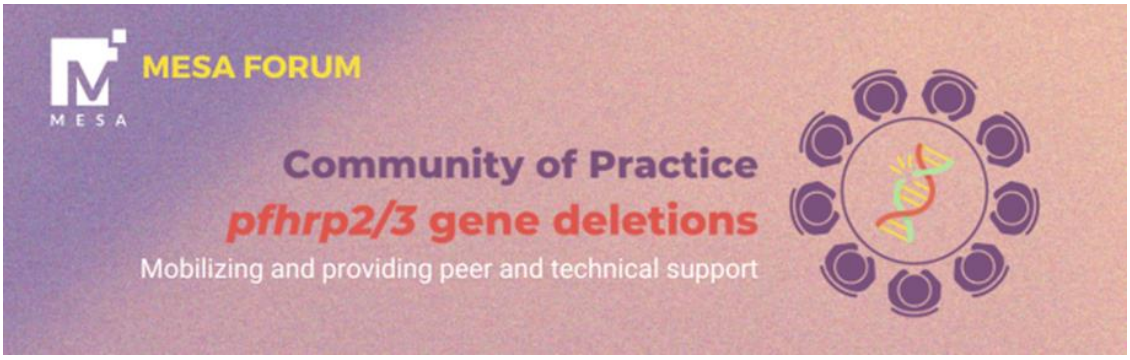

Answers to pending Questions

Field implementation

1. What is the best sample type for detection and/or monitoring of *pfhrp2/3* gene deletions in field surveys?

WHO recommends collecting dried blood spots (DBS) on filter papers from symptomatic patients. If available, whole blood can also be used. DNA can be extracted from DBS and/or whole blood for molecular detection of gene deletions and to elute protein for serological analysis to confirm lack of protein expression. If serology or *Plasmodium* antigen detection is expected to be done, collection of samples in preservatives such as RNA/DNA shield which may deactivate protein is not recommended.

2. How can we start to conduct the *hrp2/3* gene deletions surveillance in my country?

Any country interested in conducting a survey for *pfhrp2/3* gene deletions should use the WHO template of the protocol. We would recommend the following:

1. Develop a customized country protocol using the WHO template. The protocol should provide information on the number of provinces/regions to be covered and the number of health facilities to be included in the survey. The protocol should also state the test to be used as a comparator (*e.g.* microscopy if it is available at all health facilities or a non-HRP2 RDT). The team should also choose the type of survey; either research (this needs an IRB approval) or a surveillance. It is important to engage and work with WHO for technical support.
2. Sensitize and bring everyone on the same level of understanding of the *pfhrp2/3* gene deletions threat, and emphasize the need to conduct a survey - it might also involve choosing priority regions (if resources are limited and will not suffice for a national survey). This can also help mobilize resources based on the perceived risk/threat.
3. Secure funding for the survey either locally (in-country) or from external funders.
4. Obtain IRB approval if the survey will be done as a research and not a surveillance activity.
5. Develop a clear work plan for the survey.
6. Procure the materials required for the survey.
7. Identify sources of technical support if needed. Most of NMCP work with local researchers who provide them with technical support. WHO also provides technical support and should be consulted at all stages.
8. Organize training for the survey team.
9. Prepare a plan for supervision of field activities.

Once the above are in place, the team can now implement the survey and then make a plan for sample analysis and writing of and disseminating the report.

3. Are the deletions present across all the parasite species or is species-specific?

The deletions only occur in *P. falciparum* because other human plasmodium species do not have *hrp2/3* genes and do not produce HRP2 antigens.

4. Does PfHRP2/3 loci contribute to parasite virulence and fitness and is it a determinant of survival advantage?

This is an important research question. It is, however, unknown whether *pfhrp2/3* deletions provide survival advantage for the parasite under normal conditions (*i.e.* in the absence of RDT-based test and treat policy). There is a great need for field studies to demonstrate a direct relationship between *pfhrp2/3* and parasite fitness, and between *pfhrp2/3* and virulence in clinical settings.

5. Can this gene deletion affect the chemotherapy efficacy?

There is no evidence to suggest gene deletions have a direct effect on treatment efficacy. The impact of pfk13 mutations co-existing with or without *pfhrp2/3* deletions needs further investigation.

6. Does the level of HRP2 antigen production in children vary from that of adults?

Higher levels of HRP2 have been associated with greater likelihood of progression to severe malaria disease in children and adults. However, to our knowledge there is no evidence showing a difference in HRP2 levels between children and adults with uncomplicated malaria. HRP2 is a surrogate marker of parasite biomass in patients and high levels of HRP might be observed in children due to low levels of immunity.

7. Is there any point of care technology for detection of these gene deletions?

There is no point of care test for detection of the deletions or assessing them in the field. However, to capture patients who may be infected by parasites with the deletions, WHO recommends a protocol to use. The WHO protocol recommends using a two-RDT method to screen for gene deleted parasites in patients: *i.e.* use a HRP2-based RDT and a Pf-pLDH-detecting RDT to test the same patient. Patients who are positive on Pf-pLDH RDT, but negative on HRP2-based RDT are suspected to have been infected with *pfhrp2/3*-deleted parasites. Dried blood spots collected from these patients shall be sent to a qualified laboratory to confirm gene deletions using molecular and serological tests.

8. For field studies, has any group been able to determine the parasite density threshold at which single deletion (either *pfhrp2* or *pfhrp3*) results in false negative HRP2-RDT?

