nature research

Peer Review Information

Journal: Nature Microbiology
Manuscript Title: **Plasmodium falciparum is evolving to escape malaria rapid diagnostic tests in Ethiopia**
Corresponding author name(s): Jonathan Parr

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Dr Parr,

Thank you for your patience while your manuscript "Plasmodium falciparum histidine-rich protein 2 deletion is under recent positive selection in Ethiopia and threatens malaria diagnostic strategies" was under peer-review at Nature Microbiology. It has now been seen by the same 2 referees who provided input on the first version of this manuscript. You will see from their comments below that reviewer 1 offers guidance on further improvement to your paper, most of which are minor and involve clarifications, more detail in the methods and moving Supplementary items to the main text. We are very interested in the possibility of publishing your study in Nature Microbiology, and now encourage you to revise your manuscript using the comments from reviewer 1 as guidance.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/nmicrobiol/info/final-submission/

The usual length limit for a Nature Microbiology Article is 6-8 display items (figures or tables) and 3,000 words. We have some flexibility, and can allow a revised manuscript at 3,500 words, but please consider this a firm upper limit.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available

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Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see:
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Figure legends must provide a brief description of the figure and the symbols used, within 350 words, including definitions of any error bars employed in the figures.

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-- that control panels for gels and western blots are appropriately described as loading on sample processing controls

-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

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* ensure it complies with our format requirements for Articles as set out in our guide to authors at www.nature.com/nmicrobiol/info/gta/

* state in a cover note the length of the text, methods and legends; the number of references; number and estimated final size of figures and tables

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We hope to receive your revised paper within three weeks. If you cannot send it within this time, please let us know.

We look forward to hearing from you soon.

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Reviewers Comments:

Reviewer #1 (Remarks to the Author):

This article by Feleke et al. presents a comprehensive assessment of two rapid diagnostic tests (RDTs) employed to detect Plasmodium falciparum (Pf) malaria in Ethiopia. Both tests rely on the detection of of Pf HRP2 antigen in whole blood. CareStart includes a test for P. vivax LDH, whereas SD Bioline includes a test for Pf LDH. The authors report the compelling finding that nearly 10% of all Pf infections sampled in Ethiopia (in their three sites) are HRP2 negative and thus would escape RDT detection. This finding was made through a powerful combination of RDT data from the two tests, PCR, molecular inversion probes and some whole-genome data. These state of the art genetic and genomic techniques, applied to a large data set of 2,714 Pf-positive samples (from a larger screen of 12,572 participants) yielded evidence that parasites with HRP2 deletions are closely related at the population level and that HRP3 deletions, which are more prevalent, occurred earlier and are more heterogeneous. The HRP2 data suggest a selective sweep arising from parasite escape of RDT screening prior to treatment. These data highlight a critical weakness in using these RDTs to detect and treat Pf malaria.

In addition to reviewing this submission, I have read the critiques from the prior submission to Nature Medicine, and feel that the authors responded very well. Overall, I find this an important study rich in data that will be of substantial interest to the malaria control and research community and other investigators studying antimicrobial diagnostics and population structures associated with intensive pathogen screening prior to treatment. I nonetheless have a few additional queries and comments:

1. Greater clarity should be provided about the way these tests work. The CareStart literature that I saw (https://www.apacor.com/wp-content/uploads/2019/01/APA059-v8-1630-16305-CareStart-Malaria-Test-Procedure-updated-1.pdf) indicates that one band is specific to Pf HRP2 and the other will appear positive if there is detectable LDH antigen from any of the Plasmodium species. Therefore a LDH+ band combined with a HRP2- band could indicate either a non-Pf species or Pf that lacks the hrp2 gene. The manuscript states that the CareStart is Pf HRP2 / Pv-specific LDH. The authnors should clarify this apparent discrepancy. If the LDH is indeed pan specific that would change a lot of their interpretation. For the SD Bioline assay I found it surprisingly difficult to obtain information about how the assay works. Some versions on the internet show that there is only one band for Pf, which would conflate HRP2 and LDH. The manuscript indicates that the SD Bioline recognizes both Pf HRP2 and Pf-specific LDH. The authors need to clarify this. Also, they should point out that each assay has a control band. Ideally, it would help to have an additional supplementary figure that shows both types of RDTs with a description of how they work and how different banding patterns should be interpreted. The authors should also include links in their additional supplementary figure (there may be several

versions of each test, leading to the apparent discrepancy above). The issue of species specific versus pan-Plasmodium is a critical one to address.

