



False-negative RDT results and implications of new reports of *P. falciparum* histidine-rich protein 2/3 gene deletions

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INFORMATION NOTE

TARGET AUDIENCE

National malaria control programme (NMCP) managers and their implementing partners, procurement agencies, national regulatory authorities for in-vitro diagnostics and manufacturers of malaria rapid diagnostic tests (RDTs).

PURPOSE

To provide information on the implications of recent reports of histidine-rich protein 2/3 (*pfhrp2/pfhrp3*) gene deletions in *P. falciparum* parasites for case management in Africa and to advise on procedures for investigating suspected false-negative RDT results.

BACKGROUND

Most of the currently available commercial RDT kits work by detecting a specific protein expressed only by *P. falciparum*, called HRP2, in the blood of people infected with falciparum malaria. The antibodies on the test strip recognize the PfHRP2 antigen but may cross-react with another member of the HRP gene family, *pfhrp3*, due to strong similarity of the amino acid sequence. The general preference for Pf-HRP2-based RDTs in procurement is due largely to the finding in some studies that they are more sensitive and heat-stable than RDTs that detect other malaria antigens, such as plasmodium lactate dehydrogenase (pLDH) – pan (all species) or *P. falciparum*-specific – or aldolase.

It has been shown that, in certain situations, HRP2-detecting tests are less sensitive, particularly for parasites that express little or no target antigen, resulting in a false-negative result. In 2010, Gamboa et al.¹ reported the first confirmed identification of *P. falciparum* parasites with *pfhrp2/pfhrp3* gene deletions, not expressing PfHRP2 and/or PfHRP3, in the Amazon River basin in Peru. Subsequent retrospective analyses² at different sites in the Loreto region of the Peruvian Amazon showed a statistically significant increase in the number (and percentage) of parasites with gene deletions between specimens collected in 1998–2001 (20.7%) and in 2003–2005 (40.6%). The prevalence of parasites with *hrp2/hrp3* gene deletions varies, however, from locality to locality. Publications followed from other countries, such as India, Mali and Senegal, but with much lower prevalence estimates, and some studies were based on a flawed design and/or had incomplete analyses.³ There have been no reports of parasites failing to express pLDH or aldolase, the other antigens targeted by malaria RDTs, as these targets are essential enzymes for parasite metabolism and survival.

In light of pending and recent reports of HRP2 deletions in parasites in at least four African countries, including Eritrea and Ghana, WHO is providing guidance to RDT manufacturers, procurers, implementers and users on confirming (or excluding) new geographical foci of parasites with deleted *pfhrp2/pfhrp3* and on investigating other causes of suspected false-negative RDT results.

POTENTIAL CAUSES AND INVESTIGATIONS INTO SUSPECTED FALSE-NEGATIVE RDT RESULTS

In most settings, genetic mutations like deletion of *pfhrp2/pfhrp3* in parasites are not likely to be the main cause of false-negative results in RDTs, and more studies are required to determine the true prevalence of these mutations. False-negative RDT results are far more likely to be due to the procurement and use of poor-quality RDTs or use of the wrong comparator for the diagnostic test, such as poor-quality microscopy for cross-checking negative RDT results.⁴ Poor transport and storage conditions for RDTs, with sustained exposure to high temperature, can affect their diagnostic performance. More rarely, operator errors during performance and/or interpretation of RDT results can result in false-negative results. Table 1 lists the range of product, operator, supply chain, host and parasite factors that can lead to false-negative RDT results and also suggested means to investigate such cases. Many of the potential causes of false-negative results can be prevented or minimized by procuring good-quality RDTs, by improving the quality control of procured RDTs (lot verification) and by good training of users.

TABLE 1.

