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Title: PCR-correction strategies for malaria drug trials: Updates and Clarifications

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Corresponding Author: Dr. Ingrid Felger,

Corresponding Author's Institution:

First Author: Ingrid Felger

Order of Authors: Ingrid Felger; Georges Snounou, Dr.; Ian Hastings, Dr.; Joerg J Moehrle, Dr.; Hans-Peter Beck, Prof. Dr.

Abstract: Malaria drug trials conducted in endemic areas face a major challenge in their analysis: parasitaemia in blood samples collected after treatment may indicate a drug failure or originate from a new infection (NI) acquired after treatment. It is therefore vital to reliably distinguish drug failures from NI to obtain accurate estimates of drug failure rates. This is achieved in Plasmodium falciparum by comparing parasite genotypes obtained at time of treatment (the baseline) and on the day of recurring parasitaemia. Such 'PCR-correction' is required, even for new effective drugs, to obtain accurate failure rates. Despite its routine use in surveillance of drug resistance and in clinical drug trials, limitations inherent to the molecular genotyping methods has led some commentators to question the validity of current PCR-correction strategies. We describe and discuss recent developments in these genotyping strategies with particular focus on method validation and limitations. Our aim is to update scientists from public and private bodies working towards the development, deployment and surveillance of new malaria drugs. We aim to promote discussion around these issues and argue for the adoption of improved standardized PCR-correction methodologies.

Dear Editor

Thank you for sending us the reviewers' comments. We address them in the following pages. We enumerate their comments for ease of cross reference, include their comments in italics and blue font, and provide our responses in plain text and black font.

We would like to thank the reviewers for their valuable und constructive comments.

Kind regards

Ingrid Felger

(on behalf of all co-authors)

Reviewer #1: The authors present the state of the art in malaria genotyping as it relates to clinical trials for antimalarials. The message is an important one that has been overlooked by clinical trialists, regulatory authorities and policy makers: current methods are limited and may mis-attribute genotypes and inaccurately reflect drug treatments.

1.1 There are references here to unpublished results used as evidence and articles "in this issue" which I was not able to discern (pages 6 para 2, references to Jones et al to which this reviewer does not have a reference or access).

We have deleted this cross reference on page 8. It referred to a manuscript that was submitted as a joint submission to Lancet Infectious Diseases together with this personal views article. It was deemed unsuitable for LID and is now under review in Antimicrobial Agents and Chemotherapy:

Jones, S., K. Kay, E.M. Hodel, S. Chy, A. Mbituymuremyi, A. Uwimana, D. Menard, I. Felger, I. Hastings. Improving methods for analysing anti-malarial drug efficacy trials: molecular correction based on length-polymorphic markers *msp-1*, *msp-2* and *glurp*. Submitted.

The Jones et al. manuscript uses methodology previously published in:

Jaki T, Parry A, Winter K, Hastings I. Analysing malaria drug trials on a per-individual or per-clone basis: a comparison of methods. *Stat Med* 2013; **32**(17): 3020-38.

We therefore cite Jaki et al. 2013 (e.g. in caption to Figure 1) to allow readers access to the underlying methodology. On **page 8** of our article we have inserted ("Jones and Hastings, personal communication"), which will be exchanged against the correct reference, should the AAC article be published before this one.

Page 8: The paragraph on modelling has been revised as follows:

> "Modelling provides a new approach to validate potential algorithms for interpreting PCR-corrected data. Pharmacological models can simulate a population of patients in a drug efficacy trial, their therapeutic outcomes, and the genotyping results that would occur at Day 0 and Day X.16 PCR-corrected failure rates were compared using several molecular correction approaches based on simulation models from Jaki and coworkers.16 The "2/3 approach" (i.e. only using glurp if msp1 and msp2 gave concordant results) provided the best fit with the simulated treatment failure rates in a comparison to other molecular correction strategies (Figure 1). Adopting the 2/3 approach instead of the current WHO sequential typing method led to higher failure rate estimates but these were closer to the theoretical true failure rate.
>
> The current WHO/MMV method, and the new algorithms currently under investigation (Jones and Hastings, personal communication), all seek to define recurrent infections as either NI or a drug failure.

