Response plan to *pfhrp2* gene deletions

Second edition

World Health Organization

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Abbreviations

ACT	artemisinin-based combination therapy
CHAI	Clinton Health Access Initiative
COVID-19	coronavirus disease
DHS	Demographic and Health Survey
ELISA	enzyme-linked immunosorbent assay
HRP2	histidine-rich protein 2
HRP3	histidine-rich protein 3
LDH	lactate dehydrogenase
MIS	Malaria Indicator Survey
NAAT EQA	nucleic acid amplification testing external quality assurance
NMP	national malaria programme
PCR	polymerase chain reaction
pfhrp2	*Plasmodium falciparum histidine-rich protein 2*
pfhrp3	*Plasmodium falciparum histidine-rich protein 3*
RDT	rapid diagnostic test
WGS	whole genome sequencing
WHO	World Health Organization

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 implement in the decentralized settings where most malaria occurs. Therefore, the advent of disposable lateral-flow immunoassays for malaria (widely known as rapid diagnostic tests (RDTs)) has been fundamentally important to modern malaria management, enabling targeted therapy, reducing drug wastage and limiting pressure for the development of drug 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 the World Health Organization (WHO) and its partners.

The clinically relevant RDTs for malaria diagnosis detect parasite proteins circulating in the blood. Some are configured to detect only *Plasmodium falciparum*, whereas others detect other *Plasmodium* species. The tests that are most sensitive in diagnosing falciparum malaria contain antibodies to detect histidine-rich protein 2 (HRP2) and/or the related histidine-rich protein 3 (HRP3). 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 15 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 these parasites undetectable by HRP2-based RDTs. Since then, many studies have demonstrated the presence of such gene-deleted strains in other countries and regions *(1)*. 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 deleted mutants has been found to be high enough to threaten the usefulness of HRP2-only RDTs.

This updated response plan to gene deletions that limit the effectiveness of HRP2-based RDTs provides a framework intended to support national malaria programmes (NMPs) and their implementing partners to address this problem pragmatically. The original document has been updated to summarize current knowledge and critical gaps in knowledge to guide future research and product development. The four objectives of an implemented response plan are as follows:

1. Define the frequency and distribution of *pfhrp2/3* deletions causing false-negative RDT results in circulating *P. falciparum* parasites.

2. Provide concrete guidance to countries on malaria diagnosis and treatment in settings where such deletions 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.

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

Two new sections have been added to this updated version of the response plan: modelling the spread of *pfhrp2/3* gene deletions and development of an interactive map, and market size projections for RDTs.

2. Defining the issue

2.1 RDTs in malaria control

Malaria remains a huge global health risk, causing an estimated 249 million cases of febrile disease (range, 225–278 million) and 608 000 deaths in 2022 *(2)*. The greatest burden of malarial disease is in sub-Saharan Africa, where approximately 90% of all malaria cases and malaria deaths occur *(2)*. 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% *(3)*. Recent studies have demonstrated that, even in African countries with endemic malaria, the great majority of cases of febrile illness are not due to malaria *(4)*. Despite this progress in malaria control, recent events, including the coronavirus disease (COVID-19) pandemic, have led to some increases in malaria cases and deaths, as outlined in the *World malaria report* 2023 *(2)*.

Malaria does not usually present with distinct physical signs that would enable accurate clinical diagnosis. Furthermore, as the incidence of malaria drops, confirmatory testing before treatment becomes essential to prevent unnecessary treatment and reduce drug resistance. Timely, accurate diagnostic testing is the cornerstone of modern malaria control, and, since 2010, the WHO treatment guidelines have included the recommendation for all cases of suspected malaria to be tested by microscopy or an RDT *(5)*. The benefits of diagnostic testing extend beyond malaria case management. As stated in the WHO *Global technical strategy for malaria 2016–2030 (6)*, "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, and as its quality is not always guaranteed, 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 finger-stick blood samples, they offer simple, unambiguous detection of malarial infection, enabling disease confirmation prior to treatment at the primary care level. First developed in the early 1990s, malaria RDTs were initially used very little, despite published reports of good performance *(7)*. By 2002, nearly 10 million tests were being produced each year by about 24 manufacturers. In view of variations in the manufacture of RDTs 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 the 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, FIND, the United States Centers for Disease Control and Prevention and other partners instituted a quality assurance programme, which functioned between 2008 and 2018. The programme had three main elements: product testing to verify performance against a standardized panel of blood samples, lot testing to ensure that each procured lot had maintained its specified criteria before distribution, and job aids and training materials for health workers and trainers to support proper use.

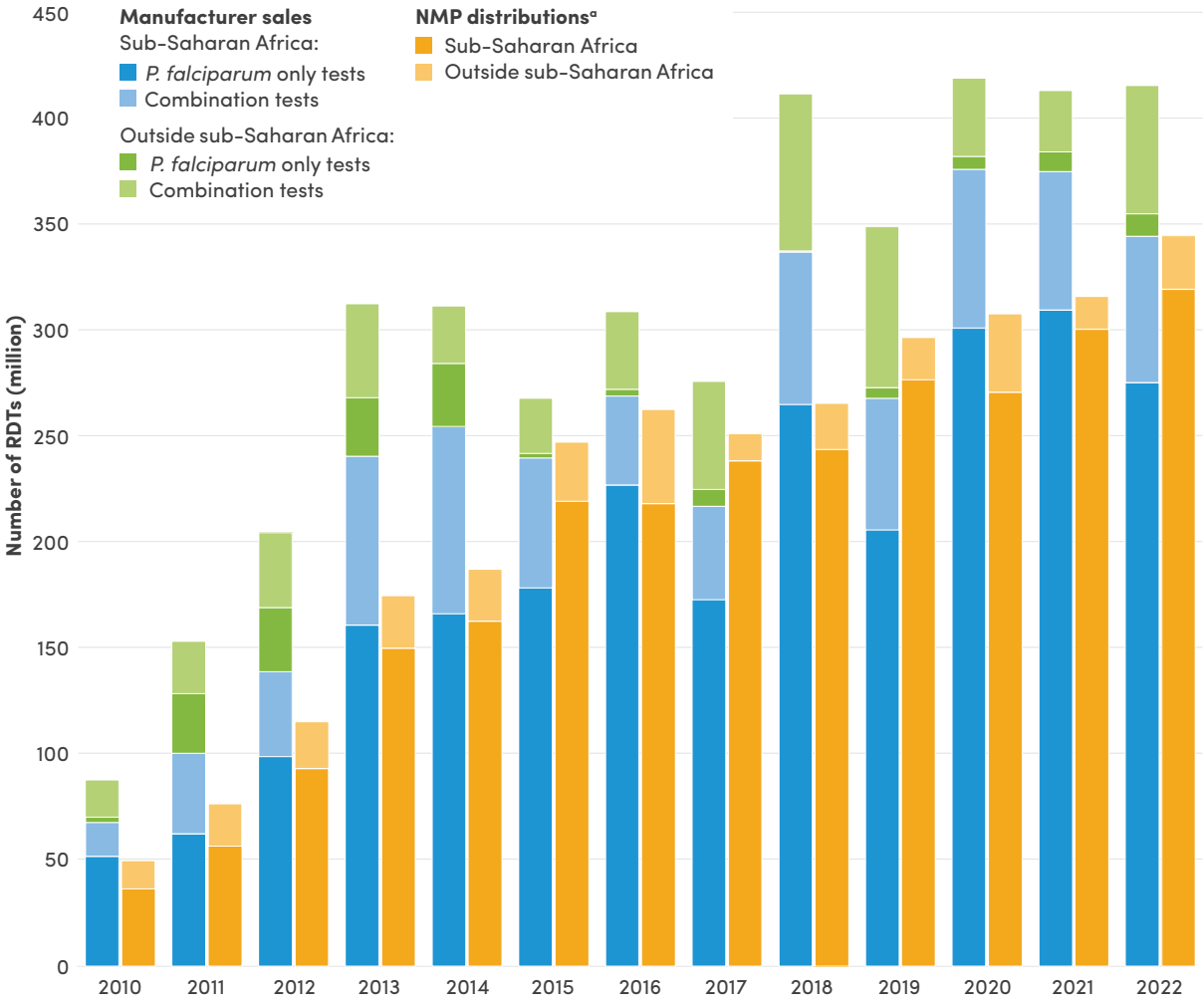
Between 2007 and 2017, all companies manufacturing malaria RDTs in conformity with the ISO 13485:2003 standard were invited to submit RDTs for testing against a large bank of geographically diverse clinical specimens and cultured parasites, in order to determine the RDTs' performance in detecting 200 or 2000 parasites/μL. The WHO and FIND malaria RDT product testing programme established performance parameters (e.g. panel detection score, false positivity, invalid rate, stability, ease of use) and evaluated 332 new or newly submitted products. For a decade, the results of product testing formed the basis of the procurement criteria implemented by 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 resulted in overall improvement in test quality *(8)*.

Since 1 January 2018, WHO has required WHO prequalification for all malaria RDTs for procurement. All products that are WHO-prequalified meet the required minimum performance criteria and are considered acceptable for procurement and for diagnosis of clinical malaria. WHO prequalification is dependent on the attainment of performance criteria, successful dossier review and inspection of the manufacturing site.

Similarly, between 2007 and 2017, WHO and FIND supported two lot-testing sites – at the Research Institute for Tropical Medicine in the Philippines and the Pasteur Institute of Cambodia – to evaluate procured lots before their distribution for use to ensure that each lot had not degraded and its performance was that determined during product testing. Currently, WHO supports lot-testing services free of charge at the Research Institute for Tropical Medicine in the Philippines. Capacity to meet national lot-testing needs has also been developed at the ANDI Centre of Excellence for Malaria Diagnosis, University of Lagos, Nigeria, and at the National Institute of Malaria Research, India. These laboratories conduct lot verification for RDT batches imported into their respective countries and should be contacted for information regarding their ongoing compliance with WHO procedures and availability of lot-testing services.

As shown in Fig. 1, the manufacture and sales of RDTs exceeded 400 million tests per year by 2020. The differences between the data from manufacturers and the data from NMPs shown in Fig. 1 are probably due to the inclusion of private-sector sales in the information supplied by manufacturers and incomplete reporting by some NMPs. RDT use in Africa accounts for the vast majority of NMP deliveries, and, as seen from the manufacturers' data, *P. falciparum*-only tests based on HRP2 detection are predominant.

Fig. 1. Data from manufacturers and NMPs on the volumes and types of RDTs delivered worldwide, 2010–2022



a NMP distributions do not reflect RDTs that are still in storage and are yet to be delivered to health facilities and to community health workers.

