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Response plan to *pfhrp2* gene deletions

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1. Introduction and objectives

Accurate, timely diagnosis of malaria is critical to case management and is a key element in national and global malaria control and strategies for elimination. Malaria microscopy, the traditional diagnostic approach, is difficult to use in the decentralized settings where most malaria occurs; therefore, the advent of disposable lateral-flow assays for malaria (widely known as rapid diagnostic tests, RDTs), has been of fundamental importance in modern malaria management, for targeting therapy, reducing drug wastage and limiting pressure towards the development of resistance. Malaria RDTs are available from many manufacturers in various conformations. Ensuring the safety and quality of the RDTs used in malaria control and case management has been a major focus of WHO and its partners.

The clinically important malaria RDTs detect parasite proteins circulating in the blood. Some are configured to detect only *Plasmodium falciparum* and others, other *Plasmodium* species. The tests that are most sensitive for detecting falciparum malaria contain antibodies to detect the histidine-rich protein 2 (HRP2) and/or the related HRP3 protein. These protein targets, which are specific to *P. falciparum*, are strongly expressed by asexual parasites and have multiple copies of the target epitopes per protein. Some 10 years ago, researchers working in the Peruvian Amazon region identified patients infected with *P. falciparum* strains that had acquired deletions in the genes that encode these proteins (*pfhrp2* and *pfhrp3*), rendering them capable of avoiding detection with HRP2-based RDTs. Since then, many studies have demonstrated the presence of such strains in other countries and regions. The frequency and global distribution of this phenomenon is not yet fully understood, but, in a limited number of countries, the relative incidence of these deletion mutants has been found to be high enough to threaten the usefulness of HRP2-only RDTs.

This Global Action Plan for a response to mutations that limit the effectiveness of HRP2-based RDTs comprises a global framework to support national malaria control programmes and their implementing partners to address this problem pragmatically. The document also summarizes current knowledge and critical gaps in knowledge to guide future research and product development. The four objectives of an implemented global action plan are to:

1. define the frequency and distribution of these diagnostically relevant mutations in circulating *P. falciparum* strains;

2. provide concrete guidance to countries on malaria diagnosis and treatment in settings where such mutations are found to be frequent;

3. identify gaps in knowledge about the genesis and spread of strains with *pfhrp2* and/or *pfhrp3* deletions and the actions required to develop new, accurate tests for malaria based on alternative target antigens; and

4. coordinate advocacy and communication with donors, policy-makers, test developers, research agencies, technical partners and disease control programmes to assist in planning.

2. Defining the issue

2.1 RDTs in malaria control

Malaria remains a huge global health risk; it causes an estimated 214 million cases of febrile disease (range, 149–303 million) and 438 000 deaths (range, 236 000–635 000) a year. Approximately 90% of all malaria deaths occur in sub-Saharan Africa and nearly 70% in children under 5 years. Nevertheless, impressive progress has recently been made in the control of malaria worldwide: between 2000 and 2015 alone, the incidence of malaria cases was reduced by 41%. Recent studies demonstrate that, even in African countries with endemic malaria, the great majority of cases of febrile illness are not due to malaria *(1)*.

Malaria does not usually present with distinct physical signs that would allow accurate clinical diagnosis; and, as the incidence of malaria drops, confirmatory testing before treatment becomes essential. Timely, accurate diagnostic testing is the cornerstone of modern malaria control, and, since 2010, WHO treatment guidelines have included the recommendation that all cases of suspected malaria be tested by microscopy or an RDT *(2)*. The benefits of diagnostic testing extend beyond malaria case management. As stated in the *WHO global technical strategy for malaria 2016–2030 (3)*, "Expansion of diagnostic testing will provide timely and accurate surveillance data based on confirmed rather than suspected cases. Additionally, it will lead to improved identification and management of the many non-malarial febrile illnesses presumed to be malaria solely on the basis of the presence of fever."

As malaria microscopy is not always feasible in primary care settings, the development of malaria RDTs based on lateral flow has been critical to current strategies for malaria control. Indeed, RDTs for malaria are one of the most successful diagnostic products in global health. With a disposable cassette to detect parasite antigens in fingerstick blood samples, they offer simple, unambiguous detection of malarial infection, allowing disease confirmation before treatment at primary care level. First developed in the early 1990s, malaria RDTs were initially little used, despite published reports of good performance *(4)*. By 2002, nearly 10 million tests were being produced each year by about 24 manufacturers. In view of variations in manufacture and in published results, WHO and partners began a quality-assurance programme to ensure procurement of high-quality tests (Box 1). Once quality assurance was in place, donors and policy-makers were confident in extending use of RDTs for confirmatory testing.

Box 1. Quality assurance for malaria RDTs

Variation in the manufacture of RDTs, between both companies and manufacturing lots, can significantly affect their performance. In view of the large number of RDTs commercially available and the relative weakness of many national regulatory systems, WHO, the Foundation for Innovative New Diagnostics (FIND), the United States Centers for Disease Control and Prevention and other partners instituted a quality assurance programme, which has been functioning since 2008. The programme has three main elements: product testing to verify their performance against a standardized panel of blood samples, lot testing to ensure that each procured lot has maintained its specified criteria before distribution, and job-aids and training materials for health workers and trainers to support proper use.

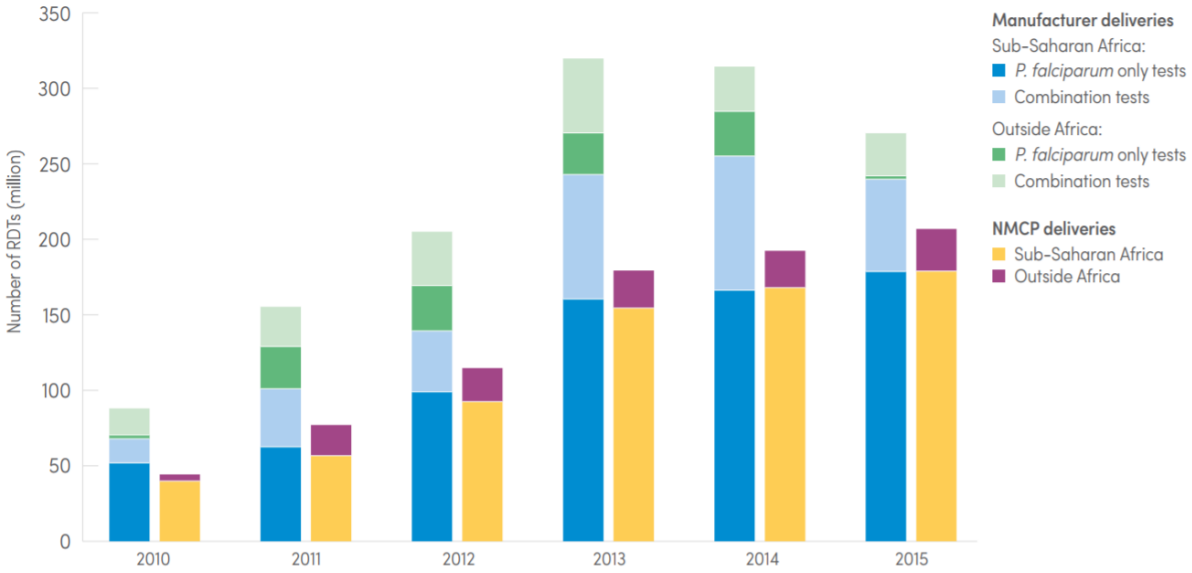
All companies that manufacture malaria RDTs in conformity with ISO13485:2003 are invited to submit RDTs for testing against a large bank of geographically diverse clinical specimens and cultured parasites to determine their performance in detecting 200 or 2000 parasites/μL. The WHO malaria RDT product testing programme has established performance parameters (e.g. sensitivity, specificity, stability, ease of use) and evaluated 297 new or newly submitted products. The results of product testing form the basis of the procurement criteria of WHO, other United Nations agencies, the Global Fund to Fight AIDS, Tuberculosis and Malaria, national governments and nongovernmental organizations. The results have shifted markets towards better-performing tests and are resulting in overall improvement in their quality. From 1 January 2018, WHO will require WHO prequalification as part of procurement recommendations. WHO prequalification requires attaining performance criteria, a successful dossier review and a site inspection.