1.2 False impressions of drug failure in a clinical trial context is a major issue. Newer methods including NGS are touched upon as possibly being the solution. The major concern I have is that the authors would do well to convene a technical consultation with experts in the field including clinicians, method developers, NGOs in trials like MMV, policy makers etc. and then publish the results of the round table rather than this somewhat superficial piece. The "2/3 approach" is mentioned as a solution but there is insufficient evidence that it should be adopted without a fuller application to larger datasets beyond what is presented in Figure 1.

We agree entirely with the comments listed by the Reviewer as "major concerns". We reassure the Reviewer as follows:

We wrote the article for LID primarily because we believe it will be of significant interest to the readership of LID, as it deals with two crucial facets of malaria control, namely drug efficacy trials and monitoring for emergent drug resistance. However, as recognised by the Reviewer, it also has a significant political aspect. We agree with the Reviewer that a "technical consultation" and "round table" discussion of key stakeholders will be necessary to reach a consensus on most appropriate methodology and we plan to organise and convene such a meeting. The problem is that such meetings require financial support and clear demonstration to donors and potential participants that there is a clear need for such a meeting; this article is designed to start this process. We are ideally placed to drive this process. Current recommendations originate from round table technical discussions held in Geneva in May 2007, supported by WHO and Medicines for Malaria Venture (MMV) and subsequently published as a WHO report i.e.

World Health Organization (2008). Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations.

H-P Beck chaired that meeting, I. Felger was rapporteur, J. Möhrle was part of the secretariat and G. Snounou was a participant. More recently I. Hastings was asked by WHO via their TEG on Drug Resistance and Containment, to asses and evaluate the literature and methodology of correction in trials. We anticipate that this submission to LID would be the first stage of the process leading to a similar meeting being convened in the near future. We recognise that support for policy change, driven by scientific analysis, is one of the prime objectives of LID, hence our decision to submit in your journal.

In addition, I. Felger and I. Hastings participated in the Technical Expert Group on Drug Efficacy and Response held at the World Health Organization, Geneva, Switzerland in June 2017. The group discussed the issue of molecular correction and their key recommendation was: ""The Technical Expert Group (TEG) recommends that once the new analysis has been completed, the guidance on P. falciparum genotyping should be reviewed and revised if necessary" (Minutes available at https://www.who.int/malaria/mpac/mpac-oct2017-teg-drug-efficacy-response-session3.pdf?ua=1.)

Page 10: As recommended by Reviewer 1 we suggest at the end of our paper that it is timely to convene another technical meeting to update recommendations for PCR-correction published in 2008 by WHO.

> "We suggest that improved understanding and technical developments since publication of the WHO/MMV consensus protocols (2008) makes it important that its recommendations should be updated. Another technical meeting should be convened, particularly to address the methodological requirements for drug registration trials that are more demanding than drug resistance monitoring studies. Regulatory trials require precise methodologies and should be implemented as state-of-the-art. The present paper will serve as a basis for discussion towards a revised consensus."

Reviewer #2: General comments: The aim of the review was to discuss the validity and limitations of genotyping strategies used to correct for cure rates in drug trials and efficacy of antimalarial drugs. This is to update stakeholders and to encourage discussions around the current challenges and advance an argument for improvement of strategies.

This very well written review sets the status-quo on PCR correction during drug trials especially in highly endemic areas and during surveillance of drug efficacy in malaria endemic areas. The authors establish the fact that the US-FDA is lagging behind even with the current PCR-corrected cure rate although there is a general consensus on the relevance of the correction. They discuss the biological principle behind PCR correction and the molecular markers used. The two major drawbacks to the currently used genotyping techniques were highlighted to be either biological or technical. Finally some very practical solutions on the way forward were offered.

2.1 Page 9: An alternative method genotypes a large number of SNPs distributed over the entire genome (molecular barcode).33 All SNPs are genotyped by individual qPCRs using allele-specific probes and high-resolution melting curve analysis to distinguish nucleotides. Multi-clonal infections will yield mostly mixed signals and haplotypes for concurrent clones cannot be established for samples of high multiplicity. It should be mentioned that the molecular barcode is most suitable for clonal infections in low transmission areas.