2. On a similar note, I think it would be very helpful to readers to have Supplementary Table 11 moved to a main Table as that shows the differences between the two assays and also documents the 332 (~9.7%) of samples that were Pf LDH+, HRP2- and Pv-LDH-. That table explicitly states that the CareStart has a Pv-specific LDH, which the authors should clarify in response to comment 1.

3. It would be helpful to know the prevalence of P. vivax in their cohort, even though I recognize this is not central to their study. They describe P. vivax data in the supplement (lines 863-878) and it would help to give a percentage (or estimate?) of the number of P. vivax samples as defined by their CareStart RDT. Were there any P. vivax mono-infections or were they always with Pf? The authors should also expand on any other evidence for P vivax that they obtained using other molecular techniques (PCR? WGS?). If the Editors allow more space, it would be useful to include this section in the main manuscript.

4. The authors make the worrying statement (lines 165-167) that sufficient cross-reactive HRP3 can trigger a positive HRP2 band. They then exclude hrp2- hrp3+ samples with sufficient cross-reactive HRP3. How do they determine which samples have sufficient cross-reactive HRP3? This seems like a difficult factor to correct. Any quantitative data in this regard or more explanation would be very helpful.

5. On page 386 the authors state that the prevalence of hrp2/hrp3-deleted parasites appears to have been stable in neighboring Eritrea despite removal of HRP2-based RDTs two years ago. The authors should indicate what is being done in their place. This also relates to their statement on lines 453-454 that refer to the deployment of alternative malaria diagnostics.

Minor comments:
6. Figure 2 has an exceptionally small font on the Y axis that should be slightly increased. Also, I feel it would be less confusing to have the control samples listed in the same vertical order below chromosome 8 (top) and 13 (bottom) data. LC and HC should be defined in the legend.

7. The Figure 3 Y axis font is even smaller and truly impossible to read on a print out.

Reviewer #2 (Remarks to the Author):

my comments and concerns have been addressed

Author Rebuttal to Initial comments

Reviewer #1 (Remarks to the Author):

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RESPONSE: Yes, we confirm that the CareStart RDT used in this survey included a *P. vivax* (not pan-species) LDH line. The provided link does not correspond to the product code used in the study (RM VM-02571). Information about this specific RDT's performance versus a standardized panel of samples can be found in the report "Malaria rapid diagnostic test performance: Summary results of WHO product testing of malaria

RDTs: round 1-8 (2008–2018)", available at:
https://www.who.int/publications/i/item/9789241514965.

2. On a similar note, I think it would be very helpful to readers to have Supplementary Table 11 moved to a main Table as that shows the differences between the two assays and also documents the 332 (~9.7%) of samples that were Pf LDH+, HRP2- and Pv-LDH-. That table explicitly states that the CareStart has a Pv-specific LDH, which the authors should clarify in response to comment 1.

RESPONSE: Thank you for this suggestion. We agree that this information is important and are grateful to the reviewer for suggesting its addition during our initial revision. We regret that we are unable to include additional tables and figures in the main text due to limits specified in the Nature Microbiology publication guidelines. However, we have added an explicit reference to Supplementary Table 11 in the discussion (line 272) to highlight the availability of these results.

3. It would be helpful to know the prevalence of P. vivax in their cohort, even though I recognize this is not central to their study. They describe P. vivax data in the supplement (lines 863-878) and it would help to give a percentage (or estimate?) of the number of P. vivax samples as defined by their CareStart RDT. Were there any P. vivax mono-infections or were they always with Pf? The authors should also expand on any other evidence for P vivax that they obtained using other molecular techniques (PCR? WGS?). If the Editors allow more space, it would be useful to include this section in the main manuscript.

RESPONSE: We have added a sentence to the Results section noting that 9.4% of subjects were positive for *P. vivax* by the Pf/Pv CareStart RDT (lines 135-136). This frequency is derived from Table 1: 593 (4.7%) subjects had CareStart RDT results consistent with *P. falciparum*-*P. vivax* co-infection and 590 (4.7%) had results consistent with *P. vivax* mono-infection. We did not perform *P. vivax*-specific PCR assays, and our sWGA primer sets and MIP probes were designed to target the *P. falciparum* genome.