Two lot-testing sites, at the Research Institute for Tropical Medicine in the Philippines and the Pasteur Institute of Cambodia, evaluate procured lots before their distribution for use to ensure that each lot is not degraded and that its performance is that determined during product testing. From 1 January 2018, WHO will pilot-test partially decentralized lot testing to national reference laboratories, which will be supported by a central reference laboratory.

As shown in Fig, 1, RDT manufacture and sales exceeded 300 million tests per year by 2013 and 2014. The differences between the data from manufacturers and that from national malaria control programmes (NMCPs) as illustrated in Fig. 1, are probably due to the inclusion of sales in the private sector sales in the information supplied by manufacturers and incomplete reporting by some NMCPs. Use in Africa accounts for the vast majority of NMCP deliveries, and, as seen from the manufacturers' data, *P. falciparum*-only tests based on HRP2 detection predominate.

FIG. 1.

Data from manufacturers and from national malaria control programmes (NMCPs) on the volume and types of RDTs delivered worldwide, 2010–2015



Source: reference *(5)*

The advent of RDTs and their widespread use, spurred by WHO policy and now adopted in the public sector by all 91 countries with continuous malaria transmission, has massively increased the fraction of all suspected cases of malaria that are confirmed. Such diagnostic certainty has averted millions of cases of mistreatment and overtreatment, has helped thousands of clinicians working in malaria-prone areas to understand that fever does not necessarily mean malaria and has provided much clearer understanding of the current epidemiology of malaria during the drive towards its elimination. Access to such testing has improved: by 2015 some 80% of all malaria suspects attending public health facilities worldwide underwent confirmatory testing rather than a syndromic diagnosis *(5)*.

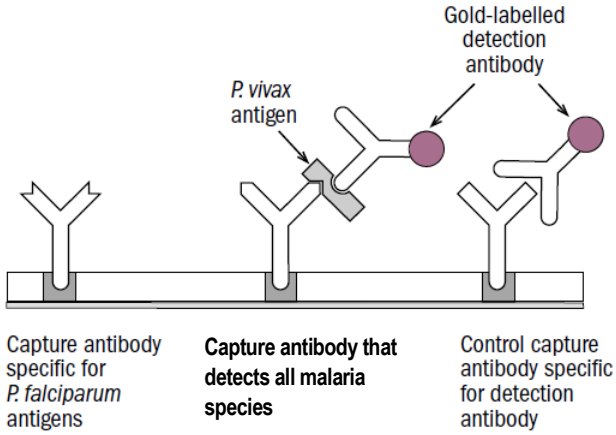
The rates of diagnostic testing vary by geographical area, however, and are lowest for febrile children in Africa.

2.2 How RDTs work

RDTs are lateral-flow immunoassays that allow visualization of specific antigen–antibody recognition events. In routine use, a specified amount of fingerstick blood is transferred to one end of the RDT, the sample pad, which is loaded with reagents that will lyse the blood cells to release any malaria antigens present and allow binding of monoclonal antibodies labelled with colloidal gold or another colorimetric indicator. Addition of a liquid buffer helps the blood to wick up through the nitrocellulose membrane towards an absorbent pad. On the way, it crosses one or more test lines on the strip, where immobilized monoclonal antibodies can bind to exposed epitopes on *Plasmodium* proteins (*P. vivax* in Fig. 2) *(6)*. In addition to test lines, which darken when malarial proteins are bound and detected, there is also a control line, which

ensures that the sample pad reagents were liquified and wicked appropriately up the RDT membrane. As each test may have a slightly different configuration and require different handling (e.g. amount of buffer to be added, time until results), the instructions accompanying the tests must be followed closely.

FIG. 2.
Immunological reaction on a positive RDT strip (example: *P. vivax* infection)



Source: New perspectives: malaria diagnosis. Report of a joint WHO/USAID informal consultation, 25–27

The *Plasmodium* species that is detected by an RDT is defined by the capture and detection antibodies. A number of different malaria antigens are target by manufacturers (Table 1), sometimes in combination on a single test. Of the 202 unique products evaluated in the WHO product testing programme to date, 71% were designed to detect and differentiate *P. falciparum* from non-*P. falciparum* malaria (either pan-specific or species-specific for *P. vivax* or *P. vivax*, *ovale* and *malariae*), 32% were designed to detect *P. falciparum* alone, 5% were designed to detect *P. falciparum* and non-*P. falciparum* malaria without distinguishing between them, and a single product was designed to detect *P. vivax* only. HRP2-based testing for *P. falciparum* is included in more than 80% of commercially available tests *(7–9)*.

TABLE 1.
Plasmodium antigens targeted by antibodies used in malaria RDTs

Target antigen	Full antigen name	Selectivity of assay	Characteristics
HRP2	Histidine-rich protein II	Detects only *P. falciparum*	Water-soluble protein of unclear function that is abundantly produced by trophozoites and young gametocytes and contains repeating epitopes. Persists in serum for days to weeks after successful treatment.
Pf-pLDH	*P. falciparum* parasite lactate dehydrogenase	Detects only *P falciparum*	Soluble glycolytic enzyme produced by trophozoites and gametocytes. Blood levels decline rapidly during therapy.
Pv-pLDH	*P. vivax* parasite lactate dehydrogenase	Detects only *P. vivax*	
Pvom-pLDH	Parasite lactate dehydrogenase from *P. vivax*, *P. ovale* and *P malariae*	Detects *P. vivax, P. ovale* and *P malariae*	
Pan-pLDH	*Plasmodium* parasite lactate dehydrogenase	Detects all *Plasmodium* spp. that infect humans	
Aldolase	*Plasmodium* aldolase	Detects all *Plasmodium* spp. that infect humans	Key enzyme in the glycolysis pathway, with relatively conserved amino acid sequences. Relatively rapid clearance after therapy.

Because of the wide prevalence and medical importance of falciparum malaria, essentially all RDTs contain antibodies that detect *P. falciparum* proteins (pan-pLDH, Pf-pLDH or HRP2). A number of factors are usually taken into account in selecting an antigen.

- Tests for HRP2 are often more sensitive than pLDH assays, in terms of both detecting smaller concentrations of protein and the clinical limit of detection (measured as parasites per microlitre) *(10)*.
- HRP2 RDTs tend to be more heat stable.
- pLDH assays more accurately identify acute infection, as the target pLDH enzyme concentration falls quickly with treatment, whereas HRPs may persist for days to weeks after treatment.
- Aldolase assays tend to be the least sensitive of the current RDTs and are rarely procured.

The sensitivity of a given RDT depends on several factors, including the accuracy of testing procedures, the characteristics of the blood sample, the age and storage conditions of the test and the specificities of its manufacture, such as the selection of capture and detection antibodies, type of nitrocellulose, label and buffer conditions. False-negative result may be due to low parasite density *(9)*, incorrect interpretation of results, gene deletion of the parasite target protein (e.g. pfhrp2) or a prozone effect *(11)*.

2.3 Quality assurance

Almost 75% of suspected cases of malaria in Africa are tested with an RDTs *(12)*, and procurement of malaria RDTs currently represents an investment of 151 million USD annually by multilateral and bilateral donors *(13)*. Anything that compromises the utility of malaria testing or the accuracy of test results threatens the value of this expenditure and the benefits of testing. RDTs that perform poorly have been excluded from the public market by dint of a large quality-control programme for RDT products before (by comparative testing of marketed products) and after (by lot-testing) procurement (see Box 1). Recently, the manufacturers of some RDT products have met the requirements of WHO prequalification. These two programmes effectively remove doubt about the quality of products. Until recently, the only remaining concern was proper clinical testing with RDTs, including storage, training, procedural correctness, accuracy of recording results and adherence to results.

2.4 Evolution of *pfhrp2/3* deletion mutants

In 2010, researchers who were characterizing malarial blood samples from the Amazon basin in Peru within the WHO product testing programme found that HRP2 was not detectable in the blood of some patients with *P. falciparum* infections that had been confirmed by microscopy *(14)*. Molecular testing by polymerase chain reaction (PCR) and gene sequencing confirmed that the genes that encode this protein (and sometimes those that encode the structural homologue HRP3) were deleted from the parasite. These genetic deletions led to false-negative results in RDTs that target this protein, raising the spectre that, if the anomaly occurred in other countries, many infections with *P. falciparum* would remain undiagnosed and untreated. Subsequent analyses at other sites in the Loreto region of the Peruvian Amazon *(15)* showed a significant increase in the frequency of parasites with gene deletions in specimens collected in 1998–2001 (20.7%) and those collected in 2003–2005 (40.6%) *(16)*.