Page 9: We have expanded our previous statement and included the point raised by Reviewer 2:

> "Multi-clonal infections will yield mostly mixed signals and haplotypes for concurrent clones cannot be established for samples of high multiplicity. Barcodes could be suitable for clonal infections in low transmission areas but their use in higher transmission areas still needs to be validated."

We fully agree with Reviewer 2 in that no haplotypes can be reconstructed for multi-clone infections.

2.2 Recommendations
For the low clone detectability (due to sequestration observed within 48 hours) can we recommend samples for days 0, 1, 2 in addition to X and X+1 even from LM-negative individuals?

We have addressed this issue in the original version of our manuscript on page 5, end of first paragraph. Owing to LID's limitation in word count, we could not elaborate further on this topic. We would be very happy to include the paragraph presented below, in case we receive permission from editors to go beyond the word limits.

Here is our response to Reviewer 2:

The practicalities of genotyping two blood samples taken 24 h apart was discussed in length at the expert meeting in 2008. From an operational aspect this is considered impracticable and not feasible by sponsors as it would require detaining patients for 24 hours post-treatment, or asking them to return next day when they may still be symptomatic. Moreover, there is invariably an ethical obligation to provide immediate treatment with rapidly acting ACTs, where parasites disappear after 8 hours post ACT treatment; the likelihood of detecting minor clones at day 1 and beyond then becomes very small. Therefore, the probability of catching what was sequestered on admission will be diminished, and this will be even more problematic for Day 2 samples. In the perspective of Single Exposure Cures that are in the pipeline, obtaining PCR samples beyond Day 0 will become even more difficult.

2.3 It is most unlikely to detect all low clones as a result of immunological processes such as sequestration; WI think we need some studies to estimate the variations in the estimates of cure rate and drug efficacy using the various PCR correction methods applied in the field. These could improve the recommended Bayesian models and incorporate the more realistic uncertainty around each approach. Also reporting of these uncertainties should be made mandatory.

The biological constraints and limitations had been discussed in detail in our manuscript. This can be found on Page 4, section "Biological constraints resulting in undetected clones".

Page 9: We have extended the original version as follows:

"Yet, biological constraints will remain the same, such as sequestration or detection limits for minority clones."

We agree in that it would be very useful to compare PCR-correction outcomes after various alternative genotyping methods have been performed in parallel on the same sample set.

Even when different algorithms for analysing genotyping data are used, we argue that the results from all PCR-correction algorithms be presented. This discussion will be in a separate manuscript that originally had been suggested as a companion paper: Jones et al. submitted to AAC (see 1.1. above).

We agree entirely with the basic point being made by the Reviewer (i.e. the need to compare and provide various methods of analysis) and we promoted this approach so that researchers have access to all different analyses and can find the one(s) most appropriate to their beliefs or which are most consistent with previous analyses.

Given this request by Reviewer 2 for more comparative studies, we find it bewildering that in section 2.7 s/he later states that molecular correction data should not be collected, a strategy that would rob future researchers of access to these different analyses.

2.4 The general problem of PCR amplification bias before sequencing needs to be addressed. Gene or genomic region capture by Molecular Inversion Probes before amplification reduces the bias in the gene or region of interest and should also be considered in the NGS approaches.

Page 9: We thank Reviewer 2 for a valuable contribution, which we have included by adding a reference to relevant literature (a recent *P. falciparum* publication on the use of Molecular Inversion Probes in genotyping: Aydemir et al. 2018)

> "Molecular inversion probes may be useful for highly multiplexed targeted sequencing.[33] "

2.5 The authors should discuss the feasibility of NGS in most disease endemic countries. If PCR-correction is made so high-tech that only a handful of labs in the North can do it, it will not adequately serve the purpose of widely informing policy in disease endemic countries.

We understand the point made by Reviewer 2 about limited access to NGS of laboratories in malaria endemic countries. Our paragraph on POSSIBLE USE OF ALTERNATIVE GENOTYPING TECHNIQUES on page 9 starts with: "This section briefly describes developments with great potential to improve genotyping of *P. falciparum* multiple-clone infections." In this section we do not argue that NGS is recommended for PCR correction. We argue that the value of alternative techniques for PCR-correction deserves to be investigated. In our view it would be a great advance for regulatory trials in endemic regions if state-of-the-art techniques are employed that finally may overcome the limitations repeatedly raised over many years and described in our Personal View manuscript in detail.