Causes of false-negative RDT results and investigative actions

CLASSIFICATION	CAUSE OF FALSE-NEGATIVE RDT RESULT	SUGGESTED ACTIONS
Operator factors	Operator error in preparing the RDT, performing the test or interpreting the result.	Verify whether RDTs are used by untrained staff; assess RDT competence on site.
Use of an imperfect "gold standard" as a comparator	Thick or thin films from a patient with a negative RDT result are incorrectly interpreted as "positive" by microscopy.	Verify microscopy procedures and interpretation by a qualified microscopist.
Product design or quality	Poor sensitivity of an RDT due to poor specificity, affinity or insufficient quantity of antibodies. Poor packaging can result in exposure to humidity, which will rapidly degrade RDTs.	Inspect the instructions for errors; inspect the integrity of the packaging, including the colour indicator desiccant for evidence of moisture. Cross-check suspected false-negative RDT results against microscopy performed by two qualified microscopists or, if microscopy is not available, against a high-quality non-HRP2-detecting RDT; retrieve RDTs from affected areas, and send for lot testing to WHO- or FIND-recognized laboratories.*
Transport or storage conditions	Poor visibility of test bands due to strong background colour on the test. Incorrect instructions for use.	Assess RDT performance and training on site; if the strong background colour persists, notify the manufacturer. Review the instructions for use for accuracy.
Parasite factors	Antibody degradation due to poor resistance to heat or incorrect transport or storage, e.g. exposure to high temperatures, freeze-thawing. Parasites lack or express low levels of the target antigen, i.e. HRP2.	Inspect temperature monitoring of RDT transport and storage chain to determine whether temperatures exceed maximum storage temperature, typically 30 °C or 40 °C or < 2 °C. If temperatures are not within those in the manufacturers instructions, send the RDTs to the WHO-FIND lot testing laboratory.* Train health workers to respect storage conditions, and improve storage places (e.g. add fans). Patient samples are negative on an HRP2 test line of at least two quality-assured malaria RDTs and positive on the pan- or pf-PLDH test line of a combination RDT and the sample is confirmed to be positive microscopically for <i>P. falciparum</i> by two qualified microscopists. If these conditions are met, place fresh blood samples or dried blood spots (50-60 µl) on Whatman® 3MM filter paper or other collection cards, in frozen storage (-20 °C) until shipment for PCR and <i>pfhrp2/pfhrp3</i> gene analysis.
Host parasite density	Variation in the amino acid sequence of the epitope targeted by the monoclonal antibody. Very low parasite density or target antigen concentration. Very high parasite load (severe malaria) causing prozone effect (hyperparasitaemia and antigen overload).	Repeat test with an RDT of a different brand or different manufacturer that targets the same antigen or an RDT that targets a different antigen, e.g. pan-PLDH or Pf-PLDH. Manufacturers may use monoclonal antibodies that target different epitopes of the same antigen. Perform high-quality microscopy, and record the parasite count; if high-quality microscopy is not available, repeat the RDT if symptoms persist. Repeat testing with a 10 x and if needed a subsequent 50 x dilution of the sample, with dilutions in 0.9% NaCl (Gillet et al. Malar J 2011;10:166).

Note:

* Information about lot testing can be found here: <http://www.who.int/malaria/areas/diagnostic-tests/evaluation-lot-testing/en/>

Thousands of febrile children with negative RDT results have been followed up in several studies,^{5,6} which showed no malaria-related deaths or hospitalizations. In many endemic areas, malaria prevalence rates have fallen to low levels, and the majority of accurately performed RDTs give negative results. Non-compliance with negative RDT results promotes drug resistance, wastes resources and can delay diagnosis of non-malaria causes of fever. In some circumstances, however, false-negative RDT results should be suspected, and an investigation should be carried out to determine the quality of the RDTs, the competence of the operator and/or investigations for *hrp2/hrp3* deletions.

When should false-negative RDT results be suspected in individual patients?

- A symptomatic patient with an initially negative RDT presenting with persistent signs or symptoms of malaria and repeat negative RDT results but a positive blood film interpreted by a qualified microscopist or a positive result with a different quality-assured RDT, which targets a different malaria antigen (e.g. pf-pLDH) or is of the same brand but from a different lot.
- A patient with signs or symptoms of malaria with a negative HRP2-based RDT result, who recently visited an area in which there are known to be *hrp2/hrp3*-deleted parasites.

When should false-negative RDT results be suspected in a population living in a certain geographical area?

- Discordance between RDT and microscopy results, with ≥ 10 –15% higher positivity rates by microscopy where routine quality control by cross-checking is implemented or when both tests are performed on the same individuals (e.g. during surveys).
- The NMCP and/or the RDT manufacturer receives multiple formal complaints or anecdotal evidence of RDTs returning inaccurate results.

WHEN AND HOW SHOULD FALSE-NEGATIVE HRP2-DETECTING RDT RESULTS DUE TO SUSPECTED *PFHRP2* DELETION BE INVESTIGATED?⁷

pfhrp2 deletion should be strongly suspected if a patient sample tests negative on an HRP2 test line of at least two quality-assured malaria RDTs⁸ **and** positive on the pan- or pf-pLDH test line if a combination test is used **and** the sample is confirmed microscopically to be positive for *P. falciparum* by two qualified microscopists.

If a *pfhrp2* gene deletion is suspected and the conditions described above are met:

- Immediately inform the National Malaria Control Programme and WHO.
- Archive the labelled RDTs and slides in a dry, clean area.
- Collect at least 50 μ L of blood (about one drop) onto filter paper (e.g. Whatman® 3MM) or appropriate collection cards optimized for DNA

analysis;⁹ filter paper/cards should be air-dried overnight in a clean environment and sealed in air-tight plastic bags with desiccant.¹⁰

- Confirm the presence of *P. falciparum* infection by PCR analysis according to established protocols and with appropriate standards and quality control measures.
- If PCR is positive, confirm *pfhrp2/hrp3* gene deletion by PCR and antigen analysis at laboratories experienced in this kind of assay. WHO/GMP can facilitate linkages with such laboratories and provide further guidance.