In a global survey of HRP2 sequence variation in 458 samples collected in 2008–2009 in 38 countries within the WHO RDT quality assurance programme *(17)*, substantial diversity was found in *pfhrp2/3* sequences, including the number and type of repeating epitopes, but no samples with *pfhrp2/3* deletions were found. Sequence variation did not substantially alter the sensitivity of RDTs to detect parasitaemia at a clinically important level (> 200 parasites/µL).

In a more recent, continuing global survey (http://www.malariagen.net/projects/parasite/pdf), *pfhrp2* and *pfhrp3* exons from 2671 blood samples collected from patients with *P. falciparum* infection were sequenced in the MalariaGEN *P. falciparum* Community Project, which is building a catalogue of variants and allele frequencies in order to characterize common genetic variations in *P. falciparum*. Samples from symptomatic and asymptomatic patients in 27 countries were contributed to the project by 32 investigators. Strong evidence of deletions of

pfhrp2 were found in 0.6% of all samples and identified in 9 of the 27 countries. The frequency of *pfhrp2* deletions in collected samples was > 5% in those from only 3 countries – Indonesia (Papua Province), Kenya and Peru. Deletions of *pfhrp3* were more common, with an overall frequency of 2.4%, and were identified in 15 countries, in 4 of which > 5% of samples contained *pfhrp3* deletions. Mutations of both *pfhrp2* and *pfhrp3* were found in 0.3% of samples from 6 countries, and dual mutations were found in more than 5% of samples only from Kenya and Peru.[1]

Thirty, mainly national studies have been carried out in about 24 countries (Table 2), which confirm that *P. falciparum* malaria strains with mutations that affect production of HRP2 and/or HRP3 proteins (hereafter called *pfhrp2/3* deletion mutants) have evolved in many regions endemic for malaria, in some cases at a prevalence that significantly reduces the effectiveness of RDTs that test for this antigen to detect falciparum malaria. Parasites that fail to express the alternative antigens pLDH or aldolase, both of which are critical for parasite survival, have not been reported. Because of the large methodological differences between studies, especially in the selection of participants, only broad conclusions can be drawn.

1. There are clear local "hot spots" where *pfhrp2/3* deletion mutants are common enough to make diagnostic testing based only on HRP2 inadequate. Specifically, relatively high rates of *pfhrp2/3* deletions were seen in the Amazonian regions of Colombia and Peru and in central coastal Eritrea.
2. The prevalence of *P. falciparum* that do not express HRP2 varies by province in any given country. Similarly, although the presence of *pfhrp2/3* deletion mutants in a neighbouring country is a risk factor, it does not guarantee local circulation of such strains.
3. *pfhrp2/3* deletion mutants can cause outbreaks, especially in low-transmission regions, which may be missed by HRP-based RDTs (see, for example, Peru in Table 2).
4. In many studies, the methods by which patients were selected resulted in overestimates of the true prevalence of *pfhrp2/3* deletion mutants. In Rwanda, for example, 23% of *P. falciparum* strains assessed by *pfhrp2* PCR were deletion mutants, but the only samples assessed were positive for falciparum malaria by microscopy and PCR and negative in HRP2 RDTs. If none of the HRP2-positive RDT results were due to *pfhrp2* deletion mutants, the true prevalence of *pfhrp2* deletions in microscopy-positive falciparum cases would be around 1%.
5. The data in Table 2 may, however, underestimate the prevalence of *pfhrp*2-deleted strains because of cross-reactivity of HRP2-based RDTs with HRP3. As well, in areas of moderate-to-high transmission, the circulation of strains with *pfhrp2* deletions may be masked by co-infection with *P. falciparum* strains without such deletions (infection with more than one strain type:multiplicity of infection > 1).
6. The absence of PCR amplification of *pfhrp2/3* may be due to an inadequate quantity of parasite DNA. In many studies, the methods used to obtain DNA did not provide enough material to detect single-copy genes like *pfhrp2/3*.

[1] Unpublished data presented at a WHO technical consultation on *pfhrp2/3* gene deletions. Geneva; 7–8 July 2016.

TABLE 2.

Published and unpublished data on the prevalence of *pfhrp2/3* deletion mutants

Region	Country or area	Source of samples[d]		Initial evidence[e]			Gene deletion analysis by PCR				Percentage (%)			
											pfhrp2 deletions	*pfhrp3* deletions	*pfhrp2* and *-3* deletions	
			Period collected	Microscopy	High-quality RDT	Species (PCR)	*pfhrp2*	*pfhrp3*	One-copy genes	No. tested				Reference
South America	Peru	S	2003–2008	D	D	D	D		3	157	43.9%	70%	24%	*14*
		S	2010–2011	D	D	D	D	D	ND	74	25.7%	44%	26%	*18*
		U	1998–2001	ND	ND	D	D		ND	92	20.7%			*15*
		U	2003–2005	ND	ND	D	D		ND	96	40.6%			*15*
		S	2010–2012	D	U	D	D		1	54	100.0%			*19*
	Honduras	S	2008–2009	D	U	D	D	D	2	68	0.0%	44.0%	0.0%	*20*
	Brazil	S		D	D	D	D	D	ND	1	Case report	Case report	Case report	*21*
	Bolivia, Brazil	S	2010–2012	D	D	D	D	D	1	223	12.6%	39.4%	11.6%	*22*
	Colombia	S	2008–2009	D	D	D	D	D	3	100	18.0%	52.0%	13.0%	*23*
		S	2003–2012	D	D	D	D	D	3	374	4.0%	42.0%	4.0%	*24*
	Guyana	S	2009–2011	D	ND	D	D	D	1	97	0.0%	0.0%	0.0%	*25*
	Suriname	S	2009–2011	D	ND	D	D	D	1	78	14.1%	3.8%	2.6%	*25*
	France (French Guiana)	S	2009–2011	D	D	D	D	D	ND	221	0.0%	4.5%	0.0%	*26*
Africa	Mali	A/S	1996	D	ND	D	D		1	480	2.0%			*27*
	Democratic Republic of the Congo	A	2013–2014	D	D	D	D	D	2	783	6.4%		0.6%	*28*
	Kenya	A	2016	U	U	D	D			50	0.0%			*b*
	Democratic Republic of the Congo, Gambia, Kenya, Mozambique Rwanda, Uganda, United Republic of Tanzania	S	2005–2010	D	U	D	D	D	ND	77	0.0%	0.0%	0.0%	*29*

	Mozambique	S	2010–2016	U	U	D	D	D	1	69	1.4%	0.0%	0.0%	[c]
	Rwanda	S	2014–2015	D	U	D	D		0	138	23.2%			*30*
	Ghana	A	2015	D	U	D	D	D	2	315	29.0%	29%	28%	*31*
		A	2016		D					165	0.0%			[b]
	Zambia	A/S	2008–2012	D	ICT	D	*pfhrp* leader	*pfhrp* leader	1	61	20.0%			*32*
	Malawi	A	2013	D	D	D	U	ND	1	80	1.3%			*33*
	Uganda	A/S	2012–2013	D	D	D	D	D	0	56	1.8%	3.8%	1.3%	*33*
	Eritrea	S	2016	D	D	D	D	D	3	50	62.0%	82.0%		*34*
		U	2013–2014	D	ND	D	D	D	1	144	9.7%	43.1%	9.0%	*35*
	Senegal	S	2009–2012	D	ND	D	D	D	1	136	2.4%	12.80%		*36*
Middle East	Yemen	S	2014	D	D	D	D	ND	1	189	4.8%	ND	ND	*37*
Asia	India	S	2010	D	D	D	D	D	3	48	4.2%	4.17%	4.17%	*38*
		S	2014	D	D	D	D	D	3	1521	2.4%	1.70%	1.60%	*39*
	China–Myanmar	S	2011–2012	D	ND	ND	D	D	3	97	4.1%	3.09%	3.09%	*40*

[a] Brazilian cohort only

[b] *Plasmodium falciparum hrp2/3* gene deletions: update, implications and response. Breakfast side meeting. At 65th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta (GA), 15 November 2016.