Page 9: We have modified the concluding paragraph in this section on alternative techniques (two sentences added are underlined):

> "Both these methods have the potential to improve detection of minority clones and overcome allelic suppression. Thus, these should be validated in clinical trials at high priority. In particular regulatory trials would benefit from state-of-the-art techniques that overcome the technical limitations stated above. Yet, biological constraints will remain the same, such as sequestration or detection limits for minority clones. For surveillance trials, optimized protocols of established typing techniques may be easier implemented than NGS-based methods in laboratories in endemic countries."

2.6 The authors discuss in details the technical limitations but fail to address the impact of the PCR-correction on the relevant downstream decisions. Indeed, the overall utility of PCR-correction is based on the assumption that new infections will cause no harm to the patient or have no impact on the disease epidemiology. This assumption is probably not adequate.

Reviewer 2 appears to have misunderstood our background section on PCR-correction. Just to be clear: we do not assume that "new infections will cause no harm to the patient". All

trials to our knowledge are ethically obliged to treat patients returning with recurrent malaria during follow-up because most researchers, ourselves included, believe that any episode subsequent to treatment, whether a new infection or a recrudescence, can cause harm. The requirement to treat a patient presenting with malaria infection x days after treatment, does not depend on the molecular analysis. Treatment should be given either according to the study protocol (in a drug trial) or treatment guideline (in efficacy monitoring).

PCR-correction has never been suggested to be used to inform treatment decisions at the point-of-care. Genotyping of recurrent parasitemia is generally performed on archived samples, weeks or months after the malaria episode. The purpose of genotyping is to better estimate drug efficacy in regions where episodes of infections during the follow-up period occur frequently.

Page 3: To rule out any such misconception among readers, we have made this point explicitly clear by inserting the following sentence:

> "Genotyping is performed on archived blood samples at a later point in time and does not inform or influence treatment of recurrent parasitemia. Treatment is always given, either per protocol in clinical trials or according to local treatment guidelines in drug efficacy monitoring, for all parasitemic episodes."

2.7 Indeed, what difference does it make for a Physician working in a remote health post in Africa to know whether the parasites his patient is carrying are new or old? A number of studies show that the so-called "new infections" that are discarded from the PCR-corrected cure rates:
1/ are likely to yield symptoms a few days after the end of the follow-up,
2/ are associated with anemia
3/may be involved in malaria transmission in the community
*Therefore, in this era of malaria elimination and ultimate eradication, and because it is technically challenging and of debatable public health benefit I am of the opinion that PCR-**correction is to be removed from trials for new antimalarial drugs**. We should get back to plain microscopy or use species-specific PCR to check whether parasites are present or not after drug treatment (recrudescent or new infection altogether).*

We concur with the three points above raised by the reviewer and re-iterate that PCR corrections are not used to inform treatment. They do, however, provide public health officials with a rationale for the anti-malarial treatment regimens they adopt, and of equal importance, an early warning of failure of these regimens. We thus, feel that removing PCR correction from trials of new antimalarial drugs would be scientifically and ethically inappropriate. This view is held by most scientists and stakeholders involved in drug

development and clinical trials, and they fully accept the usefulness of PCR-correction to obtain more precise estimates of drug efficacy.

The Reviewers comments are easily addressed, in particular with reference to comment 2.3 above, where s/he requests (correctly in our opinion) that various analytic methods should be attempted and reported. We simply state that separating new infections from recrudescences provides readers with maximal information from a trial. If readers believe, as does this reviewer, that this distinction is unimportant they can simply sum the recrudescences and new infections to get the overall "failure rate". If, like most people, they believe the two outcomes represent separate processes they have access to the corrected data. In other words, why deny readers access to such important information?