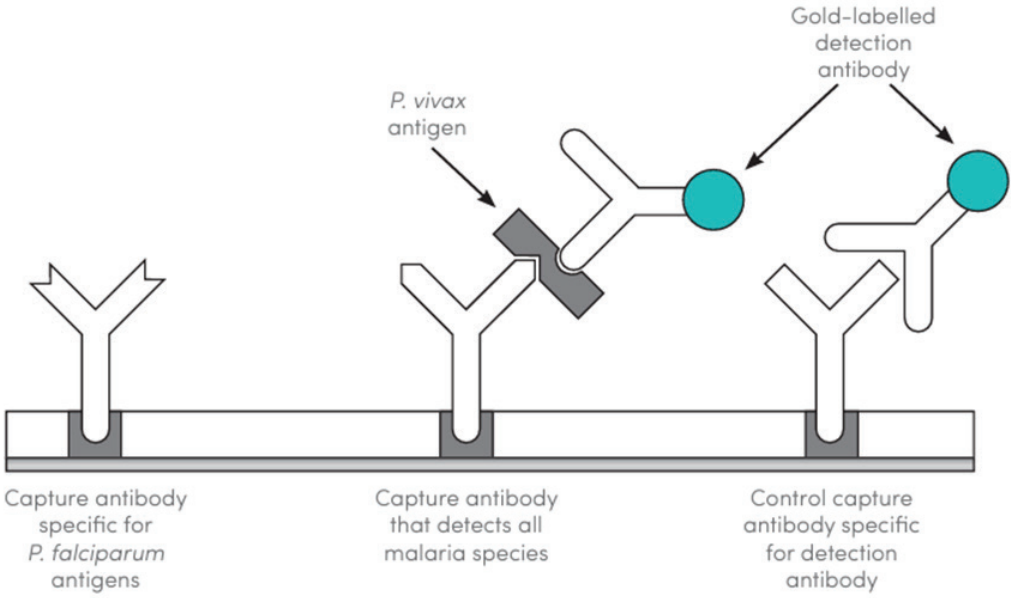
Sources: NMP reports and sales data from manufacturers eligible for the WHO Malaria RDT Product Testing Programme.

The advent of RDTs and their widespread use, spurred on by WHO policy and now adopted in the public sector by all 86 countries with continuous malaria transmission, has massively increased the proportion of suspected cases of malaria confirmed prior to treatment. In 2022, manufacturers reported about 415.5 million RDT sales. NMPs distributed 345 million RDTs in 2022. Such diagnostic expansion has averted millions of cases of mistreatment and overtreatment, helped thousands of clinicians working in malaria-prone areas to understand that fever does not necessarily signify malaria, and provided a much clearer understanding of the current epidemiology of malaria during the drive towards its elimination. Along with the expansion of sales, access to such testing has improved; by 2015, some 80% of all suspected malaria cases attending public health facilities worldwide underwent confirmatory testing instead of receiving a syndromic diagnosis *(3)*. However, the rates of diagnostic testing vary by geographical area and are lowest for febrile children in Africa. Specifically, the proportion of children under 5 years of age who received a diagnosis with a finger or heel prick increased from a median of 30% (12.2% to 38.4%) at baseline to 54% (40.7% to 63.8%) in the latest household surveys in Africa *(2)*. In addition, RDT sales are similar to sales of artemisinin-based combination therapies (ACTs), which indicates that there is significant scope for increasing the total number of RDTs used.

2.2 How RDTs work

RDTs are lateral-flow immunoassays that enable visualization of specific antigen–antibody recognition events. In routine use, a specified amount of finger-stick blood is transferred to one end of the RDT, the sample pad, which is loaded with reagents that lyse the blood cells to release any malaria antigens present and allow binding of monoclonal antibodies labelled with colloidal gold or another visible colorimetric indicator. The addition of a liquid buffer helps the blood 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) *(9)*. 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 have liquified and wicked appropriately up the RDT membrane. As each test may have a slightly different configuration and may 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 October 1999 (9)

There are a number of *Plasmodium* antigens that can be targeted by malaria RDTs (see Table 1). Because of the wide prevalence and medical importance of falciparum malaria, almost all RDTs contain antibodies that detect *P. falciparum* proteins (pan-pLDH, Pf-LDH or HRP2).

Table 1. *Plasmodium* antigens targeted by antibodies used in malaria RDTs

Target antigen	Full antigen name	Selectivity of assay	Characteristics
HRP2	Histidine-rich protein 2	Detects *P. falciparum* only	Water-soluble, non-essential protein of unclear function that is abundantly produced by all asexual stages and young gametocytes and contains repeating epitopes. Persists in serum for days to weeks after successful treatment. The crossreactive antigen HRP3 is also produced by *P. falciparum* and this antigen can also contribute to positive reactivity.
Pf-LDH	*P. falciparum* parasite lactate dehydrogenase	Detects *P. falciparum* only	Soluble glycolytic enzyme produced by trophozoites and gametocytes. Blood levels decline rapidly during therapy.
Pv-LDH	*P. vivax* parasite lactate dehydrogenase	Detects *P. vivax* only	
Pvom-LDH	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. There is relatively rapid clearance after therapy.

A number of factors are usually taken into consideration when selecting an antigen:

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

The sensitivity of a given RDT depends on several factors, including the accuracy of testing procedures, the antigen concentration and other 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 results may be due to low parasite density *(11)*, incorrect interpretation of results, poor quality and degradation of tests, procedures not followed correctly, gene deletion of the parasite target protein (e.g. *pfhrp2*) or a prozone effect *(12)*.

2.3 Quality assurance

Approximately 80% of suspected cases of malaria presenting for care in public-sector facilities in Africa are tested, and RDTs accounted for 74% of malaria testing in 2015 *(3)*. In 2014, the procurement of malaria RDTs represented an investment of US$ 103 million by multilateral and bilateral donors *(13)*. Anything that could compromise the utility of malaria testing or the accuracy of test procurement of malaria RDTs threatens the current investment of US$ 151 million annually by multilateral and bilateral agencies and the benefits of testing. RDTs that perform poorly have been excluded from the public market, likely through the influence of the WHO and FIND-sponsored large quality control programme for RDT products before (with comparative testing of marketed products) and as part of (with pre-shipment or post-shipment lot testing) procurement (see Box 1). Over the past several years, more RDT products have met the requirements of WHO prequalification *(14)*. Proper clinical testing with RDTs, including storage, training, procedural correctness, accuracy of recording results and adherence to results, remains a concern in many settings.

Currently, WHO recommends that all RDTs procured be WHO-prequalified. A full list can be found on the WHO prequalification page related to prequalified in vitro diagnostic products *(14)*. All products that are WHO-prequalified meet the required minimum performance criteria and are considered acceptable for procurement and for diagnosis of clinical malaria. Products that exceed these minimum performance criteria are not expected to have any significant clinical benefit over those that just meet these criteria. There is a public report available for all WHO-prequalified malaria RDTs that summarizes the assessment carried out and any inspection of the manufacturing site(s) where the product is manufactured *(15)*. There are currently no criteria for prequalification based on non-HRP2-expressing strains of *P. falciparum*; however, *pfhrp2*-deleted panels may be included in the prequalification process in the future to enable determination of performance against non-HRP2-expressing strains.

If no appropriate WHO-prequalified test is available to meet procurement needs (or the choice is very limited), i.e. in settings with a high prevalence (> 5%) of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions, all procured RDTs should meet the following minimum requirements:

- The product should be ISO 13485 certified.
- The product should be in the WHO prequalification pipeline and should have passed the laboratory evaluation achieving:
    - at least a 75% "panel detection score"[1] for low parasite density samples (200 parasites/µL) from the product testing evaluation panel for the detection of *P. falciparum* (HRP2-expressing and non-HRP2-expressing panels) and, if applicable, for *P. vivax*;
    - a false-positive rate of less than 10%; and
    - fewer than 5% invalid tests.

1 The panel detection score is not the same as "sensitivity", which is measured in a real population in which there is a variable mix of high- and low-density infections. Rather, the panel detection score at 200 parasites/µL reflects how the test can be expected to perform when challenged at the lower limits of clinically significant parasitaemia.

2.4 Evolution of *pfhrp2/3*-deleted variants

In 2010, researchers who were characterizing malarial blood samples from the Amazon basin in Peru as part of 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 *(16)*. Molecular testing by polymerase chain reaction (PCR) and antigen testing by enzyme-linked immunosorbent assay (ELISA) confirmed that the genes encoding this protein (and sometimes those encoding the structural homologue HRP3) were deleted from the parasite.

These gene deletions led to false-negative results in the RDTs targeting this protein, raising the fear that, if this anomaly occurred in other countries, many *P. falciparum* infections would go undiagnosed and untreated. Subsequent analyses at other sites in the Loreto region of the Peruvian Amazon showed a significant increase in the frequency of parasites with gene deletions from specimens collected in 1998–2001 (20.7%) to those collected in 2003–2005 (40.6%) *(17)*.

In a global survey of HRP2 sequence variation in 458 samples collected in 38 countries under the WHO and FIND RDT evaluation programme (Box 1) in 2008–2009 *(18)*, substantial diversity was found in *pfhrp2/3* sequences, including in the number and type of repeating epitopes; however, 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 global survey *(19)*, *pfhrp2* and *pfhrp3* exons from 2671 blood samples collected from patients with *P. falciparum* infection were sequenced in the MalariaGEN *P. falciparum* Community Project. This project is building a catalogue of variants and allele frequencies to characterize common genetic variations in *P. falciparum*. Thirty-two investigators contributed samples to the project from symptomatic and asymptomatic patients in 29 countries. Strong evidence of *pfhrp2* deletions was found in 0.6% of all samples and identified in two of the 29 countries.

The frequency of *pfhrp2* deletions in tested samples was 38% in Peru and 4% in the Papua region of Indonesia. Deletions of *pfhrp3* were more common, with an overall frequency of 2.4% identified in eight countries; there were four countries in which more than 5% of samples contained deletions. Dual mutations were found only in Peru. Deletion of both genes occurred in multiple genetic backgrounds. An earlier independent analysis of the global survey also found similar frequencies of *pfhrp2* and *pfhrp3* deletions: 0.5% and 1.4%, respectively *(20)*. However, these study findings may not be a true representation of gene deletions in these countries, as original sample collection was not designed to address *pfhrp2* and *pfhrp3* deletions as recommended by WHO and included only a limited number of samples from many countries. Nevertheless, when this type of survey confirms the presence of *pfhrp2* deletions, it should trigger a baseline survey in the country and in neighbouring countries.

A review of published reports (years 2010 to 2019) found 51 studies from 36 journal publications with data from 29 countries confirming the presence of *P. falciparum* malaria strains with mutations affecting the production of HRP2 and/or HRP3 proteins (hereafter called *pfhrp2/3*-deleted variants) in many regions that are endemic for malaria, in some cases at a prevalence that would significantly reduce the effectiveness of RDTs that test for this antigen to detect falciparum malaria *(1)*. Fig. 3 shows the geographical distribution of reported *pfhrp2/3*-deleted variants. Based on all data from publications included in the Malaria Threats Map *(21)*, as of January 2024, some form of investigation for *pfhrp2/3* deletions has been conducted in 55 countries, and the presence of deletions has been confirmed in 45. The red dots represent the location of samples reported to have *pfhrp2* deletions. Parasites that fail to express pLDH or aldolase antigens, which are enzymes critical to parasite survival, have not been reported.

Fig. 3. Distribution of reported *pfhrp2/3* gene deletions – Malaria Threats Map, as of May 2024

Source: WHO Malaria Threats Map *(21)*

Because of the large methodological differences between studies, especially in the selection of participants and/or selection of samples for analysis, only broad conclusions can be drawn.

- There are clear local "hot spots" where *pfhrp2/3*-deleted variants are common enough to make diagnostic testing based only on HRP2 inadequate. Specifically, relatively high rates of *pfhrp2/3* deletions have been seen in the Amazonian regions of Brazil *(22)*, Colombia *(23)* and Peru *(16, 17, 24, 25)*, and on the African continent in Djibouti *(26)*, Eritrea *(27, 28)*, Ethiopia *(29–31)* and South Sudan *(32)* (Fig. 3).

- The prevalence of *P. falciparum* parasites that do not express HRP2 varies by province or area in any given country. For example, the ranges of reported prevalence were 0–25% by study site in India *(33)*, 0–53.6% in Colombia *(34)* and 0–21.7% in the Democratic Republic of the Congo *(35)*. Similarly, although the presence of *pfhrp2/3*-deleted variants in a neighbouring country presents a risk factor, it does not guarantee local circulation of such strains *(36, 37)*.

