MESA FORUM

Responding to the threat of malaria parasites evading HRP2-RDTs

Answers to pending Questions

Questions for Dionicia Gamboa & Selam Mihreteab

1. **How can professionals at the Ministry of Health be motivated to adopt the WHO recommendation to switch to *Plasmodium falciparum* Lactate Dehydrogenase (pfLDH) rapid diagnostic tests (RDTs) once they have more than 5% prevalence?**

SM: This requires strong leadership by the Ministry of Health, headquarters and particularly the National Malaria Control Program (NMCP) in making policy decisions. This should be accompanied with orientation to health care professionals on the causes of the problem and the consequences if Histidine-Rich Protein 2 (HRP2)-RDTs are used in situations where *pfhrp2* gene deletions are causing > 5% false-negative RDT results. Also, if the switch to pLDH-RDTs is only required in specific country regions, the program must be careful with the different stocks and distribute the new RDTs only to these areas in order to avoid confusions. **DG:** National and local health authorities, specifically the NMCP, are key to inform, train and supervise the activities of the health workers, including the community health promotores (leaders identified for each community to perform diagnostic based on RDT and to give treatment if necessary).

2. **In rural areas where there are only RDTs that target HRP2 as a means of detecting *P. falciparum*, what has to be done when faced with false negatives of the RDT?**

The first measure is to investigate if the false negativity is due to operator errors, storage conditions or RDT quality. Then *pfhrp2* gene status can be assessed using molecular techniques. The next step would be to make corrective measures for the identified causes such as improvement in RDT operation skills, improving the storage facilities, the RDT quality itself (jointly with the manufacturer), etc. depending on the problems detected. In case it is gene deletion, and if high prevalence is confirmed, the RDTs must be immediately withdrawn and replaced with other suitable ones.

3. **What are your suggestions if there's a need to conduct *pfhrp2/3*-deletion surveillance in settings with storage challenges for pLDH mRDTs?**

In areas with storage challenges, the biggest stock of the pLDH RDTs must be stored at a Central Medical Warehouse where there is optimal temperature conditioning. Relatively less amount can move to the provinces, while the peripheries store only small volumes. Additionally, 2 or 3 partial shipments can be considered during procurements rather than a single annual delivery in order to minimize the amount to be stocked at the warehouse/s. These documents may also be useful resources:

- Transporting, Storing, and Handling Malaria RDTs at Central and Peripheral Storage Facilities
- Transporting, Storing, and Handling Malaria RDTs in Health Clinics

Questions for Eric Rogier

4. What factors drive the deletion of *pfhrp2* gene in the malaria parasite?

It is most likely that deletions happen spontaneously and then are selected for through the use of HRP2 detecting RDTs. However, there are certainly other factors at play that are giving these parasites an advantage as they have expanded in Peru and other countries in South America where there was not significant RDT pressure. Genetic relatedness studies suggest that the *pfhrp2/3* genotype of the parasites from the same geographical area show highest relatedness to background strains in the same population with intact *pfhrp2* and *pfhrp3* genes. There is no evidence of distant importation of the *pfhrp2*-deleted allele but rather de novo mutation/deletion and spread within the Horn of Africa.

5. Is it possible that this mutation is transmitted through mosquitoes, from a person infected with these deleted parasites to another human being?

Yes, all evidence indicates that Pf parasites which have deleted either (or both) of these genes still produce gametocytes which have the same infectivity to the anopheline host, and still produce sporozoites to infect humans upon mosquito bite.

6. Is there any relationship between *pfhrp2/3* gene deletion and *pfhrp2/3* variants?

As the Pf parasite can randomly delete either of these two genes, random smaller deletions and point mutations can lead to variation of the genomic sequences of *pfhrp2* and *pfhrp3*. The variation, especially in *pfhrp2* gene sequence, can hypothetically have an impact on RDT sensitivity, especially at lower parasite densities. To date, *pfhrp2/3* gene variants appear to show geographical clustering similar to other measures of Pf relatedness. Evidence has not yet been presented that Pf populations with more variation in *pfhrp2* gene sequence are likely to have more gene deletions.

7. I would like to know the maximum sequence size (bases) that can be captured by the Molecular Inversion Probes (MIPs).

For the best answer to this question, please reach out to the corresponding author of the recent Feleke et al. manuscript describing deletions in Ethiopia.

8. Did you check to see if the parasites with HRP2 deletion had another mutation such as *multidrug resistance gene 1 (mdr1)* mutations?