[c] Gupta H, Matambisso G, Galatas B, Cisteró P, Nhamussua L, Rabinovitch R et al. Molecular surveillance of *pfhrp2* and *pfhrp3* deletions in *P. falciparum* isolates from Mozambique. Presented at *Plasmodium falciparum hrp2/3* gene deletions: update, implications and response. Breakfast side meeting. At 65th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta (GA), 15 November 2016.

[d] S- symptomatic; A – asymptomatic; U – Unknown clinical status

[e] – D- done; ND- not done; U-unknown

3. Response to the diagnostic threat

There is now clear evidence from many countries of the emergence of *P. falciparum* strains that cannot be detected with the most common diagnostic tool used in primary care across Africa and beyond. The Global Action Plan proposes a multi-faceted response to this threat, which will require:

- pragmatic action by NMCPs and their implementing partners,
- strengthened laboratory networks,
- research to further understand the factors behind the development of these resistant strains and the global scope of the problem,
- research and development of improved RDTs and
- a coordinated response by donors and policy-makers to avoid interruption in malaria diagnostic services.

3.1 Pragmatic action by national malaria programmes

The evolution and circulation of *pfhrp2/3* deletion mutants will threaten malaria control, like drug resistance, and must be managed. Although there are unanswered questions about the genesis and spread of *HRP2/3*-negative strains, NMCPs can act now, while further information is being collected. The programme management of *pfhrp2/3* deletion mutants described below includes that covered in the WHO information note on false-negative results in RDTs, published in May 2016 and updated in September 2017 *(41)*.

1. *Programme response to frequent false-negative HRP2 RDT results due to pfhrp2/3 deletions.* In many areas traditionally endemic for malaria, the prevalence has fallen, and most of the RDTs used in testing febrile individuals give correctly negative results. Large studies conducted to follow the outcomes of febrile children for whom RDT results were negative *(41, 42)* found that it was safe not to treat them for malaria, with no malaria-related deaths or hospitalizations. That being said, in areas where *pfhrp2/3* deletions are found to be prevalent, as in Eritrea and Peru, NMCPs should switch to RDTs that do not rely exclusively on HRP2 for detecting *P. falciparum*. Circulation of p*fhrp2/3* deletion mutants is likely to be focal, and the introduction of a new testing strategy may be prioritized for regions or provinces with the highest prevalence of these mutants. Data from the prevalence survey recommended below will provide guidance to national programmes on changing their testing methods and the provinces or regions in which to apply the changes first.

   WHO convened a meeting of experts to prepare guidance on *pfhrp2/3* mutations in July 2016 and published the outcome as a background document for the 2016 Malaria Policy Advisory Committee meeting *(44)*. The group decided that a 5% local prevalence of false-negative HRP2 RDTs due to gene deletions would warrant a change in testing strategy. This cut-off was selected because it is at about that level that the public health impact and the proportion of cases missed by less sensitive non-HRP2-based tests are likely to be lower than those associated with continued use of HRP2-based tests. Future modelling based on

rigorously collected programme data may be useful to confirm or refine this cut-off value. It is expected that strains carrying *pfhrp2/3* deletion mutations will continue to evolve and spread, especially in areas where HRP-2 testing predominates and is used to decide on treatment. All countries should therefore consider planning a gradual transition to testing that does not rely solely on the detection of HRP2.

2. *Scouting for deletion mutants by investigating suspected false-negative RDTs.* The NMCPs and implementing partners in countries in which HRP2-based RDTs are used should support investigation of suspected false-negative RDT results, bearing in mind not only possible *pfhrp2/3* deletion mutants but also the common causes of false-negative results, including operator error, false-positive microscopy results, degradation of RDTs during transport or storage, manufacturing error or infections with a low parasite density. *pfhrp2/3* gene deletions should be suspected and the NMCP and WHO informed when:

    - a patient's sample gives a negative result on the HRP2 test line of at least two quality-assured malaria RDTs, a positive result on the pan or PfpLDH line if a combination RDT is used and confirmation of positivity for *P. falciparum* by two qualified microscopists; or

    - the rates of discordance between RDT and microscopy results in the programme are systematically ≥ 10–15%, with higher positivity rates with microscopy, and quality is controlled routinely by cross-checking or both tests are performed for the same individuals (e.g. during a survey); and/or

    - the NMCP receives many formal complaints or anecdotal evidence that HRP-based RDTs are giving false-negative results for *P. falciparum*.

3. *National assessment of the prevalence of pfhrp2/3 deletion mutants.* The interpretation of the survey data collected to date (Table 2) is limited by methodological differences in the studies performed, particularly in the selection of patients or screening methods, resulting in large variations in the estimated prevalence of *pfhrp2/3* deletion mutants. When an NMCP or other credible group detects *pfhrp2/3* gene deletions in local strains, it should determine the prevalence in the country and in neighbouring countries in order to plan an appropriate response. Although the infrastructure of activities such as malaria indicator surveys and therapeutic efficacy studies may be used to determine the distribution of these strains, it is highly recommended that a standardized enrolment protocol be used in all countries so that the results will be comparable. WHO has published a protocol on its website (http://www.who.int/malaria/areas/diagnosis/en and Box 2), which targets the most relevant group for case management and disease control: symptomatic individuals attending health facilities being evaluated for clinical malaria. The goal of the protocol, the elements of which are described below, is to determine rapidly whether the prevalence of *pfhrp2/3* deletion mutants among patients with falciparum malaria is high enough (≥ 5%) to consider changing the malaria diagnostic strategy and tools. A threshold of 5% was selected because it somewhere around this point that the proportion of cases missed by HRP2 RDTs due to non-hrp2 expression may be greater than the proportion of cases that would be missed by less-sensitive pLDH-based RDTs. A recommendation to switch is further informed by mathematical models that show whether parasites lacking *pfhrp2* genes will spread

under HRP2-only RDT pressure; a switch may also be decided because of the complexity of procuring and training in use of multiple RDTs. Any change should be applied nationwide, although roll-out might be prioritized on the basis of the prevalence of *pfhrp2* deletions. Clinical sites for enrolment of patients in surveys of the prevalence of *pfhrp2/3* deletion should be selected such that they represent the population distribution and the heterogeneity of transmission.

NMCPs may wish to establish sentinel sites to repeat estimates of the prevalence of *pfhrp* deletions over time in order to determine whether changes in diagnostic testing are effective in controlling the transmission of deletion mutants. New initiatives to find these gene deletions are not currently recommended if there are no confirmed reports of *hrp2/hrp3* gene deletions locally or in neighbouring countries.

Box 2. Standardized protocol for assessing national prevalence of *pfhrp2/3* deletion mutants among patients with falciparum malaria

Subjects: Symptomatic patients with suspected falciparum malaria among those seen at 10 health facilities in selected provinces

Screening method: A high-quality RDT for detecting HRP2 and either microscopy or a second RDT for detecting PfpLDH

Selection criteria: All falciparum patients with uncomplicated malaria, for whom a suspected false-negative HRP2 RDT was found and who agree to participate

Study method: Collect, label and dry a minimum of two fingerprick blood spots for molecular analysis. Conduct PCR testing of a dried blood spot for (i) species confirmation, (ii) level of parasitaemia, (iii) amplifiable DNA and (iv) the presence of genes that encode HRP2 and HRP3.

Sample size: 370 falciparum cases per province or region will be screened with dual RDTs. Molecular analysis will then be undertaken on the samples suspected to have *pfhrp2*/3 deletions and a statistical analysis of the prevalence with 95% CI will be computed. The analysis will result in one of three outcomes per province:

Outcome 1: That the upper limit of the 95% CI does not overlap with 5%. In this case the is a high statistical confidence that *pfhrp2/3* deletion causing false negative RDT results is below 5%

Outcome 2: The lower limit of the 95% CI is above 5%. This result means that there is a high statistical confidence that *pfhrp2/3* deletion causing false negative RDT results is greater than 5%

Outcome 3: The statistical analysis shows that it is inconclusive (5% contained within the 95% CI) as to whether or not the prevalence of *pfhrp2/3* deletion causing false negative RDT results is greater than or less than 5%.