- *pfhrp2/3*-deleted variants can cause epidemics, especially in low transmission regions, which may be missed by HRP2-based RDTs *(24)*.

- In many studies, the methods for selecting samples for molecular analysis have resulted in overestimates of the true prevalence of *pfhrp2/3*-deleted variants. In Rwanda, for example, 23% of *P. falciparum* strains identified by PCR were *pfhrp2*-deleted, but PCR was performed only on samples with false-negative HRP2-based RDTs *(38)*. If the samples with HRP2-positive RDT results and no *pfhrp2* gene deletions had also been considered in the analysis (i.e. as part of the denominator), the true prevalence of *pfhrp2* deletions in microscopy-positive falciparum cases would have been around 1%. This type of analytical limitation can be overcome by following the WHO-recommended analysis to estimate the prevalence of *pfhrp2* deletions based on the total population of people with symptomatic falciparum malaria.

- The data illustrated in Fig. 3 may, however, underestimate the prevalence of *pfhrp2*-deleted variants because of the cross-reactivity of HRP2-based RDTs with HRP3. In addition, in areas of moderate to high transmission, the circulation of strains with *pfhrp2* deletions may be masked by coinfection with *P. falciparum* strains without such deletions, as infection with more than one strain type is common in these settings. Masking due to coinfection may occur less frequently at the start or end of the transmission season in settings with seasonal malaria transmission. A modelling study showed that the prevalence of false-negative RDTs caused by *pfhrp2*-deleted parasites varies over the season, especially in moderate to high transmission settings. Therefore, to minimize the bias in estimating the prevalence of *pfhrp2*-deleted parasites, it is important to collect specimens throughout the transmission season *(39)*.

- The absence of PCR amplification of *pfhrp2/3* may be attributed to an inadequate quantity of parasite DNA or to PCR procedural issues. In many studies, the DNA extraction and purification methods used have not provided enough amplifiable DNA to detect single-copy genes such as *pfhrp2/3*. Therefore, it is critical to ensure that adequate parasite DNA concentrations are present before making deletion calls based on the successful amplification of two other single-copy genes such as *msp1* and *msp2*. Furthermore, requiring a parasite density of at least 100 parasites/µL decreases the risk of unintentional misclassification of results due to insufficient DNA template concentration. It will be important to report parasitaemia data when microscopy is used for enrolling patients, and estimated parasite density or parasite genome copy number when real-time PCR is used *(40, 41)*.

- Although longitudinal data are scarce, *pfhrp2* deletions appear to persist over time, even after the pressure of HRP2-based RDTs has been lifted *(42, 43)*. Results from the same geographical area in asymptomatic and symptomatic populations can yield very different findings *(35, 44)*.

WHO tracks and reports surveys of *pfhrp2/3* deletions using an online mapping tool: the Malaria Threats Map *(21)*. To facilitate interpretation of the results, the Malaria Threats Map enables data to be filtered based on the enrolment population (symptomatic vs. asymptomatic) and by single or double deletions of *pfhrp2* and *pfhrp3*. The Malaria Threats Map data are not independently assessed based on the quality of the studies performed; however, a 2020 systematic review *(1)* provided a laboratory comprehensiveness indicator score on the basis of the clinical and molecular testing information available in the published report. This scoring was adapted from Cheng et al.'s recommendations for standardization of testing and reporting of *pfhrp2/3*-deleted variants *(40)*. Studies were assigned a score of 1 to 7 based on evidence of the following criteria (1 point each):

- evidence of performance of quality-assured microscopy;
- use of an RDT for diagnosis that met WHO performance criteria (p. 13);
- evidence of quantification of parasitaemia by microscopy and/or real-time PCR or qPCR;
- testing for availability of amplifiable DNA using more than one single-copy *P. falciparum* gene;
- evidence of molecular species identification;
- testing for both *pfhrp2* and *pfhrp3* deletions; and
- use of another quality HRP2-based RDT, ELISA or bead-based assay to confirm molecular findings.

The fulfilment of each criterion was given a score of 1, and the study score was the sum of all criteria scores. Overall, the findings suggest that there is much room for improvement, as only three publications (6%) met all the published criteria, and another nine publications (18%) fulfilled six out of the seven criteria.

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 response plan to *pfhrp2/3* deletions proposes a multifaceted response to this threat that requires:

- pragmatic action by NMPs and their implementing partners;
- strengthened and expanded laboratory networks;
- research to further understand the factors contributing to the development of these *pfhrp2/3*-deleted variants 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 of malaria diagnostic services.

3.1 Pragmatic action by NMPs

Like antimalarial drug resistance, the evolution and circulation of *pfhrp2/3*-deleted variants will threaten malaria control and must be managed. Although there are unanswered questions about the genesis and spread of *pfhrp2/3*-deleted variants, NMPs can act now while further information is being collected. The programmatic management of *pfhrp2/3*-deleted variants described below includes guidance covered in the WHO information note on false-negative results in RDTs, which was published in May 2016 and updated in September 2017 and June 2019 *(45)*.

In many areas where malaria has historically been endemic, disease prevalence has fallen, and most of the RDTs used to test febrile individuals give correct negative results. Large studies conducted to follow the outcomes of febrile children with **negative** RDT results *(46, 47)* found that it was safe not to treat them for malaria; there were no malaria-related deaths or hospitalizations. That being said, in areas where *pfhrp2/3* deletions are found to be prevalent and are likely to cause at least 5% false-negative RDT results, such as in the Horn of Africa and most Amazonian regions of South America, NMPs should switch to RDTs that do not rely solely on HRP2 for detecting *P. falciparum (48)*. Circulation of *pfhrp2/3*-deleted variants is likely to be focal, and the introduction of a new testing strategy may be prioritized in regions or provinces with the highest prevalence of these variants. 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.

In July 2016, WHO convened a meeting of experts to prepare guidance on *pfhrp2/3* deletions and published the outcome as a background document for the September 2016 meeting of the Malaria Policy Advisory Committee (now the Malaria Policy Advisory Group) *(49)*. The group decided that a ≥ 5% local prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions would warrant a change in testing strategy. This cut-off was selected because it reflects the predicted difference in clinical sensitivity between Pf-LDH or pan-pLDH RDTs and HRP2-based RDTs, and therefore indicates when public health gains will be obtained by changing from RDTs that solely detect HRP2 to those that target pLDH antigens (alone or in combination with HRP2). Recently, a systematic analysis of published data related to differences in clinical sensitivity between HRP2-based and Pf-LDH-based RDTs was conducted to determine whether this guidance was still valid. This

investigation confirmed that the commercial Pf-LDH-based tests have lower sensitivity than HRP2-based RDTs. However, the difference varied between studies. In 24 publications from 24 countries including different endemic settings, HRP2-based RDTs or strips were 5.9% more sensitive than Pf-LDH tests, using microscopic data as the gold standard *(48)*.

As this review included studies using only Pf-LDH-based RDTs, it is not known whether similar differences should be expected with pan-pLDH tests. Overall, this review suggests that currently available Pf-LDH tests are at least 5% less sensitive than HRP2-based RDTs, and this level of difference is consistent with the current 5% threshold of false-negative test results for switching to non-HRP2-based RDTs when deletions are confirmed. Recent efforts have led to the development and introduction of new Pf-LDH-based tests that are more sensitive than current Pf-LDH tests, based on their comparative analytical sensitivity (Domingo G, unpublished data, 2022). Three such products have been submitted for WHO prequalification and have passed the independent laboratory evaluation; however, manufacturer site inspection is pending for their final approval. In addition, these products are being evaluated for their clinical sensitivity compared to WHO-prequalified HRP2-based RDTs in different endemic settings in Africa, South America and Asia. When such data become available, their impact on the current threshold (5% prevalence of false-negative RDT results caused by *pfhrp2/3* deletions) may be determined. Furthermore, future modelling based on rigorously collected programme data from the use of new Pf-LDH RDTs may be useful to confirm or refine the current 5% cut-off value.

As outlined in this document, strains carrying *pfhrp2/3* deletions will continue to expand and spread, as observed in recent years in the Horn of Africa. Therefore, it is important to monitor for the presence of *pfhrp2/3* deletions and move away from the use of HRP2-based RDTs when the prevalence of false-negative RDT results caused by *pfhrp2/3* deletions exceeds the 5% threshold set by WHO. All countries should therefore consider planning a gradual transition to tests that do not rely solely on the detection of HRP2. In the interim, NMPs should be prepared to investigate suspected false-negative RDT results due to deletions, introduce surveillance for *pfhrp2/3* deletions and act on the outcomes. Approaches are proposed below.

3.1.1 Investigating suspected false-negative RDT results for *pfhrp2/3*-deleted variants

NMPs and implementing partners in countries in which HRP2-based RDTs are used should support investigation of suspected false-negative RDT results for possible *pfhrp2/3*-deleted variants, considering 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 NMP 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 and a positive result on the pan- or Pf-LDH line if a combination RDT is used, and positivity for *P. falciparum* is confirmed 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 NMP and/or the manufacturer receives many formal complaints or anecdotal evidence that HRP2-based RDTs are giving false-negative results for *P. falciparum*.

3.1.2 National assessment of the prevalence of false-negative RDT results caused by *pfhrp2/3*-deleted variants

The interpretation of the survey data collected to date (see Fig. 3) 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*-deleted variants. When an NMP or other credible group detects *pfhrp2/3* gene deletions in local strains, it should determine the prevalence in the country in order to plan an appropriate response; NMPs in neighbouring countries should do the same. Although the infrastructure for activities such as Malaria Indicator Surveys (MIS) and therapeutic efficacy studies may be used to determine the distribution of these strains, these surveys are limited in scope; although they cannot provide accurate data on the frequency and spread of *pfhrp2/3*-deleted parasites causing false-negative RDT results, they can trigger additional investigations and surveys if *pfhrp2/3*-deleted parasites are confirmed. In this regard, these surveys can provide warning signals when deletions are identified, but cannot accurately generate population-level data (as explained in Box 2 below) and may not be adequate to inform policy decision-making.

Box 2. Limitations of alternative survey approaches for the assessment of national prevalence of false-negative RDT results caused by *pfhrp2/3* deletions

To investigate the *pfhrp2/3* deletion status of *P. falciparum* parasites, several studies have been conducted using alternative survey methodologies that differ from the WHO surveillance protocol templates *(50, 51)*. These include MIS, Demographic and Health Surveys (DHS), and therapeutic efficacy studies. When outcomes from these alternative study methodologies indicate a > 5% prevalence of false-negative RDTs caused by deletions, they can serve as red flags for planning where to target investigation of deletions and highlight the need to conduct larger surveys to determine deletion status at the national level. However, a lack of deletions in these surveys does not mean that *pfhrp2* deletions are not present at potentially clinically relevant thresholds.

Therefore, it is important to understand the limitations of the data collected from non-standardized surveys, as they may not accurately represent national/regional or local prevalence of *pfhrp2* deletions. Some of the limitations of these alternative approaches are highlighted here.

Sample size: Alternative survey designs most often fail to meet sample size requirements (300 confirmed *P. falciparum* cases per domain based on 10 health facilities per domain) recommended in the WHO surveillance protocol templates *(50, 51)*.

Screening methodology: Testing of subjects using a WHO-prequalified HRP2-based RDT and a non-HRP2-based RDT or quality-assured microscopy is recommended during enrolment. Most often, alternative survey designs do not follow these criteria and it becomes challenging to determine the number of deletions causing false-negative RDT results. In some cases, only HRP2-RDT-positive cases are enrolled.