This is certainly an area of interest/concern as parasites evading diagnosis as well as therapeutics would greatly confound case management and elimination efforts. To date, no association has been noted with *pfhrp2/3* deletions and point mutations for putative drug resistance markers (or copy number variation); however, research is underway to look for such associations.

9. Do the ultra-sensitive RDTs (based on HRP2) fail to detect deleted parasites the same way as conventional RDTs?

This depends on the deletion profile of the Pf parasites.
If Pf is deleted only for *pfhrp2*, the HRP3 antigen can still compensate to provide positive test results on HRP2-RDTs, especially at clinically-relevant, high density infections. However, at lower parasite densities, these infections with parasites only producing HRP3 will be more likely to cause false negative RDT results. As the detection limit is improved in the ultra-sensitive RDTs, these would be able to capture some amount of the lower-density infections. However, if both *pfhrp2* and *pfhrp3* are deleted from a parasite genome, no HRP2-based RDT can detect that infection regardless of test sensitivity.

10. Why do *pfhrp2/3* gene deletions seem common among the submicroscopic infections as compared to those infections detectable by the classical diagnostic tests (microscopy & RDTs)?

There's probably a few factors leading to this finding. In order to "detect" gene deletions, the deleted Pf strain must be the only genotype in the infection. If a wild-type Pf strain is also present in the infected person, even if at much lower parasite density, there is a high chance that HRP2-based RDTs will still elicit a positive result, and almost certainty that PCR-based identification of the *pfhrp2* and *pfhrp3* genes will show a "positive" result stemming from this wild-type parasite DNA.
In higher-transmission settings of the world, it is almost certain we are underestimating the prevalence of strains with deletions due to the incidence of multi-clonal infections, but fortunately, these multi-clonal infections would still provide positive HRP2-RDT results allowing for appropriate case management.

11. Do these mutations have any significance on the degree of pathology caused by the parasite?

This is a very interesting concept that has yet to be investigated.
It is well known that Pf parasites with these deletions can still cause severe and fatal malaria, including cerebral malaria. However, there is potentially a fitness cost imparted on these deleted strains, though this potential mechanism has not been elucidated yet.
There is also a potential that deleted parasites may have a reduced pyrogenic threshold – leading to reduced treatment-seeking behavior and progression to gametocytemia.
I would expect a wealth of studies investigating these important questions in the near future.

Questions for Jane Cunningham

WHO template protocol

○ Study sites and population:

12. What should be an acceptable population size of a domain from where to draw 10 Health Facilities?

Domain at state or province level is the administrative level most commonly used. District level can also be used but will require much more resources.

13. The WHO protocol indicates that you have to collect 370 samples per study area. If the study is conducted in different provinces, does it mean 370 *P. falciparum* cases in each area, or it refers to the total samples for the whole study?

To demonstrate that the prevalence of *pfhrp2/3* gene deletion (causing false negative RDTs) within symptomatic patients with *P. falciparum* is below or above 5%, a sample size based on an expected population prevalence of 3.2% (n=370) or 8.0% (n=318), respectively, per sampling domain would be adequate. **As a minimum, therefore, a sample of 370 individuals with a *P. falciparum* infection per sampling domain (e.g. province) is recommended (37 per health facility, 10 health facilities per sampling domain).** Within the domain, health facilities should be selected on the basis of probability proportional to size depending on the fever or suspected malaria caseload.
If a country already has some information about the expected prevalence, then this can be used to make more precise sampling estimates – see the survey protocol template [Table 2].

14. Why is the 5% HRP2 switching only computed for symptomatic patients? What about asymptomatic cases which are often more prevalent in endemic settings?

Currently, in the majority of settings, we only recommend diagnosis and treatment of suspected malaria cases based on presence of symptoms/clinical malaria – therefore, this is the priority group in which we must verify the performance of HRP2-RDTs. If resources are available, we recommend assessing a subset of samples from HRP2-RDT positive individuals and HRP2-RDT negative individuals to determine if *pfhrp2/3* deletions are present in these populations as mixed infections or low density infections. Data from asymptomatic cases may be useful in triggering priority areas for surveys amongst symptomatic individuals. The reasons for why *pfhrp2/3* deleted parasites may be more 'common' in low density and asymptomatic infections is an area of research.

○ Comparator tests:

15. Is using combo RDTs (as routine test) enough for HRP2/3 gene deletion surveillance, or is there still a need to conduct a survey?