In the following we provide a section on the rationale for PCR-correction. This is meant as a response to point 2.7. raised by Reviewer 2, but could also be included, rephrased, as a new section into our manuscript. **This will take us over the word limit, so we leave it as an editorial decision.**

<u>The need for molecular correction</u>

Molecular correction will not be used to guide the physician's decision on treatment of a patient presenting with recurrent malaria. Genotyping is performed because it permits to estimate the therapeutic effectiveness of a drug in clearing existing infections, separately from its prophylactic effectiveness in preventing new infections. Malaria control and eliminating programmes require estimates of prophylaxis to predict their impact. The most likely end-users of clinical trials are national programme managers who, assuming they follow WHO guidelines, are mandated to change their first line antimalarial once failure rates exceed 10%. Because uncorrected failure rates include new infections, these managers could find themselves in a position where all available drugs are 'failing' on this metric and no rational basis for drug deployment would be left. We also argue that having recruited participants into a clinical trial, we have an ethical obligation to maximise the value of their participation, particularly since no further action on their part is required for molecular correction to occur (correction uses blood samples already provided by participants at the time of recurrence and treatment). While we recognise that different views will be held on how best to utilise molecular correction, our strongly-held personal view is that to wilfully ignore molecular correction is at best a missed opportunity to understand malaria treatment, prophylaxis and resistance and, at worst, at liability for effective public provision.

To illustrate the need, we provide useful comparison (Table taken from Jones et al; see section 1.1. above) showing the difference between corrected and non-corrected estimates.

Drug tested	**Uncorrected vs corrected failure rates**	**Country/yr**	**Ref**
AR-LF	54% vs 10%	Burkina Faso, 2014	(34)

AS-AQ	42% vs 10%		
AS-AQ	17% vs 6%	Congo, 2013	(35)
AR-LF	22% vs 0%	Tanzania, 2014	(36)
AR-LF	13% vs 0%	Benin, 2016	(37)
AR-LF	9% vs 2%	Mozambique, 2015	(38)
AR-LF	2% vs 1%	India 2015	(39)
AR-LF AS-AQ	16% vs 1% 22% vs 5%	Congo 2012	(40)

AR-LF, artemether plus lumefantrine; AS-AQ, artesunate plus amodiaquine

Table: The need for molecular correction: a comparison of estimated drug failure rates obtained without correction vs with molecular correction performed according to the current WHO/MMV recommended algorithm. Failure rate was calculated as 1 minus the 28-day adequate clinical and parasitological response reported in the studies (data collated and provided by Drs Jörg Möhrle and Stephan Duparc).

2.8 The new antimalarial drugs target product profiles should be oriented towards drugs that kill the existing parasites and prevent new infections for a reasonable length of time.

We agree entirely but note that target product profiles of new drugs are not the topic of our manuscript. This has been extensively discussed in the following paper (DOI 10.1186/s12936-016-1675-x)

Burrows JN, Duparc S, Gutteridge WE, et al. New developments in anti-malarial target candidate and product profiles. *Malaria J* 2017; **16**(1): 26.
For example, their Figure 2.

As this point is not directly relevant to our manuscript and owing to constraints in word counts, we did not include this additional aspect of drug development and the mentioned citation.

2.9 I would request the authors to address the above points in the paper.

We have done so, details above.

Reviewer #3: The authors present updates on PCR correction of clinical and parasitological outcomes in studies evaluating antimalarial drug efficacy, outline major limitations of the currently recommended method, and propose alternative methods to correctly distinguish between recrudescence and reinfection.

Major comments:

3.1 The authors advance very good technical arguments to demonstrate the limitations of the WHO-recommended method to distinguish between recrudescence and reinfection. However, the authors' recommendations seem to be applicable only (or mostly) in non-malaria endemic, industrialized countries with access to the latest technologies and sophisticated laboratory equipment (for example, capillary electrophoresis). Should all blood samples be sent to reference laboratories in Europe or USA for genotyping analysis? This aspect of feasibility in countries where the assessment of antimalarial drug efficacy is performed should probably be discussed.

To our knowledge many laboratories in resource-limited endemic countries (as well as in endemic countries with well-developed economies) have the same sophisticated equipment as that in Europe/USA, and sometimes more. Second, conducting a PCR-correction is not a matter of routine to be performed under remote field conditions. Such drug efficacy studies might be conducted in hospitals where lab equipment is not advanced, but the molecular analysis of samples collected is rarely done in the same hospital (or health clinic) but is always done retrospectively and sent to a research laboratory in the same country, where the equipment needed is available. Therefore, we can reassure this reviewer that PCR-correction assays have been, are, and will be generally conducted in the same endemic country as the one where the efficacy study was conducted.