Some surveys use immunoassays such as multiplex bead assay or ELISA for determining infection status after enrolment. As sensitivity of these immunoassays differs from the RDT detection threshold and is often not standardized, it can be challenging to use the results of these immunoassays to estimate the prevalence of false-negative RDT results caused by deletions.

Symptomatic versus asymptomatic cases: Many surveys include subjects with no symptoms of malaria, which will lead to biases, as RDTs are designed to detect symptomatic cases. In addition, some data from the Democratic Republic of the Congo and Ghana suggest that *pfhrp2* deletions may be more common among asymptomatic parasitaemic individuals, as repeat surveys among clinical cases have revealed no or very low prevalence of *pfhrp2* deletions *(35, 44, 52, 53)*.

Transmission status: Many surveys focus on enrolling subjects from high transmission areas and do not include all regions with low transmission. Historically, high levels of deletions are more commonly reported in low transmission areas, and it is possible that alternative survey methods will not yield nationally representative data for assessing *pfhrp2/3* deletions.

WHO has published two surveillance protocol templates. These are the same except that one for surveillance only and one has the addition of biobanking of samples for potential additional research outputs *(50, 51)*. The protocol targets the most relevant group for case management and disease control: symptomatic individuals attending health facilities who are being evaluated for clinical malaria. The goal of the protocol, the elements of which are described below, is to rapidly determine whether the prevalence of false-negative RDT results caused by *pfhrp2/3*-deleted parasites among patients with falciparum malaria is high enough (> 5%) to consider changing the malaria diagnostic strategy and tools. Clinical sites for enrolment of patients in surveys of the prevalence of *pfhrp2/3* deletions should be selected such that they represent the population distribution and heterogeneity of transmission.

NMPs may wish to establish sentinel sites to repeat estimates of the *pfhrp2/3* deletion prevalence over time in order to determine whether changes in diagnostic testing are effective at controlling the transmission of *pfhrp2/3*-deleted variants. New initiatives to find these gene deletions are not currently recommended if there are no confirmed reports of *pfhrp2/3* gene deletions locally or in neighbouring countries.

3.1.3 Response to survey outcome: > 5% false-negative RDT results caused by *pfhrp2/3* deletions

If a survey confirms that the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* deletions is greater than 5%, then the NMP will need to take a series of actions to immediately optimize case management and plan for the cessation of HRP2 RDTs and introduction of replacement RDTs. Any change should be applied nationwide, although roll-out might be prioritized on the basis of the prevalence of *pfhrp2/3* deletions.

Step 1 Immediately introduce safeguards to reduce the impact of false-negative RDT results

A number of safeguards can be introduced and the choice and scope of implementation will be influenced by the prevalence and distribution of malaria and false-negative HRP2 RDT results caused by *pfhrp2/3* deletions, patient access to good-quality microscopy, ACT stocks, the capacity to rapidly inform end-users and the feasibility of RDT product retrieval.

In the highest risk areas, initiate an order for health workers to immediately adopt alternative diagnostic algorithms, depending on the type of HRP2-based RDT being used (a, b, c, d):

a) HRP2-only RDTs:

- Treat all positive cases as per national guidelines.
- Send RDT-negative patients for quality-assured microscopy.
- If microscopy is not available or the patient is unlikely to follow through with referral, provide presumptive treatment.

b) HRP2/pan-pLDH RDTs:

- Treat all HRP2 and/or pan-pLDH test line positive cases with ACTs to cover both HRP2-expressing and non-expressing *P. falciparum* and non-*P. falciparum* infections.
- To confirm species, send pan-pLDH test line-only positive cases for quality-assured microscopy or perform a *P. vivax*-specific RDT.

c) HRP2/Pv-LDH RDTs:

- Treat positive RDT results as per national guidelines.
- Send HRP2 test line negative patients for quality-assured microscopy.
- If microscopy is not available or if the patient is unlikely to follow through with referral, provide presumptive treatment.

> To avoid undermining confidence in RDTs, it is important for the communication accompanying the new diagnostic algorithm to explain that RDT failures were most likely attributable to parasite factors and not to RDT defects.

If the prevalence of false-negative RDT results caused by *pfhrp2/3* deletions is extremely high, as was the case in Eritrea *(28)* and Djibouti *(26, 54)*, and confidence in RDT results has been severely eroded, then the following algorithm should be considered:

d) Stop using HRP2-based RDTs and confirm diagnosis by quality-assured microscopy.

- Where quality-assured microscopy services are not available or not accessible, treat all malaria suspects presumptively with ACTs until quality-assured microscopy or other WHO-prequalified diagnostic tests can be deployed.

Step 2 | Investigate feasibility and resources for product retrieval

This decision will be based on the available resources, the interim diagnostic algorithm selected, the expiry dates of lots in the field and anticipated time before product replacement is deployed.

Step 3 | Carry out supplementary distribution +/- procurement of ACT stocks

If a decision is made to fully or partially introduce presumptive treatment of malaria suspects until replacement RDTs are distributed, the demand for ACTs will increase and will need to be met with existing or supplementary stocks.

Step 4 | Select and procure replacement RDTs

Ideally, all replacement RDTs being considered for use should be either WHO-prequalified or in the WHO prequalification assessment pipeline and meet WHO performance criteria[2] for the detection of all *P. falciparum* strains, including those with *pfhrp2/3* deletions.

Until recently, the laboratory evaluation component of the prequalification process did not include *pfhrp2/3*-deleted variants and therefore performance of non-HRP2 RDTs against HRP2-expressing *P. falciparum* panels was assumed to be representative of test performance against non-HRP2-expressing parasites. The majority of Pf-LDH-based RDTs have poorer performance against the evaluation panel of *pfhrp2/3* single- and double-deleted samples and as of July 2024 these products do not meet the prequalification criteria. Given these challenging circumstances, interim guidance on procurement in areas with a high prevalence of *pfhrp2/3* gene deletions has been provided *(55)*. This guidance will be updated as new data emerge and as next-generation RDTs are developed and approved for general international procurement.

Status updates on products in the prequalification pipeline can be found at https://extranet.who.int/prequal/vitro-diagnostics/vitro-diagnostics-under-assessment, and updates on their prequalification status can be found at https://www.who.int/teams/global-malaria-programme/case-management/diagnosis/rapid-diagnostic-tests/selection-and-procurement. Performance against *pfhrp2/3*-negative samples with antigen concentrations reflecting 2000 parasites/μL is included to inform selection of RDTs for use in surveys, as a *pfhrp2* deletion screening tool, based on the WHO survey protocol templates *(50, 51)*. More specifically, only those RDTs that have a panel detection score of > 90% against 2000 parasites/μL should be used to screen for suspected *pfhrp2/3* deletions *(5)*.

Table 2 summarizes alternative testing options for areas where the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions is > 5%.

Table 2. Alternative testing options for areas with a > 5% prevalence of false-negative RDT results caused by *pfhrp2/3* deletions

Target species	Target antigens
Detect *P. falciparum*	pan-pLDH-only RDTs
	Combination of HRP2 and pf-LDH[a]
Detect and discriminate Pf from Pv or non-Pf infections	Combination of Pf-LDH, HRP2 and pan-pLDH[a]
	Combination of Pf-LDH, HRP2 and Pv-LDH[a]
	Combination of Pf-LDH, Pv-LDH
	Combination of Pf-LDH and pan-pLDH

[a] Pf-LDH and HRP2 may be on the same test line or separate test lines

2 Panel detection score ≥ 75% at 200 parasites/μL, false-positivity rate < 10% and invalid rate < 5%

Table 3 lists the non-WHO-prequalified tests meeting critical criteria for malaria case management that are currently available.

Table 3. Available non-WHO-prequalified tests meeting critical criteria[a]

Product name	Product code	Manufacturer name
Biocredit Malaria Ag Pf (pLDH)	C14RHG25, C14RHH25	Rapigen Inc.
Biocredit Malaria Ag Pf (pLDH/HRP2)	C13RHG25, C13RHH25	Rapigen Inc.
Biocredit Malaria Ag Pf/Pv (pLDH/pLDH)	C61RHG25, C61RHH25	Rapigen Inc.
CareStart™ Malaria Pf (HRP2/pLDH) Ag RDT	RMPM-02571	Access Bio Inc.
CareStart™ Malaria PAN (pLDH) Ag RDT	RMNM-02571	Access Bio Inc.

[a] Valid ISO 13485:2003, submission of application for WHO prequalification, and acceptable diagnostic performance against both HRP2-expressing and non-HRP2-expressing at 200 parasites/µL (*pfhrp2/3* single or double deletions), based on the most recent WHO laboratory assessment performed at the United States Centers for Disease Control and Prevention.

a) Replacing RDTs for case management in areas with *pfhrp2* deletions and predominantly *P. falciparum* transmission

As of July 2024, four non-WHO-prequalified tests – pan-pLDH-only RDT (CareStart™ Malaria PAN), HRP2/Pf-LDH single-line RDT (CareStart™ Malaria Pf (HRP2/pLDH) Ag RDT) and two Pf-LDH RDTs (BIOCREDIT Malaria Ag Pf (pLDH) and dual test-line BIOCREDIT Malaria Ag Pf (pLDH/HRP2)) – meet the performance criteria on both HRP2-expressing and non-expressing performance panels and would therefore be the top choices for RDT replacement in places where it is not a high priority to discriminate between *Plasmodium* spp., i.e. in most of sub-Saharan Africa. The BIOCREDIT RDTs listed above are approved by the Global Fund Expert Review Panel for Diagnostics and can therefore be procured with Global Fund financing for use in areas where HRP2-based RDTs cannot be used.

b) Replacing RDTs for case management in areas with *pfhrp2* deletions and mixed *P. falciparum* and *P. vivax* transmission

As of July 2024, no Pf-LDH-containing combination (Pf-specific and Pv-specific) RDTs are WHO-prequalified. However, there is one product (BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH)) with laboratory testing completed that meets the performance criteria on both HRP2-expressing and non-expressing panels of *P. falciparum* and *P. vivax* panels. This product is approved by the Global Fund Expert Review Panel for Diagnostics and can therefore be procured with Global Fund financing for use in areas where HRP2-based RDTs cannot be used.

There are several Pf-LDH-based combination RDTs that do perform well at higher densities/antigen concentrations. Ultimately, the impact of using these less sensitive Pf-LDH test lines for clinical case management of *P. falciparum* in areas with *pfhrp2/3* deletions will depend on the interplay of the various factors listed below (Box 3); however, most programmes will not have the required data to assess the impact of using less sensitive Pf-LDH combination RDTs.

Box 3. Factors affecting the performance of Pf-LDH combination RDTs against *pfhrp2/3*-deleted parasites

- prevalence of *pfhrp2/3*-deleted parasites in the population
- HRP3 cross-reactivity with HRP2 RDTs
- the multiplicity of infection in the population – i.e. infections that include both HRP2-expressing and non-expressing *P. falciparum* isolates
- prevalence of lower density infections; reviews suggest that 0–30% of symptomatic *P. falciparum* cases are < 200 parasites/μL *(57)*

When better performing RDTs using Pf-LDH or other non-HRP2 antigens become available, another important consideration in selecting a replacement combination test will be the technical complexity. Although the protocols (i.e. blood volume, drops of buffer, reading time) for various RDT products will be similar and unlikely to pose major difficulties for health workers, the test interpretation does vary considerably depending on the number of test lines, their order and the target antigen. Pilot testing some of these different options with a small group of intended users could provide valuable insights for product selection.