It depends on what kind of combination test you are referring to.
If you are referring to a HRP2/pfLDH test (two separate test lines targeting Pf antigens) then this would be acceptable for surveillance. HRP2(-)/pfLDH(+) samples would be prioritized for molecular analysis (confirm Pf infection and then screen for *pfhrp2* and *pfhrp3* deletions).

If you are referring to HRP2/panLDH combination tests, then some *pfhrp2/3* deleted parasites may be picked up by the panLDH test line but either a second RDT (with pvLDH test line) or microscopy would be needed to determine the species causing the panLDH reaction, *i.e.* differentiate between Pf (*pfhrp2*-deleted) and minor species (Pv (if pvLDH RDT) or Pv, Po, Pm (if microscopy)). Also keep in mind that based on this RDT, HRP2(-)/panLDH(+) individuals will be classified as non-falciparum malaria and could potentially be treated inappropriately depending on national guidelines for treatment of Pf *vs.* Pv.

16. How to select an adequate (and good performing) LDH-based RDT for the survey?

For surveys it is ideal to use a Pf specific LDH RDT – there are limited options but a list is included in the survey protocol template [Table 1].

Table 1. *Non-HRP2-based RDT options that can be used for the survey and their corresponding performance characteristics based on rounds 1-8 of WHO malaria RDT product testing.*

Performance criteria (highlighted in green if met):

A: *P. falciparum* panel detection score (PDS)a ≥ 75% at 200 parasites/µL
B: *P. vivax* panel detection score (PDS) [a] ≥ 75% at 200 parasites/µL
C: false-positive (FP) rate against clean negatives < 10%
D: invalid rate (IR) < 5%
E: *pfhrp2* negative *P.falciparum* panel detection score (PDS) > 75% at 200 parasites/uL (in areas where *pfhrp2* deletions are prevalent)

			P.falciparum HRP2 expressing, *P.vivax* and malaria negative panels					*P.falciparum* HRP2 non-expressing panel				
Product		Manufacturer	PDS [a] A [b]	B [c]	FP C [d]	IR D [e]	Meets WHO procurement criteria [h]	PDS E [i]	Pf @2000p/µL [j]	Meets WHO procurement criteria for detection of *pfhrp2/3* deleted *P.falciparum* [k]	Applicable for use with a HRP2 RDT, as screening tool for surveys of *pfhrp2* deletions [l]	Round
Pf only												
CareStart™ Malaria Pf (HRP2/pLDH) Ag Combo 3-line RDT [f]	RMSM-02571	Access Bio Inc.	82 (81/40) [f]	NA	0.5	0.0	No (not WHO prequalified)	12.5 (0/12.5) [f]	100	No	Yes	8
SD BIOLINE Malaria Ag P.f (HRP2/pLDH) [g]	05FK90	Standard Diagnostics Inc. (Alere)	90 (88/71) [f]	NA	0.0	0.1	Yes [g]	32.5 (0/32.5) [f]	100	No	Yes	8
Pf, Pf and Pv												
SD BIOLINE Malaria Ag P.f/P.f/P.v [g]	05FK120	Standard Diagnostics Inc. (Alere)	89 (89/62) [f]	97.1	0.0	0.0	Yes [g]	20 (0/20) [f]	100	No	Yes	8

UK – unknown; Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*

a - A sample is considered detected only if all RDTs from both lots read by the first technician, at minimum specified reading time, are positive
b - Round 1, n=79; Round 2, n=100; Round 3, n=99; Round 4, n=98; Round 5, n=100; Round 6, n=100; Round 7, n=100; Round 8, n=100
c - Round 1, n=20; Round 2, n=40; Round 3, n=35; Round 4, n=34; Round 5, n=35; Round 6, n=35; Round 7, n=35; Round 8, n=35
d - Round 1, n=168; Round 2, n=200; Round 3, n=200; Round 4, n=232; Round 5, n=236; Round 6, n=208; Round 7, n=220; Round 8, n=208
e - Round 1, n=954; Round 2, n=1240; Round 3, n=1204; Round 4, n=1192; Round 5, n=1214; Round 6, n=1210; Round 7, n=1210; Round 8, n=1210
f- PDS presented in the table is based on a positive Pf test line (either HRP2 or Pf-LDH). The results in brackets are the PDS based alone on HRP2 and Pf-LDH test lines, respectively.
g - Indicates a WHO prequalified product (as 15 February 2019), see updates at: https://www.who.int/diagnostics_laboratory/evaluations/pq-list/malaria/public_report/en/
h- https://www.who.int/malaria/news/2019/rdt-procurement-criteria/en/
i Round 8, n=40 (18 double deletion: pfhrp2-/pfhrp3 -; 22 single deletion; pfhrp2-/pfhrp3+)
j - Results (PDS) of adhoc assessment of pfLDH containing round 8 RDTs against high density HRP2 negative panel: n=40 (18 double deletion: pfhrp2-/pfhrp3 -; 22 single deletion; pfhrp2-/pfhrp3+)
k- These results should be considered when procuring RDT for use in areas where pfhrp2 + or - pfhrp3 deletions are prevalent.
l - RDTs including pf-LDH individual test lines that have a PDS >90% against pfhrp2 deleted parasite samples of 2000 parasites/µL may be used to screen for pfhrp2 deletions as per this WHO survey protocol template