Testing location: RDT and/or microscopy testing will be performed at local health facilities, with appropriate quality control. Molecular analysis may be performed at regional or international reference laboratories.

4. *Selection of new RDTs in countries or provinces where pfhrp2/3 deletions are prevalent.* It is difficult to select RDTs to replace or augment HRP2-based assays in settings where *pfhrp2/3* deletion mutants are common. Ideally, all tests being considered for procurement and use should (i) meet WHO-recommended procurement criteria based on their performance in WHO malaria RDT product testing (panel detection score, ≥ 75% at 200 parasites/µL (Pf or Pv); false-positivity rate, < 10% and invalid rate < 5%); (ii) as of 1 January 2018, to have achieved WHO prequalification; and (iii) have been validated for use in detecting *P. falciparum* strains, including those with *pfhrp2/3* deletions. Unfortunately, no tests currently meet all these criteria. The development of tests that better meet the criteria is discussed in section 3.4 below. Practical advice is given here on interim considerations until RDTs of a new generation are developed and approved for general international procurement.

Table 3 lists the RDTs evaluated in the WHO malaria RDT product testing programme for the diagnosis of *P. falciparum* malaria by detection of non-HRP2 antigens, namely pan (all species)-pLDH or Pf-pLDH. The table shows their performance against prepared panels of cultured parasites and clinically collected blood samples diluted to 200 parasites/µL These tests were not, however, evaluated against *pfhrp2/3* deletion mutants, so that the reported detection performance may not be representative of that seen in populations in which these mutations are common. As can be seen in the table, the panel detection score of the HRP2 line for *P. falciparum* is generally higher than that of the Pf-pLDH line. In tests with combined Pf-pLDH and HRP detection in the same line, it is not possible to determine the relative contribution of each to overall detection performance. The ongoing round 8 of product testing will include wild-type and cultured *pfhrp2/3* gene-deleted parasites and will therefore result in refined recommendations on use of these tests. It is important to remember that the panel detection score referred to in the table is not synonymous with clinical or analytical sensitivity and that all the tests that meet current WHO procurement criteria for performance (panel detection score, ≥ 75) are expected to detect essentially all clinically significant cases of malaria. Further details can be found in the report of round 7 of product testing *(9)*. Product Testing available at http://www.who.int/malaria/publications/atoz/9789241510035/en/.

TABLE 3.

RDTs not based on HRP2 for the detection of *P. falciparum* malaria

	Product	Product code	Manu-facturer	Panel detection score [a]										Round	Meets WHO procurement criteria
				200 parasites/µl					2000 parasites/µl						
				Pf samples [c]				Pv samples[d]	Pf samples[c]				Pv samples[d]		
				HRP2/pf-pLDH (dual antigen single test line)	HRP2 test line	pf-pLDH test line	pan-pLDH only test		HRP2/pf-pLDH (dual antigen single test line)	HRP2 test line	pf-pLDH test line	pan-pLDH only test			
	Pf only														
1	CareStart™ Malaria Pf (HRP2/pLDH) Ag RDTf	RMPM(U)-XXX7X	Access Bio, Inc.	91.0				NA	99.0	NA	NA	NA	NA	6	Yes , but only for HRP2 based Pf diagnosis; unknown performance of pf-pLDH alone for detection of *pfhrp2* deleted parasites
2	CareStart™ Malaria Pf (HRP2/pLDH) Ag Combo 3-Line [e]	RMSM-05071	Access Bio, Inc.	NA	94.0	38	NA	NA	NA	99.0	92.0	NA	NA	7	Yes, but only for HRP2 based detection of Pf or as screening test in surveys for *pfhrp2* deleted parasites
3	SD Bioline Malaria Ag P.f (HRP2/pLDH) [e, f]	05FK90	Standard Diagnostics, Inc.	NA	87.0	52.0	NA	NA	NA	100.0	97.0	NA	NA	6	Yes, but only for HRP2 based detection of Pf or as screening test in surveys for *pfhrp2* deleted parasites
4	SD BIOLINE Malaria Ag P.f. (HRP2/pLDH) 2 Lines	05FK130-40-0	Standard Diagnostics, Inc.	90.0	NA	NA	NA	NA	100.0	NA	NA	NA	NA	7	Yes, but only for HRP2 based Pf diagnosis; unknown performance of pf-pLDH alone for *pfhrp2* deleted parasites
	Pf and Pan														
5	CareStart™ MALARIA Pf/PAN (pLDH) Ag RDT	RMLM-05071	Access Bio, Inc.	NA	NA	73.0	NA	0.0	NA	NA	100.0	NA	71.4	7	No for Pf or Pv detection
6	CareStart™ Malaria Screen RDT	RMAM-05071	Access Bio, Inc.	93.0	NA	NA	NA	94.3	99.0	NA	NA	NA	97.1	7	Yes, but only for HRP2 based Pf diagnosis; unknown performance of pf-pLDH alone for detection *pfhrp2* deleted parasites

	Product	Product code	Manu-facturer	Panel detection score [a]										Round	Meets WHO procurement criteria
				200 parasites/µl					2000 parasites/µl						
				Pf samples [c]				Pv samples[d]	Pf samples[c]				Pv samples[d]		
				HRP2/pf-pLDH (dual antigen single test line)	HRP2 test line	pf-pLDH test line	pan-pLDH only test		HRP2/pf-pLDH (dual antigen single test line)	HRP2 test line	pf-pLDH test line	pan-pLDH only test			
	Pf and Pv														
7	BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH)	C60RHA25	RapiGEN Inc.	NA	NA	75.0	NA	100.0	NA	NA	98.0	NA	100.0	7	Yes
	Pf, Pf and Pv														
8	SD Bioline Malaria Ag P.f/P.f/P.v [e]	05FK120	Standard Diagnostics, Inc.	NA	84.0	36.0	NA	91.4		100.0	98.0		100.0	6	Yes, but only for HRP2 based detection of Pf or as screening test in surveys for *pfhrp2* deleted parasites
	Pan only														
9	CareStart™ Malaria PAN (pLDH) Ag RDT [f]	RMNM(U)-XXX7X	Access Bio, Inc.	NA	NA	NA	84.0	88.6	NA	NA	NA	99.0	97.1	5	Yes
10	Advantage Pan Malaria Card	IR013025	J. Mitra & Co. Pvt. Ltd.	NA	NA	NA	77.0	100.0	NA	NA	NA	98.0	100.0	5	Yes

a According to methods of WHO malaria RDT product testing a sample is considered detected only if all RDTs from both lots read by the first technician, at minimum specified reading time, are positive

b The total number of times a positive result for malaria was generated when it should not have been

c Round 1, n=79; Round 2, n=100; Round 3, n=99; Round 4, n=98; Round 5, n=100; Round 6, n=100; Round 7, n=100

d Round 1, n=20; Round 2, n=40; Round 3, n=35; Round 4, n=34; Round 5, n=35; Round 6, n=35; Round 7, n=35

e PDS presented in the table is based on a HRP2 test line and Pf-pLDH test line. The overall result at 200 p/µl based on positive HRP2 or pf-pLDH test line is 88 for 05FK90; 85 for 05FK120 and at 2000p/µl it is 99 for RMSM-05071; 100 for 05FK90; 100 for 05FK120

f Indicates a WHO prequalified product

Given the relatively low frequency of *pfhrp2/3* deletion mutants in most settings in which it has been assessed, there is significant value in retaining capacity to detect HRP2 in selecting RDTs, especially because these assays tend to be more sensitive and heat-stable. The advantage of assays in which HRP2 and Pf-pLDH are detected on the same line is the simplicity of read-out; however these tests pose challenges to current independent performance evaluation schemes. Where microscopy is available, the services should be strengthened to ensure that parasitological confirmation of malaria continues during the transition to new RDTs and to support investigations of new foci of suspected *pfhrp2/3* deleted parasites.