Step 5 | Re-training and roll-out

While awaiting the arrival of the replacement RDT(s), plans should be developed for re-training and supervision, including relevant adaptations of training materials, standard operating procedures, job aids, and so on. Routine reporting forms may also need to be revised to most accurately reflect the results of one or more new RDTs. The roll-out of training and replacement RDTs should be prioritized from high to low prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* deletions. Coupling re-training on RDT use with refresher training on microscopy should be strongly considered in areas with both *P. falciparum* and non-falciparum species, and where programmes will be reliant on microscopy for species confirmation.

Ultimately, encouraging manufacturers to submit their products for WHO prequalification and evaluating currently prequalified products against a larger, more geographically diverse panel of clinical *pfhrp2/3*-deleted isolates are top priorities. Longer term, the development of tests that can meet the full set of criteria should be actively pursued; opportunities are discussed in section 3.5 below.

3.2 Strengthened and expanded laboratory networks

Strengthening laboratory capacity for the detection of malaria has been a critical feature of national and international malaria control. The emergence and spread 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 have been difficult to establish and maintain in peripheral settings, and many reports have documented poor sensitivity and specificity in the field detection of malaria by microscopy *(57–61)*. Meeting the capacity to assess suspected false-negative HRP2-based RDTs will require quality-assured microscopy and/or staff trained and ready to correctly use non-HRP2-only RDTs that are not in routine use in the NMP.

In addition to assessing individual reports of suspected false-negative RDT results, national surveys should be conducted to establish the prevalence of false-negative RDT results caused by *pfhrp2/3* deletions *(50, 51)*, which would require more training and perhaps staff recruitment, depending on local workloads. Survey protocols will also require procurement and distribution of Pf-LDH-based RDTs. As each RDT has specific instructions for use, performance of multiple testing methods in 10 health facilities per province – at least during a survey for *pfhrp2/3*-deleted variants – is not a trivial task.

Discordant test results between two different RDTs may be attributable to many factors, and not all HRP2-negative, Pf-LDH-positive RDT results will be due to *pfhrp2/3* deletions (see Table 4).

In some settings, less than half of all suspected false-negative HRP2 RDT results are found to be due to *pfhrp2/3* deletions *(35, 64, 65)*, while in others, the predictive value of false-negative HRP2 RDT results for gene deletion is much greater. Molecular analysis is essential for investigating causes of false-negative HRP2 RDT results and confirming deletions. Confirming the presence of gene deletions requires sampling, labelling and preparation of dried blood spots for shipping and multiple molecular analyses in regional or international laboratories. This work should be done in a timely manner so that the NMP can plan for possible procurement of new types of RDTs.

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 (41)*. As *pfhrp2/3*-deleted variants can be detected only as the absence of amplified products of pfhrp exons, rigorous control must be used to ensure the presence of undegraded, amplifiable parasite DNA and lack of PCR contamination. Molecular analysis 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 result be sent for molecular analysis. Therefore, the number of samples to be genetically analysed will depend on the prevalence of *pfhrp2/3*-deleted variants (and the frequency of other events causing false-negative results).

Table 4. 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, perform-ing the test or interpreting the result	Verify whether RDTs were used by untrained staff; assess RDT user competence on site.
Use of an imperfect "gold standard" as a comparator	Thick and thin films from a patient with a negative RDT result incorrectly interpreted as "positive" by microscopy	Verify microscopy results and interpretation by a qualified micros-copist.
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 with microscopy performed by two qualified microscopists or, if microscopy is not available, with a high-quality non-HRP2-detecting RDT; retrieve RDTs from affected areas and send them to a WHO-recognized laboratory for lot testing.[a]
	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 the range in the manufacturer's instructions, send the RDTs to the WHO lot-testing laboratory.[a] Train health workers to respect storage conditions, and improve storage facilities (e.g. add fans).
Parasite factors	Parasites lacking or expressing 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-LDH test line if a combination RDT is used, **and** the sample is confirmed microscopically to be positive for *P. falciparum* by two qualified microscopists. If these conditions are met, place fresh blood sam-ples or dried blood spots (50–60 μL) on Whatman® 3MM filter pa-per or other collection cards, in 4 °C or frozen storage (-20°C), if possible, until shipment for molecular analysis of *P. falciparum* and *pfhrp2/3* gene deletion status.
	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-LDH. 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× dilution and, if needed, a subsequent 50× dilution of the sample, with dilutions in 0.9% NaCl *(62)*.

a Information about lot testing can be found on the WHO website *(63)*.

A number of international reference laboratories with experience in *pfhrp2/3* molecular analysis are already collaborating with WHO (see Table 5). Although these laboratories have shown willingness to contribute to a 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 reagents for the predicted workload are not currently funded or guaranteed.

Table 5. List of international laboratories participating in the WHO reference laboratory network supporting *pfhrp2/3* deletion surveillance

Country	Name of institute
United Kingdom of Great Britain and Northern Ireland	Malaria Reference Laboratory/Faculty of Infectious Diseases/ London School of Hygiene and Tropical Medicine
United States of America	University of North Carolina at Chapel Hill
Australia	Australian Defence Force Malaria and Infectious Disease Institute and QIMR Berghofer Medical Research Institute
United States of America	Centers for Disease Control and Prevention
Senegal	Université Cheikh Anta Diop de Dakar, and International Center for Research and Training in Applied Genomics and Health Surveillance
Peru	Universidad Peruana Cayetano Heredia
India	National Institute of Malaria Research

National programmes themselves may have an interest in using or strengthening their local capacity for molecular analysis; however, the lack of standardization of molecular methods (in terms of both the protocol and reagents used) and lack of a malaria molecular assay that has been approved by a stringent regulatory authority make the comparison of results between studies and between laboratories problematic *(66)*. The consequences of false-positive and false-negative results for *pfhrp2/3* gene deletions will have serious negative impacts. WHO is therefore committed to working with expert laboratories and donors to strengthen the global capacity for detecting *pfhrp2/3* deletions through linking with reference laboratories and facilitating the sharing of protocols. Where capacity exists, regional and national laboratories with molecular expertise could play an important role, but it will be incumbent upon countries embarking on national *pfhrp2* deletion surveys to have a molecular assessment plan that includes the capacity and agreements to ship samples internationally to collaborating laboratories with the necessary capacity and quality control.

All laboratories that conduct molecular analysis for detection of malaria parasites are encouraged to participate in the WHO external quality assurance scheme for malaria nucleic acid amplification testing (WHO malaria NAAT EQA scheme), established in 2017 *(67, 68)*. Under this scheme, participants receive proficiency testing panels twice a year that include all *Plasmodium* species in a range of parasite densities, with *pfhrp2/3*-deleted parasites available in the panel as well.

3.3 New research

3.3.1 Molecular function of HRP2 and HPR3

Although the precise physiological function of HRP2 and its structural analogue HRP3 is still largely unknown, some studies have shown that HRP2 plays a potential role in haemozoin formation *(69)* and cerebral malaria pathogenesis *(70, 71)*. The structure and variability of the genes that encode HRP2 and HRP3 proteins have been described previously *(72, 73)*. 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. 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 *pfhrp2* genes often have variable genetic sequences. In a study of 458 *P. falciparum* strains collected globally, 315 different *pfhrp2* genetic sequences were found. Of the subset of 80 strains in which the *pfhrp3* gene was also sequenced, 42 different sequences were found *(18)*. Although there is some evidence that such sequence variation can affect the clinical sensitivity of tests based on specific monoclonal antibodies, this is seen only near the limit of detection and has a limited effect on the overall clinical sensitivity (except in the case of outright gene deletion) *(74)*. Deletions that halt the expression of HRP2 or HRP3 may occur at various locations around the *pfhrp2/3* exons and are frequently large, involving not only the relevant *pfhrp2/3* genes but also the upstream and downstream flanking genes *(16, 17, 20, 75)*. It has been reported that there are random deletions of certain segments of chromosomes in the *P. falciparum* genome; these two genes are located in such hotspots and become targets of such random deletions *(76)*. A recent analysis of publicly available genomic sequences suggests that duplication-mediated interchromosomal translocation in these chromosomal segments plays a key role in *pfhrp3* deletions in Africa and South America *(77)*.

3.3.2 Transmissibility of *pfhrp2/3*-deleted parasites

There are no conclusive data on the transmissibility of *pfhrp2/3*-deleted variants, compared to that of wild-type parasites; however, these variants are clearly transmitted from person to person and may be responsible for epidemics 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 the area had been malaria-free for the preceding four years *(24)*. Genotyping of 188 *P. falciparum* strains with *pfhrp2* deletions collected over seven years in areas of Peru with ongoing transmission showed increasing clonal diversity, with clear evidence of the evolution of new strains carrying deletions *(17)*. A subsequent longitudinal study conducting temporal analysis of *pfhrp2* deletions in Peru from 2011 to 2018 showed continued expansion of parasites with dual deletions of *pfhrp2* and *pfhrp3 (42)*. Overall, *pfhrp2* and *pfhrp3* dual gene deletions were detected in 67% of parasite samples. Prevalence of gene deletions varied across study sites. There was a trend towards an overall increase in the prevalence of dual *pfhrp2/3*-deleted parasites between 2011 (14.3%) and 2016 (88.39%), stabilizing at around 65% in 2018. A single clonal lineage (H8) contributed to the expansion and spread of dual-deleted parasites. Interestingly, participants infected with dual *pfhrp2/3*-deleted parasites had significantly lower parasitaemia than those without gene deletions in this cohort, suggesting that there may be a potential fitness cost for *pfhrp2/3*-deleted parasites. As expansion of double-deleted parasites occurred in the absence of RDT pressure, it appears that other factors linked to the *pfhrp2/3* deletions provide a selective advantage over non-deleted strains. Future studies are needed to better understand the role of other biological/environmental factors driving the selection of deleted parasites in the absence of RDT pressure.

3.3.3 Deteminants of spread of *pfhrp2/3*-deleted parasites

As pointed out earlier, the specific factors driving the evolution and spread of *pfhrp2/3*-deleted variants are not clear, although it is reasonable to consider that selective pressure from HRP2 detection plays an important role; however, this is not the only factor. Data from a DHS in the Democratic Republic of the Congo suggest a link between a higher prevalence of *pfhrp2* deletions and earlier introduction of HRP2-based RDTs *(35)*. It is unclear whether the strictness of adherence to diagnostic results in providing therapy helps to drive the emergence of deleted variants; however, the predominance of these strains in Eritrea, where NMP guidelines are followed closely, is noteworthy. In two studies, mathematical models were used to characterize the effect of introducing HRP2-based RDTs on the emergence and spread of *pfhrp2/3*-deleted variants *(78, 79)*. These models show that there is a high potential for *pfhrp2*-deleted parasites to spread through a community when the detection of *P. falciparum* malaria relies solely on HRP2-based RDTs. Although further studies in different endemic settings are needed to test the role of HRP2-based RDT testing in driving the selection of *pfhrp2/3*-deleted parasites, it is worth pointing out observations from recent studies *(43, 80)*. In Eritrea, two years after HRP2-based RDTs were replaced with non-HRP2-based RDTs, the prevalence of *pfhrp2*-deleted parasites was significantly lower at multiple survey sites than at the original sites. This finding suggests that the removal of selective pressure caused by HRP2-based RDTs may have contributed to a decline in *pfhrp2*-deleted parasites. Consistent with this hypothesis, the genetic diversity of the gene-deleted parasites increased after the change of RDTs. In Ethiopia, molecular inversion probe sequencing was used to identify genetic signatures of recent evolutionary selection favouring the expansion of *pfhrp2*-deleted parasites *(30)*. A follow-up study *(80)* assessed the genetic background of *pfhrp2/3*-deleted and non-deleted parasites from three regions. The findings revealed that the majority of *pfhrp2/3* deletions occurred in monogenomic infections and such parasites were highly related, with evidence of multiple emergences and clonal spread in specific localities. At the same time, *pfhrp2/3* non-deleted parasitic infections were more often polygenomic and were less closely related. These findings suggest that HRP2-based RDTs may be driving some genetic selection in this population. However, there is no conclusive empirical evidence that the choice of RDT influences the prevalence of *pfhrp2/3*-deleted variants in a community.