Page 3: We have added a sentence (at end of paragraph) to clarify that genotyping for PCR-correction is generally performed within country.

> "Many laboratories in endemic countries already have the necessary equipment for genotyping so that molecular analyses can be routinely performed within country to monitor the effectiveness of their first-line antimalarials."

Minor comments:

3.2 Page 3, line 7: recrudescence typing and PCR-correction have become
Corrected

3.3 Page 5, line 10 from the top, LM: Does it stand for light microscopy?
Yes, corrected.

3.4 Page 5, line 13 from the top, NI: "the more likely NIs are acquired"
Corrected.

3.5 Page 7, line 11 from the top; also line 4 from the bottom, page 8, line 1, "labs": laboratories

All corrected.

3.6 Page 8, line 13: Rwanda

Corrected.

3.7 Page 8, "pharmacological models can simulate a population of patients in a drug efficacy trial…": I am not sure if this statement refers to "Jones et al. in this issue." If not, please develop further how pharmacological models can improve analysis of genotyping data.

We now cite Jaki et al. 2013, which is precursor to this work and describes the basic methodology.

Page 8: This request of Reviewer 3 was addressed as follows:

> "Pharmacological models can simulate a population of patients in a drug efficacy trial, their therapeutic outcomes, and the genotyping results that would occur at Day 0 and Day X.[16] PCR-corrected failure rates were compared using several molecular correction approaches based on simulation models from Jaki and coworkers.[16] "

3.8 Page 9, last paragraph from "The following technical recommendations…": The authors present their recommendations here, but their recommendations sound like they are mandatory ("should be universally adopted" "mandatory use"). I suggest revising the wording of these statements.

Page 10: We have revised the recommendation section as follows:

> "We propose to adopt the following technical procedures: (i) separate PCR reactions for each allelic family, rather than multiplex reactions (for msp1 and msp2), which improves detection of minority clones; (ii) use of capillary electrophoresis by automated sequencer in combination with fluorescently-labelled primers for better accuracy of fragment sizing; "

Page 10: As already suggested by Reviewer 1, we have added the following paragraph, to call for a wider discussion of experts about what should be "universally adopted".

> "We suggest that improved understanding and technical developments since publication of the WHO/MMV consensus protocols (2008) means its recommendations should be updated. Another technical meeting should be convened, particularly to address the requirements for drug registration trials that are more demanding than drug resistance monitoring studies. Regulatory trials require precise methodologies and should be implemented as state-of-the-art. The present paper could be a basis for the discussion."

3.9 Ref 20 and 32: Please complete the reference volume and/or pages.

The original citations (numbers of articles in e-Journals) were correct according to PubMed.

Personal View

PCR-correction strategies for malaria drug trials: Updates and Clarifications

Ingrid Felger[1,2*], Georges Snounou[3], Ian Hastings[4], Joerg J. Moehrle[5], Hans-Peter Beck[1,2]

[1]Swiss Tropical and Public Health Institute, Socinstrasse 57, 4002 Basel, Switzerland
[2]University of Basel, Petersplatz 1, 4001 Basel, Switzerland
[3]CEA-Université Paris Sud 11-INSERM U1184, Immunology of Viral Infections and Autoimmune Diseases (IMVA), IDMIT Department, IBFJ, DRF, Fontenay-aux-Roses, France
[4]Liverpool School of Tropical Medicine, Pembroke Place, L3 5QA UK Liverpool, UK
[5] Medicines for Malaria Venture, ICC, Route de Pres-Bois 20, 1215 Geneva 15, Switzerland

***Corresponding author:**

Prof. Ingrid Felger
Swiss Tropical and Public Health Institute
Dept. Medical Parasitology and Infection Biology
Molecular Diagnostics Unit
Socinstrasse 57
P.O.Box
CH-4002 Basel
Switzerland
Tel.: +41-61-284 8117
Fax: +41-61-284 8101
email: ingrid.felger@unibas.ch