3.2 Strengthened laboratory networks

Strengthening laboratory capacity for the detection of malaria has been a critical feature of national and international malaria control. The advent of *P. falciparum* strains that cannot be detected with HRP2-based RDTs will further stretch local laboratory capacity, both for microscopy and RDT testing. Although expert microscopy has repeatedly shown good performance, microscopy services in peripheral settings have been difficult to establish and maintain, and many reports document poor sensitivity and poor specificity in the field detection of malaria by microscopy *(45–48)*. Meeting the requirement for capacity to assess suspected false-negative HRP2-based RDTs will require quality-assured microscopy and/or staff trained and ready to use correctly non-HRP2-only RDTs that are not in routine use in the NMCP.

In addition to assessing individual reports of suspected false-negative RDT results, national surveys should be conducted to establish the prevalence of *pfhrp*2/3 deletion mutants, which would require more training and perhaps strengthening staff, depending on local workloads. Survey protocols will also require procurement and distribution of Pf-pLDH-based RDTs. As each RDT has specific instructions for use, maintenance of multiple testing methods in 10 health facilities per province – at least during a survey for *pfhrp2/3* deletion mutants – will not be trivial.

Discordant test results between two different RDTs may be due to many factors, and not all HRP2-negative, Pf-pLDH-positive RDT results are due to *pfhrp2/3* deletions (see Box 3 **Causes of false-negative RDT results and investigative actions**).

Classification	Cause of false-negative RDT result	Suggested actions
Operator factors	Operator error in preparing the RDT, performing the test or interpreting the result	Verify whether RDTs are used by untrained staff; assess RDT competence on site.
Use of an imperfect "gold standard" as a comparator	Thick or thin films from a patient with a negative RDT result are incorrectly interpreted as "positive" by microscopy.	Verify microscopy procedures and interpretation by a qualified microscopist.

Classification	Cause of false-negative RDT result	Suggested actions
Product design or quality	Poor sensitivity of an RDT due to poor specificity, affinity or insufficient quantity of antibodies. Poor packaging can result in exposure to humidity, which will rapidly degrade RDTs.	Inspect the instructions for errors; inspect the integrity of the packaging, including the colour indicator desiccant for evidence of moisture. Cross-check suspected false-negative RDT results against microscopy performed by two qualified microscopists or, if microscopy is not available, against a high-quality non-HRP2-detecting RDT; retrieve RDTs from affected areas, and send for lot testing to WHO- or FIND-recognized laboratories.*
	Poor visibility of test bands due to strong background colour on the test	Assess RDT performance and training on site; if the strong background colour persists, notify the manufacturer.
	Incorrect instructions for use	Review the instructions for use for accuracy.
Transport or storage conditions	Antibody degradation due to poor resistance to heat or incorrect transport or storage, e.g. exposure to high temperatures, freeze-thawing	Inspect temperature monitoring of RDT transport and storage chain to determine whether temperatures exceed maximum storage temperature, typically 30 °C or 40 °C or < 2 °C. If temperatures are not within those in the manufacturers instructions, send the RDTs to the WHO-FIND lot testing laboratory.* Train health workers to respect storage conditions, and improve storage places (e.g. add fans).
Parasite factors	Parasites lack or express low levels of the target antigen, i.e. HRP2	Patient samples are negative on an HRP2 test line of at least two quality-assured malaria RDTs **and** positive on the pan- or pf-pLDH test line of a combination RDT **and** the sample is confirmed to be positive microscopically for *P. falciparum* by two qualified microscopists. If these conditions are met, place fresh blood samples or dried blood spots (50-60 µL) on Whatman® 3MM filter paper or other collection cards, in frozen storage (-20 °C) until shipment

Classification	Cause of false-negative RDT result	Suggested actions
		for PCR and *pfhrp2*/*pfhrp3* gene analysis.
	Variation in the amino acid sequence of the epitope targeted by the monoclonal antibody	Repeat test with an RDT of a different brand or different manufacturer that targets the same antigen or an RDT that targets a different antigen, e.g. pan-pLDH or Pf-pLDH. Manufacturers may use monoclonal antibodies that target different epitopes of the same antigen.
Host parasite density	Very low parasite density or target antigen concentration	Perform high-quality microscopy, and record the parasite count; if high-quality microscopy is not available, repeat the RDT if symptoms persist.
	Very high parasite load (severe malaria) causing prozone effect (hyperparasitaemia and antigen overload)	Repeat testing with a 10 × and if needed a subsequent 50 × dilution of the sample, with dilutions in 0.9% NaCl **

Note:
*Information about lot testing can be found here: http://www.who.int/malaria/areas/diagnosis/rapid-diagnostic-tests/evaluation-lot-testing/en/
** Gillet et al. Prozone in malaria rapid diagnostics tests: how many cases are missed? Malar J 2011;10:166. https://doi.org/10.1186/1475-2875-10-166

In some settings, less than half of all suspected false-negative HRP RDTs are found to be due to *pfhrp2/3* deletions, while in others the predictive value of a false-negative HRP RDT for genetic deletion is much greater. Molecular analysis will be essential. Confirming the presence of genetic deletions will require sampling, labelling and preparation of dried blood spots for shipping and multiple PCR analyses in regional or international laboratories. This work should be done in a timely manner so that the NMCP can plan possible procurement of new types of RDT.

Table 4 shows estimates of the volume of conventional (RDTs and microscopy) and molecular testing required to perform surveys in all provinces of countries that have reported the presence of *pfhrp2/3* deletion mutants and in neighbouring countries. The numbers in the table are based on the assumption that malaria is transmitted in all provinces, which will not be true in many countries with areas of interrupted transmission. The goal of a national HRP2 survey is to determine whether the prevalence of *pfhrp2* in any province is ≥ 5%, the cut-off recommended for local use of non-HRP2-only diagnostics for falciparum malaria. The

prevalence is calculated as the number of *pfhrp2/3* deletion mutants divided by the total number of cases of falciparum malaria.

TABLE 4.
Estimated numbers of samples from patients with falciparum malaria to be screened for false-negative HRP RDT results and numbers with suspected *prhrp2* deletion mutations, requiring molecular analysis, in all countries in which these mutations have been reported and in neighbouring countries

Countries with *pfhrp2* deletions reported	Neighbouring countries	Number of administrative divisions	Minimum number of falciparum cases to identify	Number of molecular assessments at 2% HRP RDT discordance	Number of molecular assessments at 5% HRP RDT discordance	Number of molecular assessments at 10% HRP RDT discordance
Peru		26	9620	192	481	1924
	Ecuador	24	8880	178	444	1776
	Bolivia	9	3330	67	167	666
	Chile	15	5550	111	278	1110
Brazil		27	9990	200	500	1998
	Venezuela	24	8880	178	444	1776
	Guyana	10	3700	74	185	740
	French Guiana	24	8880	178	444	1776
	Uruguay	19	7030	141	352	1406
	Paraguay	18	6660	133	333	1332
	Argentina	24	8880	178	444	1776
Columbia		33	12210	244	611	2442
	Panama	10	3700	74	185	740
Surinam		10	3700	74	185	740
Mali		9	3330	67	167	666
	Mauritania	15	5550	111	278	1110
	Algeria	48	17760	355	888	3552
	Niger	16	5920	118	296	1184
	Burkina Faso	13	4810	96	241	962
	Côte d'Ivoire	14	5180	104	259	1036
	Guinea	7	2590	52	130	518
	Senegal	14	5180	104	259	1036
DRC		29	10730	215	537	2146

Mozambique		26	9620	192	481	1924
	Angola	18	6660	133	333	1332
	Tanzania	31	11470	229	574	2294
	Burundi	18	6660	133	333	1332
	South Sudan	28	10360	207	518	2072
	CAR	17	6290	126	315	1258
	Repub. of Congo	12	4440	89	222	888
Rwanda		5	1850	37	93	370
Ghana		10	3700	74	185	740
	Togo	5	1850	37	93	370
Zambia		10	3700	74	185	740
	Mozambique	11	4070	81	204	814
	Zimbabwe	10	3700	74	185	740
	Botswana	17	6290	126	315	1258
	Namibia	14	5180	104	259	1036
Malawi		28	10360	207	518	2072
Uganda		4	1480	30	74	296
Eritrea		6	2220	44	111	444
	Kenya	47	17390	348	870	3478
Senegal		14	5180	104	259	1036
	Guinea-Bissau	9	3330	67	167	666
	The Gambia	8	2960	59	148	592
Yemen		22	8140	163	407	1628
India		36	13320	266	666	2664
	Pakistan	34	12580	252	629	2516
	Nepal	7	2590	52	130	518
	Bangladesh	8	2960	59	148	592
	Bhutan	20	7400	148	370	1480
Myanmar		7	2590	52	130	518
	Laos	18	6660	133	333	1332
	Thailand	77	28490	570	1425	5698
	China	27	9990	200	500	1998
Total			385540	7711	19277	77108