Some clinical evidence suggests that *pfhrp2/3*-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 *(35, 81)*. Although studies with cultured parasites are inadequate for drawing precise conclusions about the fitness of *pfhrp2/3*-deleted variants, one study suggested that these strains showed reduced fitness in vitro *(82)*. A recent study directly compared the fitness costs of *pfhrp2* and *pfhrp2/3* gene deletions by removing these genes in an African parasite and comparing the parasite's growth characteristics in in vitro cultures *(83)*. This study found that these gene deletions incurred fitness costs in vitro compared to wild-type parasites, a finding that would not favour the selection advantage of *pfhrp2*- or *pfhrp2/3*-deleted parasites without the use of RDTs. Although such a hypothesis remains to be validated in natural populations, which are subject to numerous other selective pressures, including interactions with antimalarial drug resistance *(84)*, the data from this study can be used to improve modelling studies to predict the impact of *pfhrp2/3* deletions on selection and spread.

3.3.4 Detection and surveillance of *pfhrp2/3*-deleted parasites

In addition to research meant to understand the factors that drive the evolution and spread of *pfhrp2/3*-deleted variants, 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 variant strains is complex and requires some clinical research infrastructure and sophisticated confirmatory testing. Surrogate markers that are easier to use are needed. For example, there is as yet no information compiled on the predictive value of suspected false-negative HRP2-based RDT results

for gene deletions in different settings. Even if the predictive value were relatively low, if there was a fixed difference between RDT and molecular results in given settings, it could serve as a useful marker to track trends.

In recent years, there have been significant improvements in molecular methods and immunoassays for characterization of *pfhrp2/3* deletions and detection of HRP2 protein. These developments are summarized in a recent study outlining the various molecular and immunological assays available and their advantages and limitations for supporting *pfhrp2* deletion surveillance *(41)*. Significant improvements in molecular methods include the development of multiplex real-time PCR methods *(85–87)* and digital PCR *(88)* for the detection of *pfhrp2* deletions. Multiplex real-time PCR assays are high-throughput assays that utilize probes for detecting PCR-amplified products, although the target regions for PCR and internal reference controls vary. Unlike initially developed conventional PCR methods, these assays can detect *pfhrp2/3* deletions in multiclonal infections. Digital PCR is one of the most recently developed methods for accurate detection of *pfhrp2/3* deletions in multiclonal infections *(88)*. In this method, the reaction volume is partitioned into approximately 15 000 droplets/partitions, and each droplet functions as an individual PCR reaction. Accordingly, this assay enables precise quantitative measures of deleted parasites in multiclonal infections. In this respect, digital PCR is more precise than real-time PCR methods for quantifying deletions in multiclonal infections. Digital PCR does not require external standards and is not affected by amplification efficiency. However, the initial cost of establishing this assay is higher than for real-time PCR, and it is less commonly available in African countries. However, with the help of international donors, some malaria-endemic countries have developed capacity for this assay.

Whole genome sequencing (WGS) has been used to characterize *pfhrp2/3* gene deletions, most commonly involving short sequencing reads *(89)*. This approach requires well developed bioinformatic pipelines for assembling sequencing reads to determine genetic sequences. Since *pfhrp2* and *pfhrp3* genes have highly repetitive genome sequences that are variable between different strains, assembly of these sequences requires considerable bioinformatics expertise. In this context, long-read sequencing platforms may offer an alternative approach for correctly assembling sequences and overcoming some of the challenges associated with short-read sequences. WGS methods are useful for determining the exact location of deletion points within chromosomes, determining the genetic diversity in populations, and understanding evolutionary patterns of deletion. However, WGS methods are not cost-effective for determining *pfhrp2/3* deletions compared to PCR methods and are more suitable for research laboratories. Recently, an amplicon-based deep sequencing method has been developed, which uses molecular inversion probes to amplify hundreds of targets, including *pfhrp2/3* genes and their flanking regions *(30)*. This method has been successfully used for a large *pfhrp2* deletion survey in Ethiopia. It is a cost-effective and high-throughput alternative to WGS that enables evolutionary analysis using genetic signatures of the parasite population. In general, long-read and short-read amplicon deep sequencing and WGS have limitations, as both depend on read coverage to determine the absence of *pfhrp2/3* genes. It is well known that read coverage can also be affected by factors other than deletions. Amplicon deep sequencing tools should include steps to ensure that the PCR-negative results are true negatives before proceeding to the sequencing steps. Careful optimization of sequencing and bioinformatic analysis will be required for collecting reliable data. These new technologies should be rigorously validated under both laboratory and field conditions before they are deployed as tools to aid in changing policies.

Targeted nanopore sequencing using MinION *(90)* offers a portable sequencing platform that can be relatively easier to use in developing countries and can be less expensive than WGS. This mobile technology is promising for use in gene deletion investigations. A recent study reported the development of a targeted genome

sequencing protocol for amplification of *pfhrp2/3* genes along with some candidate vaccine antigens, using specimens stored in dried blood spots *(91)*. This study reported performance of this method using mock-prepared known laboratory strains and field-collected specimens from Zambia. This was an initial proof of concept demonstration study and the current limitations of this technology were acknowledged, namely that specimens with high-density parasitaemia (> 1000 parasites/µL) are required and low-quality reads are generated for *pfhrp3*. In another study, portable MinION was used to characterize *pfhrp2* sequences, using artemisinin-resistant genetic marker Kelch 13 as a control *(92)*. Sequencing of *pfhrp2* was successful in 93 out of 152 field samples. A subset of these specimens were compared using long-read sequencing technology and there was perfect concordance. Nevertheless, there were certain limitations identified, including quantitative ambiguity, *pfhrp2*-specific issues that affected the consistency and degree of barcode performance, and the impact of low quality on the de novo assemblies. As a positive development, an open-source software has been described and standardized protocols openly shared. One particular advantage of this portable platform is that it can provide real-time results and has the potential for use in remote settings if basic molecular laboratory capacity is available. Targeted nanopore sequencing may be a solution for countries/regions from which specimens cannot be shipped for deletion investigation and could reduce time delays in such investigations.

High-throughput assays that can detect malaria parasite antigens in blood specimens, including dried blood spots, are available for laboratory use and investigation of *pfhrp2* deletions. These include ELISA, chemiluminescent assays and bead-based assays. Among these methods, the multiplex bead-based assay has been used in multiple surveys for *pfhrp2* deletion analysis *(93)*. This approach has been used to create assays that can detect the HRP2 protein at sub-picogram levels and can be used for moderate- to high-throughput testing *(94)*. This assay has been used as an alternative to PCR to confirm RDT results, as well as to validate the absence of HRP2 during *pfhrp2* deletion investigation studies and surveys *(93)*. This method is cost-effective, highly sensitive and quantitative. Currently, this method is used in selected laboratories, including in Africa, and antibody-coated beads are made in selected laboratories. When these beads become commercially available, this method can be extended broadly and quality standards can be implemented to compare results between laboratories.

As there is an increased need for conducting surveillance for *pfhrp2/3* gene deletions, it is important to enhance laboratory capacity in malaria-endemic countries. The COVID-19 pandemic and Ebola virus disease epidemic response efforts have highlighted the need to build laboratory capacity that can broadly support all infectious disease related threats and response. International donors and multinational agencies are recognizing the importance of cross-cutting investments in public health laboratory capacity, data management, and implementation of quality management programmes and standards. The Bill & Melinda Gates Foundation has made significant investments in building laboratory capacity for malaria surveillance and monitoring of *pfhrp2/3* deletions and drug resistance through large grants to African countries. The Bill & Melinda Gates Foundation and other donors are also making significant investments in building genomics laboratory capacity across Africa in collaboration with the Africa Centres for Disease Control and Prevention and multiple national research establishments. These investments have led to collaboration between established international laboratories and laboratories in developing countries for the transfer of technical capacity and training. Practical considerations for reference laboratories, testing requirements and validation of test results related to HRP2 surveys have been summarized in a recent review *(41)*. WHO offers a malaria NAAT EQA scheme that enables implementation and monitoring of molecular assay quality for diagnosis and provides well characterized *Plasmodium* panels, including panels of *pfhrp2/3*-deleted parasites *(67, 68, 95)*.

3.4 Diagnostics research and development

3.4.1 Alternative biomarkers of *P. falciparum* malaria

The occurrence of strains that do not express HRP2/3 increases the likelihood that some infected patients will be missed by conventional RDT testing. 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 using RDTs or ELISA were identified during research, including vaccine development, that was not intended to develop antigen immunocapture assays, and there has been little work focusing on this area in recent years.

Glutamate dehydrogenase, a cytosolic protein of *P. falciparum*, was an early target for malaria antigen detection *(96, 97)*, but was never used in a commercialized assay. Interest in the histidine-rich family of proteins of *P. falciparum* grew from the finding that knob-associated histidine-rich protein, also known as HRP1, was an important protein in knob formation on erythrocytes, a virulence characteristic of *P. falciparum (98)*. The finding that HRP2 was secreted, abundant and antigenic, indicated its possible utility as a diagnostic target *(99)*. HRP2 was first reported to be detectable (byELISA) in the plasma of malaria patients in 1991 *(100)*, and by 1993, a lateral-flow immunochromatographic assay suitable for field use had been developed *(7, 101)*.

pLDH 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 *(102)*.

Other *Plasmodium* proteins, such as dihydrofolate reductase–thymidylate synthase, haem detoxification protein, glutamate-rich protein *(103)* and glyceraldehyde-3-phosphate dehydrogenase *(104)*, have been studied for their diagnostic potential but never used in a commercialized assay. A recent quantitative study of the proteins expressed during the intraerythrocytic development cycle of *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 *(105)*. Using proteomic analysis of saliva samples from *P. falciparum*-infected African patients, 35 potential protein targets for use in saliva-based RDTs have been identified *(106)*. One of these markers, a gametocyte-specific marker called PSSP17, has been further characterized as a suitable target for identification of gametocytes in saliva samples and further development as a prototype RDT in collaboration with commercial partners. It remains to be determined if any of the other 35 markers identified in this study can become useful targets for commercial RDTs using saliva or blood as biological specimens. Field evaluation of a new magneto-optical device using haemozoin as a target for diagnosis of malaria has shown it to be comparable to microscopy and RDTs *(107)*. This assay platform uses a battery-operated device with digital interface for recording and reporting. In a Brazilian study, investigators identified a unique *P. vivax* protein (Vir14) by conducting proteomic analysis of urine samples from patients infected with *P. vivax*. This protein was not found in other human *Plasmodium* species and the authors proposed that it could be used for developing a *P. vivax*-specific RDT *(108)*. These investigators have reported that they are exploring partnerships with RDT manufacturers for developing a new *P. vivax*-specific RDT.