IMPLICATIONS OF PFHRP2/HRP3 MUTATIONS OR DELETIONS FOR PROGRAMMES

Attributing false-negative results to *pfhrp2/pfhrp3* deletion has significant implications for public health. Alternative RDTs will have to be procured, and case management decisions will have to be revised, with re-training in algorithms and RDTs. Therefore, all investigations must be carried out systematically and accurately.

Following confirmation of *pfhrp2* deletions in initial case investigations, blood collection surveys should be made of confirmed *P. falciparum* cases in the specific geographical region to determine the prevalence of parasites carrying gene deletions. Representative samples are required to establish reliable estimates of the prevalence of these parasites.

ALTERNATIVES TO HRP2-BASED RDTs

If *pfhrp2/pfhrp3* deletions are found to be prevalent, as, e.g. in Eritrea and Peru, country programmes will have to switch to RDTs that do not rely exclusively on HRP2 for *P. falciparum* detection. Such a change may be required only in particular areas of a country and not necessarily nationwide.

Table 2 lists RDTs evaluated in the WHO malaria RDT product testing programme¹¹ for the diagnosis of *P. falciparum* malaria by detection of non-HRP2 antigens, namely plasmodium lactate dehydrogenase (pLDH), pan (all species) or *P. falciparum*-specific. The table shows their performance; only products that meet current WHO-recommended procurement criteria are listed. Further details, e.g. on heat stability, false-positive results for non-*P. falciparum* infections and test band intensity, are included in (<http://www.who.int/malaria/publications/atoz/9789241510035/en/>). It is important to recall that the *P. falciparum* culture and clinical samples used in the WHO malaria RDT product testing programme express HRP2. In addition, the performance of products that have HRP2 and pf-pLDH on the same test line cannot be evaluated for both antigens; this can be done only when the two antigens are shown on separate test lines.

Where microscopy is available, the services should be strengthened to ensure that parasitological confirmation of malaria continues during the transition to new RDTs and to support investigations of new foci of suspected *pfhrp2/pfhrp3*-deleted parasites.

TABLE 2

Non-HRP2-based RDT options for detection of *P. falciparum* malaria

PRODUCT	PRODUCT CODE	MANU-FACTURER	PANEL DETECTION SCORE				PANEL DETECTION SCORE				TOTAL FALSE POSITIVE RATES (%)	INVALID RATE (%)	
			200 parasites/ml		2000 or 5000 parasites/ml		200 parasites/ml		2000 or 5000 parasites/ml				
			Pf samples		Pv samples		Pf samples		Pv samples				
		HRP2/ pf-PLDH	HRP2	pf- PLDH	pan- PLDH	HRP2/ pf-PLDH	HRP2	pf- PLDH	pan- PLDH	Clean negative samples	False positive <i>Plasmodium</i> spp. infection		
Pf only													
SD Bioline Malaria Ag P.f (HRP2/ pLDH)	05FK90	Standard Diagnostics, Inc.	NA	87	52	NA	NA	NA	NA	NA	NA	0.0	0.0
CareStart™ Malaria HRP2/ pLDH Pf test	RMPM(U)-XXX7X/ RMPM(U)-XXX9X (old codes: G0181/ G0181-ET)	Access Bio, Inc.	91	NA	NA	NA	NA	NA	NA	NA	NA	0.0	0.0
Pf/pan													
CareStart™ Malaria pLDH 3 Line Test	G0121	Access Bio, Inc.	NA	NA	88.9	NA	91.4	NA	NA	100.0	NA	0.5	0.0
CareStart™ Malaria Screen	G0231	Access Bio, Inc.	86.9	NA	NA	NA	88.6	100.0	NA	100.0	NA	2.5	0.1
Pf and Pf and Pv													
SD Bioline Malaria Ag P.f/P.f/P.v	05FK120	Standard Diagnostics, Inc.	NA	84	36	NA	91.4	98.0	NA	100.0	NA	0.0	0.0
Pan only													
CareStart™ Malaria pLDH (PAN)	G0111	Access Bio, Inc.	NA	NA	NA	84	88.6	NA	NA	99.0	97.1	0.0	0.0
Advantage Pan Malaria Card	IR013025	J. Mitra & Co. Pvt. Ltd.	NA	NA	NA	77	100.0	NA	NA	98.0	100.0	0.4	0.0