Molecular analysis can be performed on dried blood spots, but the technical work is complex and requires PCR for species confirmation, quantification, extraction and recovery of sufficient undegraded *Plasmodium* DNA and analysis of the exons and flanking genes of *pfhrp2* and *pfhrp3*. As deletion mutations can be detected only as the absence of amplified products of *pfhrp* exons, rigorous control must be used to ensure the presence of non-degraded, amplifiable parasite DNA and lack of amplicon contamination. PCR to detect the absence of amplification can be confounded by multiple factors, including the specific reaction conditions, a concentration of target genetic sequences below the limit of detection, degradation of the target DNA, or presence of contaminating native or amplicon DNA. It is recommended that all samples from patients in the survey that are found to have a suspected false-negative HRP2 RDT be sent for molecular analysis. Thus, the number of samples to be analysed genetically will depend on prevalence of *pfhrp*2/3 deletion mutants (and frequency of other events causing false-negative results). As illustrated in Table 4, if 10% of falciparum patients in HRP2 surveys are suspected of having false-negative HRP2 RDT results, the total number of samples that require molecular analysis would reach 77,108.

A number of international reference laboratories with experience in *pfhrp2/3* molecular analysis are already collaborating with WHO. Although these laboratories have shown willingness to contribute to better understanding of the causes and distribution of these mutated falciparum strains and to perform molecular testing of samples obtained in national HRP surveys, the manpower and the reagents for the predicted workload are not currently funded or guaranteed. National programmes themselves may have an interest in using or strengthening local capacity for PCR; however, the lack of PCR standardization and of a malaria molecular assay stringently approved by a regulatory authority will make comparison of results between studies and between laboratories problematic *(49)*. The consequences of false-positive and false-negative results for *pfhrp2/3* gene deletions will have serious negative consequences. WHO is therefore committed to working with expert laboratories and donors to strengthen global capacity for detection of *pfhrp2/3* deletions. Where the capacity exists, regional and national laboratories with molecular expertise could play an important role, but it will be incumbent upon countries embarking on national HRP surveys to have a molecular assessment plan that includes the capacity to ship samples internationally to collaborating laboratories with the necessary capacity and quality control. All laboratories that conduct malaria PCR are encouraged to participate in the WHO external quality assurance scheme for malaria nucleic acid amplification testing, established in 2017 *(50)*. Under this scheme, participants receive proficiency testing panels twice a year that include all *Plasmodium* species in a range of parasite densities.

3.3 New research

Although the precise physiological function of HRP2 and its structural analogue HRP3 is still unknown, much is known about the structure and variability of the genes that encode them. Both HRP2 and HRP3 are encoded by single-copy genes located in subtelomeric regions of chromosomes 8 and 13, respectively. These regions near the end of the chromosome are known to have multiple repeating elements and are hot spots for mutations, and these qualities are used by some parasites (e.g. *Trypanosoma brucei*) to generate variable surface antigens in order to escape the host immune system. *P. falciparum* strains containing intact *HRP2* genes often have variable gene sequences. In a study of 458 *P. falciparum* strains

collected globally, 315 different *HRP2* gene sequences were found. Of the subset of 80 strains in which the *HRP3* gene was also sequenced, 42 different sequences were found *(51)*. Although there is some evidence that such sequence variation can affect the clinical sensitivity of tests based on specific monoclonal antibodies *(52)*, this is seen only near the limit of detection and has a limited effect on overall clinical sensitivity (except in the case in outright gene deletion) *(52)*. Deletion mutations that halt the expression of HRP2 or HRP3 may occur at various locations around the *pfhrp* exons and are frequently large, involving not only the relevant *pfhrp* genes but also upstream and downstream flanking genes.

There are no conclusive data on the transmissibility of *pfhrp* deletion mutants as compared with that of wild-type parasites; however, they are clearly transmitted from person to person and may be responsible for outbreaks that could be missed in areas in which HRP2-only RDTs are used. In 2010, an outbreak of 210 cases of genotypically identical falciparum malaria cases occurred in the Tumbes region of northern Peru, where autochthonous transmission had been stopped and which had been malaria-free for the previous 4 years *(20)*. Genotyping of 188 *P. falciparum* strains with *pfhrp2* deletions collected over 7 years in areas of Peru with ongoing transmission showed increasing clonal diversity, with clear evidence of the evolution of new strains carrying deletions *(15)*.

The specific factors that drive the evolution and spread of *pfhrp* mutations are not clear, although it is sensible to consider that selective pressure due to HRP2 detection plays an important role; however, this is not the only factor. For example, in Peru, where HRP2-only RDTs were not used routinely, the prevalence of *pfhrp2* deletion mutants among cases in the area of Iquitos increased from 13% in samples collected between 1998 and 2001 to 40.6% among those collected between 2003 and 2005. Data from a demographic and health survey in the Democratic Republic of the Congo indicate a link between a higher prevalence of *pfhrp2* deletions and earlier introduction of HRP2-based RDTs *(29)*. Whether the strictness of adherence to diagnostic results in providing therapy helps drive the emergence of deletion mutants is unclear, although the predominance of these strains in Eritrea, where NMCP guidelines are followed closely, is noteworthy. In two recent studies, mathematical models were used to characterize the effect of introducing HRP2-based RDTs on the emergence and spread of *pfhrp2/3* deletion mutants *(53, 54)*. There is no conclusive empirical evidence that the choice of an RDT influences the prevalence of *pfhrp2/3* deletion mutants in a community, although such data may well emerge after widespread replacement of HRP2-only RDTs in Eritrea and their limited use in Peru.

Some clinical evidence suggests that *pfhrp*-deleted strains have reduced fitness. Cohorts infected with non-HRP2-expressing strains, which included young children, showed a lower parasite density than geographically matched cohorts infected with wild-type parasites *(29)*. The results of studies with cultured parasites are inadequate to draw precise conclusions about the fitness of *pfhrp*2/3 deletion mutants, although one study suggested that these strains showed reduced fitness in vitro *(55)*.

In addition to research meant to understand the factors that drive the evolution and spread of *pfhrp*2/3 deletion mutants, operational and technical research is needed to simplify the process of identifying and tracking the distribution of these strains. The currently proposed process for identifying these mutant strains is complex and requires some clinical trial

infrastructure and sophisticated confirmatory testing. Surrogate markers that are easier to use are needed. For example, there is as yet no compiled information on the predictive value of a suspected false-negative HRP RDT for genetic mutations in different settings. Even if the predictive value were relatively low, if the difference between RDT and molecular results were fixed in given settings, that could serve as a useful marker to use to track trends. On a technical level as well, more research is needed. The current molecular methods require a substantial number of controls, as the readout is a negative one (deletion mutations are identified by the absence of a PCR result, which could be caused by many factors). A method that could detect mutations with a positive result would simplify molecular testing. Similarly, there may be simpler centralized testing methods that could provide results of adequate accuracy for epidemiologic studies. A sensitive and quantitative method to measure the concentration of several proteins in multiplex might provide a cheaper and simpler reference method than molecular testing. Liquid array technologies, such as the Luminex platform, have already been used to create assays that detect HRP2 protein at sub-picogram levels and can be used for moderate to high-throughput testing *(56)*. Optimization and multiplexing of such assays could provide a useful alternative to PCR to confirm RDT results.

3.4 Diagnostics research and development

The clinical sensitivity of all malaria diagnostics has decreased slightly with decreased transmission intensity and the greater likelihood of low-density infections. The occurrence of strains that do not express HRP2/3 further increases the likelihood that some infected patients will be missed in conventional RDT testing. As countries move towards an elimination phase of malaria control, tests are required to detect malaria at low parasite densities ideally targeting proteins with conserved sequences or critical roles in parasite metabolism. The manufacture of RDTs and their components has been refined over the past 20 years, but there has been little change in *Plasmodium* protein targets. Many of the antigens evaluated in RDTs or enzyme-linked immunosorbent assays (ELISAs) were identified during research, including vaccine development, that was not intended to develop antigen immunocapture assays, and little work has focused on this area in recent years.