3.4.2 Optimization of RDTs targeting Pf-LDH

An additional strategy warranting exploration is the 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, heat stability and specificity to existing targets that can be species-specific) would also be valuable. In the short term, perhaps the most pressing need is for non-HRP2-based RDTs targeting Pf-LDH or another antigen that are more sensitive and heat-stable than the non-HRP2-based tests currently available. In terms of analytical sensitivity, there is roughly a 10-fold gap between the detection capacities of HRP2 and pLDH assays. A more sensitive pLDH assay, especially one that targets Pf-LDH, would have great benefits. Most importantly, it would enable countries to phase out HRP2-based assays if *pfhrp2/3*-deleted variants reached important thresholds and replace those tests with assays of comparable performance. Countries where *P. falciparum* is prevalent that wish to keep testing and result recording as simple as possible could use tests with a single test band that bears both antigens, without fear of missing cases carrying deleted variants or low-density infections. There are several new RDT products that have been submitted for WHO prequalification that are appropriate for use in areas affected by *pfhrp2/3* deletions (Table 3), with more in the pipeline.

Unfortunately, RDT manufacturers are working within very tight profit margins. Despite this, WHO is aware of at least three manufacturers that are developing new Pf-LDH-based RDTs. If these tests meet the improved sensitivity threshold, they will be valuable for use in areas where HRP2-based RDTs are no longer suitable. 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. Policy-makers and independent donors should consider innovative ways to fund the science that is most urgently required to meet public health goals in the short term. It is discouraging that 2021 saw the lowest level of total malaria research funding support allocated to diagnostics research since 2013, as outlined in the *World malaria report 2022 (109)*. Diagnostics received only 2.5% of total malaria funding (US$ 16 million out of US$ 626 million spent on research), representing a nearly 50% reduction from peak funding support for diagnostics research between 2017 and 2019 (US$ 30 million).

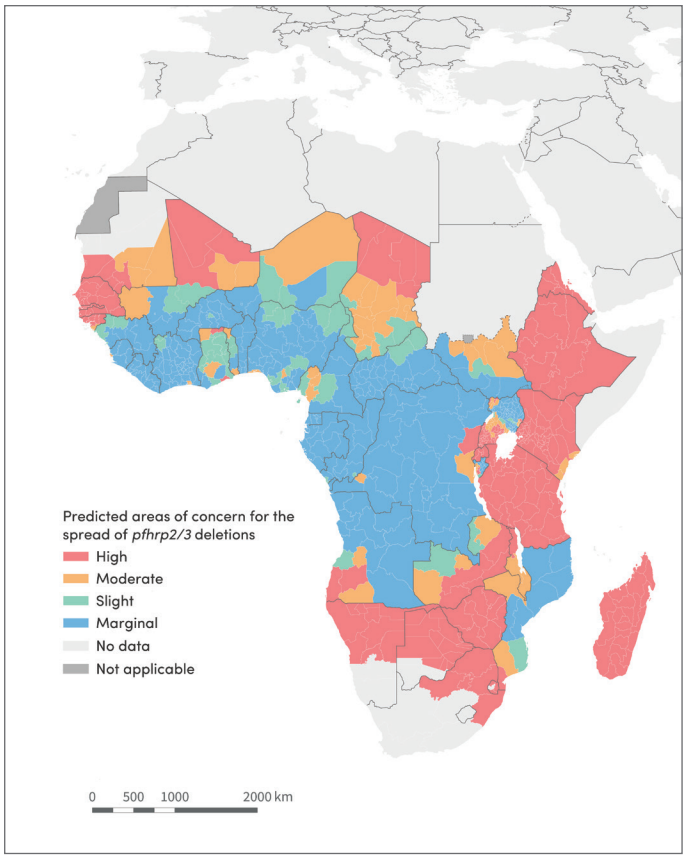
Since 2016, WHO has required companies that manufacture malaria RDTs to submit their products for assessment by the WHO prequalification of in vitro diagnostics programme. The WHO and FIND RDT evaluation programme did not include *pfhrp2/3*-deleted variants in the cultured or clinically collected reference specimens until round 8, and this inclusion of *pfhrp2/3*-deleted variants was continued under WHO prequalification independent laboratory evaluation procedures. There is an urgent need to collect additional *pfhrp2/3*-deleted parasite panels from African countries for use in the RDT evaluation programme, as the deleted panels currently available are cultured or from South America. Availability of a robust set of deleted parasite panels from different parts of Africa will be essential for conducting more stringent RDT evaluations. Continuous dialogue among manufacturers, WHO and procurement agencies is necessary to ensure that NMPs can procure products with performance that they can continue to rely on in a timely manner.

3.5 Modelling for future planning

3.5.1 Global risk of selection using an interactive *pfhrp2/3* risk explorer

The likely timeline for countries to transition away from the use of RDTs that rely solely on HRP2 for the detection of *P. falciparum* towards alternative diagnostic methods is dependent upon both epidemiological and economic factors. These factors include malaria prevalence, the prevalence of *P. vivax* malaria, the size of the private drug market, current treatment-seeking rates and the cost of alternative RDTs. A number of countries have already transitioned away from sole reliance on HRP2 to detect *P. falciparum*, and further modelling *(110)* has provided insight into which countries in each WHO region have the highest risk of *pfhrp2/3* deletions becoming established due to selective pressure from HRP2-based RDTs. Modelling suggests that in low transmission settings of the WHO African Region, those countries with high treatment-seeking rates, high levels of testing, and high adherence to RDT outcomes are at the highest risk of *pfhrp2/3* deletions (Fig. 4). An interactive application has been developed to enable users to vary these factors and generate maps accordingly (https://worldhealthorg.shinyapps.io/DeletionRiskExplorer/).

Fig. 4. Predicted areas of concern from the spread of *pfhrp2/3* deletions



Note. Once deletions have become established in a region (defined as 1% of clinical cases missed due to *pfhrp2/3* deletions), regions are classified by how quickly deletions will continue to increase. High, moderate and slight risk represents > 5% of clinical cases being misdiagnosed due to *pfhrp2/3* deletions in six, 12 and 20 years, respectively, and marginal risk represents less than 5% of cases by 2040.

Estimating the timeline for RDT transition is hindered by uncertainties in critical parameters, namely malaria prevalence, the coverage of treatment and testing, the prevalence and fitness phenotype of *pfhrp2/3*-deleted parasites, and the speed at which deleted parasites migrate between countries (Table 7). Additional research is needed to refine our understanding of the effect size associated with each of these factors on selection for *pfhrp2/3* deletions, and an improved understanding of these

factors will enhance modelling estimates for when countries may need to transition away from sole reliance on HRP2-based RDTs. Central to this is the need for routine genetic surveillance, which both serves to provide early warning of *pfhrp2/3* deletions and enables the rate of spread of *pfhrp2/3* deletions to be estimated.

Despite these uncertainties, modelling can still provide insight into possible timelines for countries to switch RDTs. These scenario modelling exercises project how *pfhrp2/3* deletions may continue to spread in Africa based on the current understanding of the factors associated with *pfhrp2/3* selection and spread. Modelling has identified 10 countries at the highest risk of deletions spreading there and subsequently being rapidly selected for; these countries are projected to reach the 5% threshold within the next six years. Of these, three have already switched and three other countries (Kenya, Senegal and Zambia) may need to consider switching RDTs in response to current trends in the spread of *pfhrp2/3* deletions. While countries with low malaria prevalence, such as most endemic countries in the Americas and South Asia, are expected to experience quick selection for *pfhrp2/3* deletions, there is considerable uncertainty as to whether imported deletions will become established following introduction due to the inherent transmission stochasticity in low-prevalence settings. Furthermore, while numerous African countries with high malaria prevalence are currently at low risk of selection for *pfhrp2/3* deletions, this assumes that conditions will remain unchanged; deletions may be rapidly selected for if malaria control initiatives reduce prevalence. Accordingly, this modelling emphasizes the need for regular generation of *pfhrp2/3* risk maps in response to updated malaria prevalence and treatment estimates.

The threat of *pfhrp2/3* deletions has significant implications for the continued use of effective RDTs, necessitating an increase in the research and development of RDTs that target alternative antigens alone or in combination with HRP2, as well as alternative diagnostic methods over the next 15 years. While the available data are insufficient to provide accurate timelines for *pfhrp2/3* deletion spread, modelling underscores the need for more data on malaria prevalence and treatment coverage, as well as longitudinal surveys to refine estimates of the effective fitness costs associated with *pfhrp2/3* deletions. It is highly likely that many countries will need to switch from HRP2-based RDTs, highlighting the need for alternative RDTs to be available at scale in the future. The modelling serves the needs of countries, donors and test developers in different ways. For countries, it enables them to assess their risk and prioritize surveillance accordingly; for donors, it supports procurement planning; and for manufacturers, it enables them to predict where market needs are likely to shift and the approximate timeline in order to support their research and development planning.

Table 6. Key drivers of pfhrp2/3 selection and their impact on the speed of selection

Drivers of *pfhrp2/3* selection	Impact on speed of selection for *pfhrp2/3* deletions	Data sources
Malaria prevalence	Lower prevalence will increase selection by increasing the likelihood that individuals will be infected by only *pfhrp2/3*-deleted parasites and will be less likely treated because of a false-negative HRP2 RDT result.	Malaria Atlas Project maps of blood slide positivity for ages 2 to 10 ($PfPR_{2-10}$) *(111)*
Microscopy-based diagnosis	The use of microscopy for malaria diagnosis will negate the advantage conferred by *pfhrp2/3* deletions.	WHO World Malaria Report "proportion of cases confirmed by diagnostic" table
Treatment-seeking rate for fever	Increased treatment seeking and testing with HRP2-based RDTs (which is desirable) will actually increase the rate at which the selective advantage conferred by *pfhrp2/3* is realized by these parasites evading diagnosis and treatment.	Commodities forecast dashboard by the Malaria Atlas Project (case management commodities) *(112)*, which uses DHS, MIS, Multiple Indicator Cluster Surveys and AIDS Indicator Surveys in a generalized additive mixed model to predict treatment-seeking patterns over time
Proportion of treatment seeking for fever in the private sector	Low use of malaria RDTs has been shown to exist in the private market in a number of locations *(113)*. If the use of RDTs is lower in the private market than in the public sector, then selective pressure will decrease with an increasingly large private drug market.	DHS/MIS used in a generalized additive mixed model for estimating treatment seeking from any (medical) source and for estimating treatment seeking in the public sector
Proportion of individuals seeking care who receive diagnostic test	Low use of any diagnostic test for guiding treatment decisions will reduce selective pressure for *pfhrp2/3* deletions.	DHS data (surveys in Africa asking if care-seeking febrile children received a finger/heel prick)
Non-adherence to RDT outcomes	Increased non-adherence (which is not desirable) to RDT outcomes (i.e. treating an RDT-negative individual) will negate the selective advantage of *pfhrp2/3* deletions.	Commodities forecast dashboard by the Malaria Atlas Project (case management commodities) *(112)*, which uses a statistical model of the probability of care-seeking fevers receiving any antimalarial, informed by DHS and MIS data
RDT brands	The use of non-HRP2-based RDTs will negate the selective advantage conferred by *pfhrp2/3* deletions.	Global Fund price and quality reporting and U.S. President's Malaria Initiative data on volumes of RDT types and brands used
Cross-reactivity of HRP3 epitopes	HRP3 is known to cross-react and may yield a positive HRP2-based RDT result, even if *pfhrp2* is deleted, which will decrease the selective advantage.	Estimate based on WHO Malaria Threats Map data *(21)* and studies reporting the performance of HRP2-based RDTs on *pfhrp2-/pfhrp3+* samples *(30, 54, 114)*
Fitness costs associated with *pfhrp2/3* gene deletions	Fitness costs associated with *pfhrp2/3* gene deletions will reduce the transmissibility of gene-deleted parasites; therefore, low fitness is a selective disadvantage.	Based on an in vitro laboratory assessment of fitness *(83)* and *pfhrp2/3* surveillance data from Eritrea and Ethiopia

3.5.2 Market size projections

For NMPs, donors and manufacturers to optimally plan for malaria RDT research and development investments, manufacturing and procurement needs, it is important to know how quickly the need for alternative RDTs that are not solely reliant on HRP2 for *P. falciparum* diagnosis might arise over the next 5–10 years, and what volumes of these alternative RDTs may be needed.