Summary

Malaria drug trials conducted in endemic areas face a major challenge in their analysis: parasitaemia in blood samples collected after treatment may indicate a drug failure or originate from a new infection (NI) acquired after treatment. It is therefore vital to reliably distinguish drug failures from NI to obtain accurate estimates of drug failure rates. This is achieved in *Plasmodium falciparum* by comparing parasite genotypes obtained at time of treatment (the baseline) and on the day of recurring parasitaemia. Such 'PCR-correction' is required, even for new effective drugs, to obtain accurate failure rates. Despite its routine use in surveillance of drug resistance and in clinical drug trials, limitations inherent to the molecular genotyping methods have led some commentators to question the validity of current PCR-correction strategies. We describe and discuss recent developments in these genotyping strategies with particular focus on method validation and limitations. Our aim is to update scientists from public and private bodies working towards the development, deployment, and surveillance of new malaria drugs. We aim to promote discussion around these issues and argue for the adoption of improved standardized PCR-correction methodologies.

INTRODUCTION

Cure rates in clinical trials of antimalarial drugs are based on clinical assessments and microscopy conducted during several weeks of post-treatment follow-up. These rates may be corrected after genotyping parasites in patient blood samples to distinguish recrudescent infections (i.e. those containing parasites that survived drug treatment) from new infections (NI) acquired after treatment. In the past years recrudescence typing and PCR-correction have become an integral part of malaria drug efficacy trials. The 'PCR corrected efficacy' excludes all NI from treatment failures and is essential when trials are conducted in high transmission areas with frequent NI because, in those areas, even a perfectly efficacious drug would have a high apparent failure rate as, without correction, NIs would be mistaken for drug failures.

PCR-corrected cure rates and adequate clinical and parasitological response (considered as primary endpoints by European Medicines Agency and World Health Organization (WHO) are now routinely reported as primary endpoints in regulatory trials of new drugs, yet the United States Food and Drug Administration (FDA) requires uncorrected cure rates as primary endpoints and FDA's assessment of PCR-corrected rates is still pending. Nonetheless, there is wide consensus that the best available genotyping methodology, following a critical and systematic validation, would be highly relevant for regulatory trials of antimalarial drugs, because this enables more precise efficacy estimates.

PCR-adjusted outcomes are also accepted as end-points for monitoring drug resistance. The WHO widely implements 'PCR-correction' in surveillance of drug efficacy in malaria endemic areas and recommends changing first-line antimalarial therapy if the PCR-corrected failure rate exceeds 10%. Without 'PCR-correction' this threshold would be reached in high transmission areas even with highly efficient drugs because NIs would be mistaken as drug failures. Genotyping is performed on archived blood samples at a later point in time and does not inform or influence treatment of recurrent parasitemia. Treatment is always given, either per protocol in clinical trials or according to local treatment guidelines in drug efficacy monitoring, for all parasitemic episodes. Many laboratories in endemic countries already have the necessary equipment for genotyping so that molecular analyses can be routinely performed within country to monitor the effectiveness of their first-line antimalarials.

PRINCIPLE OF PCR-CORRECTION

High frequency of multi-clonal infections is a hallmark of *Plasmodium falciparum* epidemiology, whereby genetically distinct parasite clones persist concurrently and over long periods of time.[1] The number of concurrent infections in a host (multiplicity of infection)

depends on transmission intensity, acquired immunity and other host factors. Patients in high-transmission areas harbour a mean of about five parasite clones, and one or two clones in regions of intermediate or low transmission.[2,3] Highly length-polymorphic *P. falciparum* genes can differentiate these co-infecting clones.

Three molecular markers are routinely genotyped to distinguish NI from recrudescent infections in clinical trials of antimalarial drugs, the *merozoite surface proteins 1* (*msp1)* and *2* (*msp2)* and *glutamate-rich protein* (*glurp)*. In 2007 a group of experts, convened by WHO and the Medicines for Malaria Venture (MMV), released recommendations for genotyping protocols, and a consensus for the analysis of genotyping data and outcome classification was presented.[4] Any microscopy-positive parasitaemia recurring seven or more days after treatment (Day X) is genotyped and compared to the baseline sample (Day 0). Recrudescence is defined by a genotype that had already been detected in the blood sample taken prior to treatment (i.e. alleles are shared at Day 0 and Day X at all three loci). A NI is defined by the absence of a shared allele between Days 0 and X at any of the three loci. Molecular correction allows statistical estimation of drug efficacy via survival analysis or the WHO 'per protocol' method to censor new infections.[5]