Glutamate dehydrogenase, a cytosolic protein of *P. falciparum*, was an early target for malaria antigen detection *(57, 58)* but was never used in a commercialized assay. Interest in the histidine-rich family of proteins of *P. falciparum* grew from the finding that HRP1 was an important protein in knob formation on erythrocytes, a virulence characteristic of *P. falciparum (59)*. The finding that HRP2 was secreted, abundant and antigenic indicated its possible utility as a diagnostic target *(60)*. HRP2 was first reported to be detectable (by ELISA) in the plasma of malaria patients in 1991 *(61)*, and by 1993 a lateral flow immunochromatographic assay suitable for field use had been developed *(4, 62)*.

Plasmodium LDH became an attractive target for malaria diagnostics when it was realized that the protein had both species-specific and pan-specific epitopes against which monoclonal antibodies could be developed. Furthermore, pLDH was found to be cleared from the blood much more rapidly than HRP2 after effective malaria treatment, rendering it a more specific target for the diagnosis of acute infection, especially in high-transmission areas *(63)*.

Other plasmodium proteins, such as dihydrofolate reductase–thymidylate synthase, haem detoxification protein, glutamate-rich protein *(64)* and glyceraldehyde-3-phosphate dehydrogenase *(65)*, have been studied for diagnostic potential but never used in a commercialized assay.

Although there has been abundant work on the proteomics of malaria, much of it has focused on understanding the fundamental biology of the organism, such as the events that mediate stage maturation of the parasites. Most studies have been done on cultures, and only some have been rigorously quantitative. A recent quantitative study of the proteins expressed in the intraerythrocytic *P. falciparum* parasites which are abundant, soluble and unlikely to be confused with human proteins, identified three that deserve further research as diagnostic targets: phosphoethanolamine *N*-methyltransferase, hypothetical protein PFI1270w and a protein disulfide isomerase *(66)*. Although proteins such as HRP2 are present in concentrations of nanograms to micrograms per millilitre, there have been virtually no quantitative proteomic studies of the comparative abundance of plasmodial proteins in human clinical samples.

An additional strategy that deserves exploration is use of existing reagents and targets in new assay configurations that have advantages in terms of sensitivity, quantification and ease of use. Greater optimization of monoclonal antibodies or other ligands, to increase their robustness, thermostability and affinity (e.g. monoclonal antibodies with high binding affinity to both HRP2 and HRP3) would also be valuable.

In the short term, perhaps the most pressing need is non-HRP2 RDTs that target Pf-pLDH or another antigen target that are more sensitive and heat stable than the non-HRP2 tests currently available. In terms of analytical sensitivity, there is a roughly 10-fold gap between the detection capacity of HRP2 and pLDH assays. A more sensitive pLDH assay, especially one that targets Pf-pLDH, would have great benefits. Foremost, it would allow countries to phase out HRP2-based assays if *pfhrp2/3* deletion mutants reach important thresholds and replace them with assays of comparable performance. Secondly, countries that wish to track the prevalence of potential *pfhrp2/3* deletion mutants or to distinguish between new and recent infections could use assays with separate test lines for HRP2 and Pf-pLDH. Countries in which *P. falciparum* is prevalent and that wish to maintain testing and result recording that are as simple as possible could use tests with a single test band that bears both antigens, without fear of missing cases carrying deletion mutants or infections at low parasite density.

Unfortunately, RDT manufacturers are working within very tight profit margins. The market is so competitive and the tests so inexpensive that even critical quality control cannot always be funded. Manufacturers are therefore unlikely to fund even translatable research on reagent optimization and certainly not on the identification of improved biomarkers; external funding will be needed. The funding model of most national research institutes rewards the most original and elegant science and not necessarily that which is most urgently required to meet public health goals in the short term. Policy-makers and independent donors should consider innovative ways to fund such targeted science.

In May 2016, WHO announced that the companies that manufacture malaria RDTs must submit their products to be assessed in the WHO prequalification of in-vitro diagnostics programme. To date, the WHO prequalification programme, and the WHO–FIND RDT evaluation programme

before it, have not included *pfhrp2/3* deletion mutants in the cultured or clinically collected reference specimens. This change is now being made to the product testing programme, and round 8 (laboratory evaluation for WHO prequalification) will include such specimens. Continuous dialogue among manufacturers, WHO and procurement agencies is necessary to ensure that NMCPs can procure in a timely manner products with performance that they can continue to rely upon.

3.5 Coordination of response

There are many different interests involved in the discovery, development, quality control, selection, procurement, distribution, storage and use of RDTs. Without a coherent and coordinated response, there is a risk of inefficiency, delay, and missed opportunity to continue with the recent gains in malaria control. An effective response to this challenge will require specific work to coordinate the actions of the multiple agencies and governments involved. Given the strength and interest of partners, a small secretariat, perhaps hosted by WHO, could provide structure (communication, development of workplans, financing forecasts, etc.) to a time-limited collaboration or consortium intended to harvest the individual capabilities of partners and ensure harmonized action.

An example list of near term products and activities for the consortium might include:

- Market forecasting for commercial manufacturers
- Refinement and maintenance of the RDT quality assurance testing
- Registry of *pfhrp*2/3 prevalence surveys
- Ongoing global mapping of data from prevalence surveys
- Nominated and funded network of reference labs
- Annually updated policy reviews, especially around the prevalence cut-offs for changing RDTs in use and the recommendations on test selection
- Centralized procurement assistance for countries changing RDTs
- Short-term operational and technical research agendas with clear timelines and deliverables and financing needs
- Target product profiles published on ideal RDT configuration for now and 5 years from now

4. Conclusions

The emergence of *P. falciparum* strains that no longer express the HRPs that are the targets of the most commonly used malaria diagnostic tool globally is an extraordinary event, which threatens the utility of a critical weapon in the fight against malaria. The full extent of that threat is not yet known and the alternative RDT options ie. pf-pLDH RDTs are extremely limited and currently have inferior performance to HRP2 RDTs for *P.falciparum* detection. It is already a

matter of urgent concern in the disparate regions of the Amazon basin and Eritrea, where the prevalence of false-negative HRP2 RDTs is forcing changes in diagnostic strategy. Information for much of the rest of the world is spotty, and the occurrence of these deletion mutants is known to be highly focal. It is likely that the problem will not go away and that, under continued selective pressure from testing and treatment strategies based on HRP2-only RDTs, it will continue to grow.

Managing the response will require needs-based prioritization – it would be counterproductive to attempt to change diagnostic test selection across Africa if unwarranted. National and global response must balance the risk of missed cases of falciparum malaria due to *pfhrp*2/3 deleted strains against the equally real risk of missing cases by changing to a less sensitive RDT and the longer term risk of eroding confidence in antigen-based confirmatory testing for malaria.

Several types of work are needed urgently:

- mapping the distribution and frequency of *pfhrp2/3* deletion mutants with harmonized protocols;
- building an international network of laboratories to perform the complex molecular confirmation required for mapping;
- supporting countries in the selection and procurement of new RDTs when a change of testing is warranted;
- advising commercial manufacturers of the priorities for new tests and providing the best available market forecasts;
- adapting the WHO malaria RDT product testing programme, which constitutes the laboratory evaluation component of WHO prequalification, to ensure proper validation of tests for the detection of *pfhrp2* deletion mutants as part of their intended use;
- working with donor agencies and research institutes to devise a funding plan to support (i) the interim costs for prevalence surveys and the necessary molecular testing and (ii) the search for improved diagnostic targets and high-affinity reagents; and
- strengthening coordination among policy-makers, NMCPs and their implementing partners, molecular testing laboratories, diagnostic industry representatives, donors and technical agencies to maximize efficiency in tracking and responding to this novel situation.

Achieving these goals within the time frame necessary to satisfy the needs of National Malaria Control Programs and the populations they serve will require a focused, staffed, and budgeted effort, and a mechanism for programme management.

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