To create a demand forecast for alternative malaria RDTs, WHO commissioned a prospective risk analysis to estimate the time taken for each first-level administrative unit (i.e. admin-1 unit – generally corresponding to provinces within a country) to reach the WHO-defined threshold of 5% false-negative RDT results caused by *pfhrp2/3* gene deletions (explained above). The risk analysis estimated three scenarios – "best", "central" and "worst" – representing the outer ranges of the set of parameters used for the modelling; each scenario represents, respectively, the slowest, central and fastest speeds of *pfhrp2/3* selection and therefore of the admin-1 unit reaching the 5% threshold. A complete overview of the analysis and methods can be found in Watson et al. (unpublished data repository, 2023; https://github.com/OJWatson/hrpup/tree/main/analysis/data_out).

WHO then commissioned the Clinton Health Access Initiative (CHAI) to combine the outputs of the "central" scenario analysis with the forecasted public-sector malaria RDT volume estimates produced by CHAI.[3] A forecast for alternative RDT demand was created under the assumption that countries would begin to switch to Pf-LDH+/-HRP2 RDTs once 10% of their admin-1 units reached 5% false-negative RDT results caused by *pfhrp2/3* deletions. For countries deciding to switch, it was assumed that 50% of their forecasted public-sector RDT volumes would switch to the alternative RDTs in the first year after making the decision, and 100% in the second year onwards. This is because malaria RDT procurement is typically conducted through annual tenders for a specific RDT type and awarded to a single manufacturer *(116)*.

Fig. 5 provides the forecast for alternative RDTs needed in countries that i) are predicted to already need to switch to alternative RDTs and ii) are expected to need to switch to alternative RDTs within the next 5–10 years under the "central" scenario, assuming that countries will switch when at least 10% of their admin-1 units reach the 5% threshold. Fig. 5a shows which countries are likely to switch under this scenario, and Fig. 5b provides the demand forecast for RDTs.

3 CHAI's forecasted volumes of malaria RDT demand in the public sector span the years 2023–2032 and consist of short-term procurement forecast volumes (2023–2025) projected to 2032 based on country-level trends in malaria testing. The short-term procurement volume projections are calculated by trending out future country disbursements for RDTs based on historical disbursements, then dividing by projected average prices per RDT per country. The data for these come from Global Fund grant allocations and commitments data and U.S. President's Malaria Initiative country funding data; more details on these methods are available *(115)*. The short-term procurement volumes are projected into long-term forecasts based on population growth and trends in malaria testing in the public sector estimated by the Malaria Atlas Project. It was assumed that the total RDT volumes procured per country would change in line with estimates of country-level changes in the number of public-sector malaria tests consumed, taking into account country-specific estimates for all-cause fever prevalence, public sector care-seeking rates and public sector testing rates through 2032. These estimates can be found in the Malaria Atlas Project database *(112)*.

Fig. 5. Demand forecast for alternative RDTs by country and by year

Fig. 5a: Countries expected to reach 5% threshold in at least 10% of their admin-1 units by 2023, 2028, 2033 or later

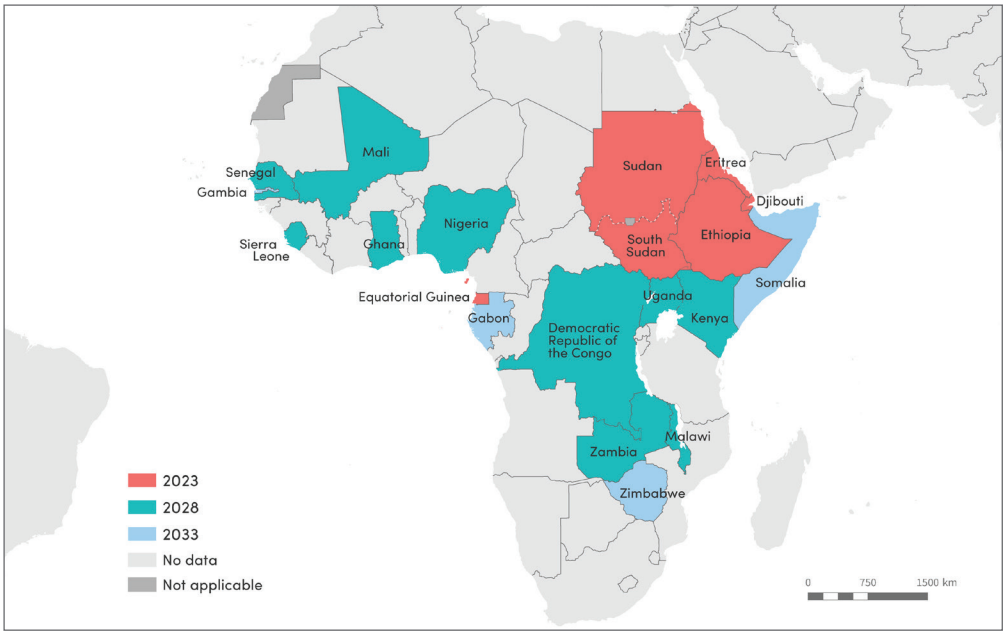
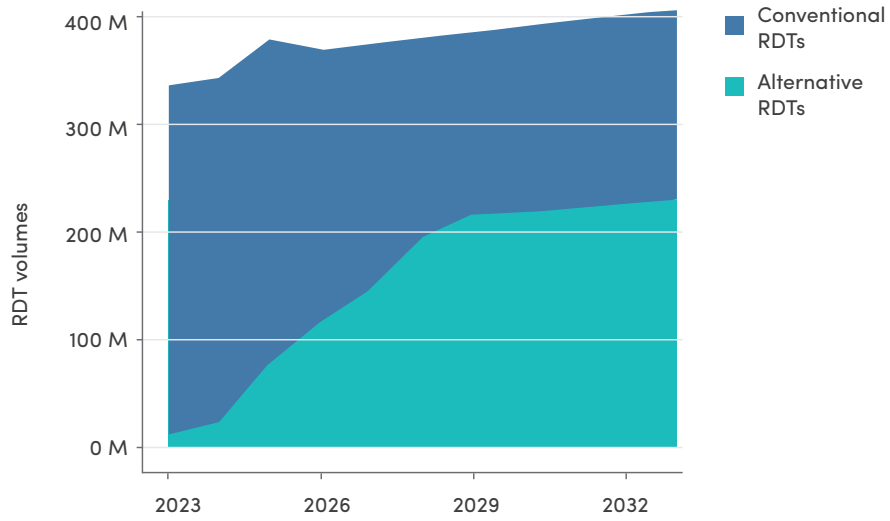


Fig. 5b: Global demand forecast for alternative RDTs if countries that reach 5% threshold in at least 10% of their admin-1 areas switch



Certain limitations of this model must be acknowledged. The forecasting exercise was conducted for Africa only, as no other regions are expected to reach the threshold of 5% false-negative RDT results caused by *pfhrp2/3* gene deletions within the next 40 years, according to even the most pessimistic modelling scenarios. The forecasted volumes of RDTs are limited to the public sector only, given the relatively small volumes of RDTs in the private sector and the uncertainty of how quickly switching may occur in private-sector settings.

3.6 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 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. Currently, the role of WHO in this response plan is limited to the following:

- conducting ongoing global mapping of data from prevalence surveys available through the WHO Malaria Threats Map *(21)*;
- nominating and funding an expanded network of reference laboratories;
- providing centralized procurement assistance to countries that are changing RDTs, especially in the Horn of Africa, which is an important need at this time;
- conducting risk-based transition planning to alternative RDTs;
- harmonizing survey protocols through the establishment and update of survey templates;
- facilitating and assisting NMPs to conduct surveillance surveys; and
- facilitating WHO prequalification of new products.

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 that threatens the utility of a critical weapon in the fight against malaria. Ever since this threat was recognized, WHO has taken action to mitigate this threat, issuing guidance on accurate reporting of *pfhrp2/3* deletions, issuing information notes and launching the Malaria Threats *Map (21)* in 2017. These efforts were followed by a global response plan and establishment of a reference laboratory network in 2019, and publication of *pfhrp2/3* gene deletion surveillance and biobanking protocol templates in 2020, both of which were updated in 2024 *(50, 51)*.

The full extent of that threat is still being investigated. It is already a matter of urgent concern, especially in the disparate regions of the Amazon basin and countries in the Horn of Africa, where a > 5% prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* deletions is forcing changes in diagnostic strategy. However, the choices for replacing HRP2-based RDTs are limited. Several new RDTs have been submitted for WHO prequalification evaluation, and at least three tests that include Pf-LDH lines have passed the laboratory evaluation component. The most critical timely need is for alternative RDTs to achieve WHO prequalification and be rapidly released for diagnostic use.

Managing the response will require needs-based prioritization and risk-based transitioning. It would be counterproductive to attempt to change diagnostic test selection across Africa simultaneously. National and global responses must identify hot spots and balance the risk of missed cases of falciparum malaria due to *pfhrp2/3*-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. Currently, the WHO threshold for a change to non-HRP2 test is warranted, when *pfhrp2/3* deletions lead to > 5% false-negative HRP2 RDT results in a country.

As new RDTs become available, attention needs to focus on considerations, such as acceptance of new product lines in the real world; how the need to change RDTs due to diagnostic inaccuracies of existing tests impacts confidence in RDTs as reliable diagnostic tools among health care workers and communities; supply chain management and security; stability of new products; and price changes and their impact on the affordability of new tests by public health programmes.

Several types of work must continue and are urgently needed:

- Map the distribution and frequency of *pfhrp2/3*-deleted variants with harmonized, high-quality protocols. In the past, many studies have used non-WHO-recommended survey protocol approaches; this has not led to nationally representative data collection and must be discouraged in the future.
- Build and expand the international network of laboratories to perform the complex molecular confirmation required for mapping, with adequate funding.
- Encourage all laboratories conducting molecular assays in endemic countries to participate in the WHO malaria NAAT EQA scheme.
- Continue supporting countries in the selection and procurement of new RDTs when a change of testing is warranted.
- Advise commercial manufacturers of the priorities for new tests, and provide and update the best available market forecasts.

- Include *pfhrp2/3*-deleted parasites of clinical samples from different countries in Africa in the evaluation panel for conducting stringent evaluation of new non-HRP2-based RDTs, as only a limited number of *pfhrp2/3*-deleted parasites are available as reference panels, mainly from cultured parasites or from one geographical region.

- Work with donor agencies (at a time when overall investment in diagnostic product development is at its lowest level) and research institutes to devise a funding plan to support i) the interim costs for prevalence surveys and the necessary molecular and other laboratory testing, and ii) the search for improved diagnostic targets and high-affinity reagents, and facilitate new sustainable product development opportunities using a fast-track approach.

- Improve local communication and training for health care professionals, laboratory staff, policy-makers and community stakeholders when implementing the switch from HRP2-based to non-HRP2-based RDTs.

- Facilitate deployment of a new generation of point-of-care tests that use different sample types (saliva or other biological fluids), non-invasive tests and other test platforms for point-of-care diagnosis.

- Strengthen coordination among policy-makers, NMPs 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 NMPs and the populations they serve will require a focused, staffed and budgeted effort, and a mechanism for programme management.

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