In addition to these, there are 3 other pfLDH products in the WHO Prequalification pipeline (2 target Pf only, and 1 targets Pf & Pv) which have passed the lab evaluation component and have Expert Review Panel for Diagnostics (ERPD) approval – see Table 2 below. As these products meet performance criteria against HRP2 expressing and non-expressing parasites, they can also be used for case management. While the ones in Table 1 above can only be used for surveillance as the performance of the pfLDH test lines does not meet WHO performance requirements.

Table 2. *pfLDH products in the WHO Prequalification pipeline that passed the lab evaluation component and have ERPD approval.*

Product name	Product code(s)	Manufacturer name
BIOCREDIT Malaria Ag Pf (pLDH)	C14RHG25 and C14RHH25	RapiGen Inc.
BIOCREDIT Malaria Ag Pf (pLDH/ HRP Ⅱ)	C13RHG25 and C13RHH25	RapiGen Inc.
BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH)	C61RHG25 and C61RHH25	RapiGen Inc.

Epidemiology and impact on the population

17. What is the importance of these deletions in case management *vs.* epidemiological surveys?

The deletions are most critically important because of their impact on case management as they can lead to missed or delayed diagnosis and associated morbidity and mortality. If HRP2 RDTs are used for epidemiological surveys then deletions may result in an underestimate of parasite prevalence. Therefore, surveys for *pfhrp2/3* deletion will inform procurement of tools for case management and epidemiological surveys.

18. What is the evidence known to date and the possible implications in terms of scale of impact?

The evidence to date is insufficient to determine the scale of the impact because of the limited number of surveys that have been completed.

19. Could implementing Mass Drug Administration (MDA) not mitigate the effect of *pfhrp2/3* deletions?

In theory, yes, MDA may mitigate the effects of *pfhrp2/3* deletions by clearing HRP2 expressing and non-expressing parasites in the population; however, it will be incomplete and reemergence due to spontaneous mutations (and selection with HRP2-RDTs, plus other drivers), as well as importation, is likely to threaten the durability of such an intervention. We do not currently recommend this approach for managing *pfhrp2/3* deletions.

20. What are the reasons or factors for HRP2 deletion spreading across the Horn of Africa? Any explanation? Could it be a sign of malaria control success?

Mutations naturally occur and as the parasite can survive without HRP2 and HRP3 we suspect that strict compliance with testing using HRP2-based RDTs and treatment only of HRP2-positive cases may have contributed to emergence of *pfhrp2/3* deletions in countries in the Horn of Africa. Regardless of the *pfhrp2/3* genotype the parasites from the same geographical area show highest relatedness so regional spread/importation is also likely to be a factor, but there is no evidence of distant importation from South America (Peru). Furthermore, in low transmission settings, individuals are more likely to be infected with fewer parasite strains and monoinfections with *pfhrp2/3*-deleted parasites will cause negative RDTs. In addition to selection pressure, we suspect there are other factors giving these parasites an advantage particularly since we see them expanding in countries where there is very little HRP2-RDT pressure, *i.e.* Peru.

21. How to manage the transmission of deleted parasites from people entering cross-border from countries with a high percentage of deletions? How do we make routine surveillance for gene deletions in the affected and neighboring countries?

One strategy to manage cross-border transmission where high risk groups are crossing is through use of combined HRP2/pfLDH RDTs in these locations. The survey protocol template is designed to be able to be incorporated into the routine workflow – parallel testing with a second pfLDH RDT (or microscopy) and collection of 2 blood spots on filter paper. Informed consent should not be required for *pfhrp2/3* surveillance purposes. There is an international network of laboratories that can support the required molecular analysis. Global Fund will support applications for funding to conduct surveillance. WHO is working on a sentinel surveillance approach to implement following baseline surveys.