This revision required moving sections from the main text to the supplement to comply with word limits specified in Nature Microbiology's publication guidelines. As a result, we regret that we could not move the *P. vivax* section into the main text.

4. The authors make the worrying statement (lines 165-167) that sufficient cross-reactive HRP3 can trigger a positive HRP2 band. They then exclude hrp2- hrp3+ samples with sufficient cross-reactive HRP3. How do they determine which samples have sufficient cross-reactive HRP3? This seems like a difficult factor to correct. Any quantitative data in this regard or more explanation would be very helpful.

RESPONSE: We agree with the reviewer that it is not an easy task to determine which samples have sufficient cross-reactive HRP3. Discerning HRP2 antigenemia from HRP3 cross-reactivity is not feasible using existing RDTs or immunoassays. At present, the

general school of thought is that infection by *pfhp2-/3+* parasites with >1,000 parasites/μL will trigger a positive HRP2 band due to HRP3 cross-reactivity. However, this threshold is not well defined and will inevitably vary based on the antibodies used in the RDT test bands and the infecting parasite strain.

To overcome this, we focused on the prevalence of the phenotype of greatest importance to programs (false-negative HRP2-based RDT results due to infection by parasites with *pfhrp2* deletion). We did not attempt to incorporate *pfhrp3* PCR genotype into this estimate to avoid ambiguity about cross-reactivity. We include explicit discussion of our approach and its limitations in lines 170-174. Our approach provides actionable information to programs but may underestimate the true prevalence of *pfhrp2/3*-deleted parasites.

5. On page 386 the authors state that the prevalence of hrp2/hrp3-deleted parasites appears to have been stable in neighboring Eritrea despite removal of HRP2-based RDTs two years ago. The authors should indicate what is being done in their place. This also relates to their statement on lines 453-454 that refer to the deployment of alternative malaria diagnostics.

RESPONSE: We removed this line in the process of reducing the word count to comply with Nature Microbiology guidelines. Eritrea has transitioned to use of a Pf-LDH-based RDT. In the Discussion, we have added information about the only Pf-LDH/Pv-LDH RDT product suitable for Ethiopia that is approved for purchase using Global Fund financing (lines 273-277).

Minor comments:
6. Figure 2 has an exceptionally small font on the Y axis that should be slightly increased. Also, I feel it would be less confusing to have the control samples listed in the same vertical order below chromosome 8 (top) and 13 (bottom) data. LC and HC should be defined in the legend.

RESPONSE: Thank you for this suggestion. We have revised the font size, re-ordered the controls, and defined the LC and HC controls in the legend as suggested.

7. The Figure 3 Y axis font is even smaller and truly impossible to read on a print out.

RESPONSE: We have increased the font size to improve readability as suggested.

Reviewer #2 (Remarks to the Author):

my comments and concerns have been addressed

Decision Letter, first revision:

Dear Dr. Parr,

Thank you for submitting your revised manuscript "Plasmodium falciparum histidine-rich protein 2 deletion is under recent positive selection in Ethiopia and threatens malaria diagnostic strategies" (NMICROBIOL-21051266A). I've check the tracked version and we are now ready to proceed to finalize your manuscript for publication in Nature Microbiology, pending minor revisions to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology. Please do not hesitate to contact me if you have any questions.

Decision Letter, final checks:

Dear Dr. Parr,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Microbiology manuscript, "Plasmodium falciparum histidine-rich protein 2 deletion is under recent positive selection in Ethiopia and threatens malaria diagnostic strategies" (NMICROBIOL-21051266A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks if not before). Please get in contact with us if you anticipate delays.

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In recognition of the time and expertise our reviewers provide to Nature Microbiology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Plasmodium falciparum histidine-rich protein 2 deletion is under recent positive selection in Ethiopia and threatens malaria diagnostic strategies". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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Final Decision Letter:

Dear Dr Parr,

I am pleased to accept your Article "Plasmodium falciparum is evolving to escape malaria rapid diagnostic tests in Ethiopia" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Before your manuscript is typeset, we will edit the text to ensure it is intelligible to our wide readership and conforms to house style.

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