INTERIM WHO RECOMMENDATIONS

1. Suspected false-negative RDT results should be investigated.
2. Pf-*hrp2/hrp3* gene deletions should be suspected and the NMCP and WHO informed when:
 - on an individual basis, a patient sample tests negative on the HRP2 test line of at least two quality-assured malaria RDTs **and** positive on the pan- or pf-pLDH test line of a combination RDT **and** the sample is confirmed by microscopy to be positive for *P. falciparum* by two qualified microscopists;
 - on a programmatic basis, the rates of discordance between RDT and microscopy results are systematically $\geq 10\text{--}15\%$, with higher positivity rates with microscopy, where routine quality control is done by cross-checking or both are performed on the same individuals (e.g. during surveys)and/or when the NMCP receives multiple formal complaints or anecdotal evidence of RDTs returning false negative results for *P.falciparum*.
3. Where *hrp2/hrp3* gene deletions have been reported, the prevalence should be determined in the affected country and neighbouring countries. This may require specific surveys or adaptation of planned surveys, such as malaria indicator surveys or therapeutic efficacy studies.
4. Analysis of well-preserved archived specimens may be useful for identifying the existence and geographical location of *hrp2/hrp3*-deleted parasite populations.
5. In the absence of confirmed reports of *hrp2/hrp3* gene deletions, new initiatives to find these gene deletions are not recommended, unless they are prompted by findings for programmes described under 2.

WHO/GMP RESPONSE

Given the complexity of investigating suspected false-negative RDT results and the risk that parasites that do not express HRP2/HRP3 emerge but are not detected, WHO and its collaborating laboratories are conducting the following activities:

- providing technical support to confirm and map HRP2/HRP3-deleted parasites where they are suspected on the basis of discordance between the results of HRP2-RDTs and high-quality microscopy;
- working with relevant groups to adapt planned malaria indicator surveys and demographic and health surveys to include collection of blood samples for molecular testing for malaria, including analysis of *pfhrp2/pfhrp3*. Areas affected by these mutations, including neighbouring countries, will be a priority;
- working with research groups that hold collections of recently archived samples to screen for the presence of HRP2/HRP3-deleted parasites;
- rigorously reviewing manuscripts submitted for publications and published reports of *pfhrp2/pfhrp3* deletions to determine the accuracy of claims; and
- encouraging test developers and RDT manufacturers to improve the performance of pLDH based tests and identify new target antigens.

Endnotes

1. Gamboa D, Ho MF, Bendezu J, Torres K, Chiodoni PL, Barnwell J, et al. A large proportion of *P. falciparum* isolates in the Amazon region of Peru lack *pfhrp2* and *pfhrp3*: implications for malaria rapid diagnostic tests. *PLoS One* 2010;5:e8091
2. Akinyi S, Hayden T, Gamboa D, Torres K, Bendezu J, Abdallah JF, et al. Multiple genetic origins of histidine-rich protein 2 gene deletion in *Plasmodium falciparum* parasites from Peru. *Sci Rep* 2013;3:2797.
3. Cheng Q, Gatton M, Barnwell J, Chiodini P, McCarthy J, Bell D, et al. *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. *Malar J* 2014;13:283.
4. Kahama-Maró J, D'Acremont V, Mtasiwa D, Genton B, Lengeler C. Low quality of routine microscopy for malaria at different levels of the health system in Dar es Salaam. *Malar J* 2011;10:332.
5. Senn N, Rarau P, Manong D, Salib M, Siba P, Robinson, L J et al. Rapid diagnostic test-based management of malaria : an effectiveness study in Papua New Guinean infants with *Plasmodium falciparum* and *Plasmodium vivax* Malaria. *Clinical infectious diseases* 2012; 54, H. 5. S. 644-651;
6. Baiden F, Webster J, Tivura M, Delimini R, Berko Y, et al. Accuracy of Rapid Tests for Malaria and Treatment Outcomes for Malaria and Non-Malaria Cases among Under-Five Children in Rural Ghana. *PLoS ONE* 2012;7(4): e34073.
7. Cheng Q, Gatton M, Barnwell J, Chiodini P, McCarthy J, Bell D, et al. *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. *Malar J* 2014;13:283.
8. Quality-assured RDTs are selected on the basis of WHO-recommended procurement criteria (<http://www.who.int/malaria/publications/atoz/rdt-selection-criteria.pdf>), lot-tested before field deployment by a WHO- or FIND-recognized laboratory (<http://www.who.int/malaria/areas/diagnosis/rapid-diagnostic-tests/evaluation-lot-testing/en/>) and transported and stored in accordance with the manufacturer's recommendations.
9. Stored blood slides and used RDTs could be used as sources of DNA, but they are not ideal.
10. The desiccant in the RDT cassette packaging can be used.
11. Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 6 (2014–2015) . Geneva: World Health Organization; 2015.