Generally, *pfhrp2* deletion leads to false negative HRP2-RDT result if *pfhrp3* is present but parasitaemia is less than 1000 parasites per microlitre (Beshir *et al.* Sci. Rep. 2017). In the absence of *pfhrp3*, it is possible to obtain false negative HRP2-RDT results if *pfhrp2* is present and parasitemia is below detection limit of RDT (unpublished data). Usually, this false negative result is interpreted as “low parasite density” rather than caused by *pfhrp2/3* gene deletions. This should be excluded from the analysis to determine the 5% threshold.

9. How can we detect deletions in multiclonal infections with wild and mutated parasites?

Both multiplex qPCR and digital PCR methods are able to detect gene deleted parasites in a sample when they are mixed with wild type parasites and are dominant. The accuracy of these methods in detecting *pfhrp2/3*-deleted variants in multigenomic infections need to be further validated. The qPCR can detect when the deletions are more than 80% (Grignard *et al.* EBioMEdicine, 2020) and the dPCR can detect as low as 10% in multigenomic infections (unpublished data). QPCR detects the deletions in multigenomic infections based on differences in Cq or Ct values. Usually, positive controls are used to adjust Cq/Ct values before determining the Cq/Ct value of the samples.

10. How comparable were the results on the non WHO validated PCR assays?

There are differences in terms of detection limit, calling deletions in multigenomic infections and determination of a "negative" result. Validation of every assay (qPCR or PCR or dPCR) will be required before the assays are routinely implemented in any lab. Participation in the WHO EQA scheme is a good way of ensuring the data generated in your lab is reliable.

11. What do you advise for the use of 18S which is a multicopy gene in multiplex qPCR for *pfhrp2* gene deletion?

When using a conventional PCR method to confirm *pfhrp2/3* deletions, it is recommended to use two single copy genes as controls for quality of DNA in the sample. While 18S RNA gene is a good marker for plasmodium species confirmation, it is not an appropriate control for *pfhrp2/3* deletions because it is a multicopy gene while *pfhrp2* and *pfhrp3* are single copy genes. The same principle applies to multiplex qPCR in that it is preferable a single copy gene is used as the normaliser in the assay.

12. What's the cost difference between using nested PCR or qPCR for the detection of gene deletions?

If the real-time PCR equipment already exists, the qPCR assay would be approximately $10 USD per sample which is inclusive of DNA extraction and the PCR assay. The nested PCR assay would cost slightly more since multiple PCR reactions need to be run to amplify different gene products.

13. In case of antigen screening, what is the cost/sample as compared to the qPCR?

If the equipment already exists, the cost is approximately $1-2 USD per sample for detection of *Plasmodium* antigens. This estimation applies both for a multiplex bead-based assay (with multiple antigens per run), or to conduct two separate ELISA runs (*e.g.* LDH and HRP2).

Policy / guidance (protocols)

14. What's the recommendation when estimating the survey sample sizes in countries with heterogeneous deletion patterns or in very low transmission settings?

The WHO surveillance template protocol for *pfhrp2* and *pfhrp3* deletions recommends sample sizes required for surveillance. The protocol is being updated currently and the updated version of the protocol will include recommendations/suggestions for sample sizes for settings with heterogeneous deletions patterns and/or very low transmission.

15. Implications of deletions on procurement decisions: what info do countries need to support their decision making?

When survey outcomes show >5% (above the confidence interval) *pfhrp2* deletions causing false negative RDTs, the WHO recommends the country switches from HRP2-based RDTs to Pf-pLDH-detecting RDTs. See the WHO Response Plan to *pfhrp2* deletions for recommended actions. Note: the document is being updated.

16. What would be the alternatives in the face of this mutation to ensure the diagnosis of Pf?

Please refer to the WHO Response Plan to *pfhrp2* deletions. It outlines response steps to be taken when survey outcomes show >5% *pfhrp2* deletions causing false negative RDTs. Note: the document is being updated. New Pf-pLDH RDTs are in the WHO prequalification pipeline and are approved by the Global Fund.

General

17. How often will CoP meetings be held and what impact will it have on the health services issues in our country?

We will have regular meetings, some of which will focus on specific topics (thematic meetings). The dates of these events will be shared in advance. The meetings and other activities of the CoP will provide peer support and a space for interaction to support National Malaria Programs, researchers and implementing partners working with NMCP/NMEP on how to implement surveillance activities of *pfhrp2/3* gene deletions for policy and decision making. The surveillance will ensure RDTs used for malaria diagnosis are performing optimally and malaria case management services are not disrupted by reduced performance of these important tests caused by the deletions.

18. Does MESA have Market Research data to assess how much NMCPs think *pfhrp2/3* deletion is a threat in their respective countries?

No, we have not performed such an assessment. One of the main goals of the first MESA Forum (organized in June 2022) was to increase the awareness of this biological threat among the malaria community. Before the event, targeted e-mails were sent to NMCPs from all malaria endemic regions, informing about this emerging threat and inviting them to join the virtual event. The Forum counted on more than 130 registrants from NMCPs or MoHs from 45 malaria-endemic countries.

This Q&A document has been prepared through the kind contribution of the following experts: Qin Cheng (ADFMIDI, Australia), Khalid Beshir (LSHTM, UK), Deus Ishengoma (NIMR, Tanzania), Bosco Agaba (NMCP, Uganda) and Eric Rogier (CDC, USA) - *[**August 2023**]*