAA TCA TTT CCT TTT ACA CTA GTG C 3'

2nd reaction

485F1: 5' GTT ACT ACA TTA GTG ATG CAT TC 3'

485R: 5' AAA TCA TTT CCT TTT ACA CTA GTG C 3'