Alternatives to HRP2-based RDTs

22. Why do we maintain the HRP2-based RDTs, is it more affordable than the pfLDH RDTs?

Yes, HRP2 RDTs have been less expensive and better performing (more sensitive) than pfLDH RDTs. There are also many more suppliers of HRP2-RDTs compared to pfLDH RDTs.

23. Is support provided for innovation in malaria RDTs?

WHO has been advocating for better performing pfLDH RDTs for nearly 15 years and in the past few years some companies have invested in better tests that are not exclusively based on HRP2, *i.e.* RapiGen. Also, research donors like Bill & Melinda Gates Foundation are funding companies to develop better pfLDH RDTs which are in field trials this year (2022).

24. Is there a plan to create a Target Product Profile (TPP) for alternative tests that could replace HRP2-RDTs?

No, there are no plans on WHO's part to develop a TPP for non-HRP2 or non-exclusive HRP2 based RDTs. Already there are RDTs that target antigens other than HRP2 and meet minimum performance criteria for detection of HRP2 expressing and non-expressing Pf parasites. We need more products to ensure a healthy market and supply security.

25. If an alternative diagnostic approach was implemented, would the problem of gene deletion still persist?

Given that *pfhrp2/3* deletions have persisted and expanded geographically and in frequency in Peru without any change in diagnostic approach suggests that there are other factors beyond the diagnostic tool that are drivers. Follow up surveys in countries post shifts away from HRP2-RDTs may be useful in explaining the contribution of HRP2-RDT pressure to persistence. Data from Eritrea suggests that these parasites persist albeit at lower prevalence, after 2+ years post transition to pan or pfLDH based diagnosis.

Way forward

26. How widespread are the deletions right now, and what can we do preemptively to avoid the worst effects?

Refer to the Malaria Threat Maps for a summary of where samples have been interrogated for *pfhrp2/3* deletions, and where they have been identified or not identified. Many countries have not been surveyed or surveys have been limited in scale and scope.
Countries need to get ahead of the problem through surveillance to ensure HRP2-RDTs are still working effectively. R&D is needed to identify and validate alternative antigens or biomarkers that are essential to parasite survival so that this issue can be avoided in the future. Manufacturers need to shift toward non-exclusive HRP2-RDTs so that options will remain and prices can stay competitive.

27. Does the deletion of this protein mean that the HRP2-RDTs will be discontinued in these areas?

No, not all *pfhrp2* deletions will result in negative RDTs and thereby lead to missed or delayed diagnosis.
The impact of *pfhrp2* deletions may be reduced at least temporarily through:

- Multiclonal infection with wild-type and *pfhrp2*-deleted *P. falciparum*
- Possible to detect using multiplex real-time or digital drop PCR but not conventional PCR
- Residual HRP2 from previous Pf infection and current infection with deleted parasites
- *Pfhrp3* is present and antibodies on the RDT strip react with common epitopes

We envision that eventually, as alternatives with comparable performance and price become available, reliance exclusively on HRP2 for Pf diagnosis will come to an end and all countries will adopt alternatives.

28. WHO diagnosis guidelines are currently under revision, will it be emphasized that some parasite strains with *pfhrp2/3* deletions can go undetected by RDTs?

Yes.

29. If a new technology, not limited to RDT based, is able to detect parasites regardless of its deletion, is it just fine? Or is there still a need to do surveillance of deletions?

Surveillance is to inform continued use of HRP2-RDTs, when affordable and prequalified alternatives are available an empirical shift away from exclusive HRP2-RDTs may be warranted and surveillance would no longer be indicated.

30. Is it safe to assume that the spread/increase of these specific deletions are a result of selective pressure placed on the parasite as a result of the RDTs being used to diagnose (and then treat) malaria infections? If so, should we be viewing the spread/increase of deletions as evidence of success of control or elimination programs (i.e. they were able to put enough pressure on a particular feature), is this an inevitable outcome in the quest to find and treat cases, or does it suggest some sort of strategy failure (and we will be here in 15 years learning about another parasite feature causing patients falsely testing negative)?

Selection pressure on deleted parasites because of strict use of HRP2-based test and treat strategy probably played and is playing a role in the emergence of this problem; however, other drivers are likely involved. These have not yet been elucidated. In the future, it would be desirable to target biomarkers that are essential to parasite survival and stable / not prone to frequent mutations – so that if they were lost the parasite would no longer be able to cause disease or the epitopes targeted by the antibodies on the test strip are not likely to lose affinity.