Inherent limitations of currently used genotyping techniques and reluctance of laboratories to adopt more precise methods have prompted calls for caution in adopting PCR-correction as endpoint.[6,7] Two technical problems were singled out as major draw-backs: first, the difficulty in detecting all parasite clones present in a blood sample, notably so-called minority clones, and second, the limited discriminatory power of gel-electrophoresis to distinguish PCR fragments similar in size.[6]

LIMITATIONS INHERENT IN THE CURRENT, WHO/MMV-RECOMMENDED METHOD OF PCR-CORRECTION

In this section, we review limitations of 'PCR-correction' and summarize the impact of parasite biology on clone detectability. These limitations are often insufficiently acknowledged by users and in publications.

A. Biological and epidemiological limitations

Biological constraints resulting in undetected clones

Parasite clones occasionally remain undetected by PCR despite their presence in the host, an observation denoted as 'imperfect clone detectability'. Imperfect detectability is attributed

to sequestration of late *P. falciparum* stages, a biological characteristic of this species, and to naturally acquired immunity leading to fluctuations of parasite densities around the PCR detection limit. For example, clone detectability in a host has been estimated at 79% based on samples collected 24 hours apart.[8] Daily samples of infected children over 14 days showed a 48-hour periodicity for some clones.[9] Thus, the near-complete sequestration of late stages prevents the detection of some clones. One way to improve detectability is by taking blood samples on consecutive days. The effect of analyzing consecutive samples (Day 0 + Day 1 and Day X + Day X+1) was evaluated[10] and shown to significantly increase recrudescences. Nevertheless, the WHO/MMV consultation of experts did not recommend including extra samples[4] as these would be redundant with fast-acting drugs, as most patients would be negative by light microscopy on Day 1 and equally at Day X+1 after receiving rescue treatment on Day X.

Effects of long follow-up periods and local transmission intensity: the need to genotype several markers

The longer the follow-up period, the more likely NIs are acquired. Thus, PCR-correction becomes increasingly important. For drugs with a long half-life, follow-up periods may last for 63 days. NI accumulating during that period, may contain, purely by chance, an allele identical to that present at baseline. This mimics a recrudescence. To counter this effect, typing of more markers is crucial.

Even when three markers were analysed, extremes in both low and high transmission cause problems for PCR-correction. In intermediate or high malaria transmission areas, a genotype can reach 16% allelic frequency,[3] whereas in low transmission the parasite population is genetically less diverse and the frequency of the most abundant allele may reach 25%. Consequently, independent infections can share the same genotype by chance. In the context of a drug trial, NIs carrying by chance the same genotype might therefore be misclassified as recrudescence leading to overestimation of drug failure.[11,12] To prevent this error caused by limited genetic diversity in molecular markers, the WHO recommendations suggested typing of three markers to confirm a recrudescence.[4] In areas of known low endemicity or when PCR-corrected failure rates exceed 10%, baseline frequencies should be determined from a representative set of admission samples to statistically calculate the likelihood of misclassifications. Despite a multi-marker strategy, genotyping methods will suffer from lack of discriminatory power in areas with almost clonal parasite population structure. In the past, conducting trials under diverse transmission intensity has been considered important, but from a genotyping perspective, sites with moderate transmission would be ideal.

The dangers of low-level genetic signals post-treatment: gametocytes, dying and dead asexual parasites

Early gametocyte stages are susceptible to (some) antimalarials, but only 8-aminoquinolines (*i.e.* primaquine, tafenoquine[13]) are effective against circulating stage V gametocytes. Most current drugs (artemisinin combination therapies; ACTs) are given without 8-aminoquinolines (although this may change), so mature gametocytes may persist for few weeks after asexual forms have been cleared.[14,15] Gametocytes are detectable by PCR and could lead to incorrect classification as treatment failures. The WHO/MMV consultation recognised this threat and recommended that genotyping should only be performed if asexual parasites had been observed by microscopy. The persistence of gametocytes alone was not considered a criterion for treatment failure.[4] Unpublished results from simulations based on published models[16] support this recommendation by suggesting that genetic signals from gametocytes will have a negligible impact on PCR-correction provided only patients are genotyped that are microscopy-positive at Day X. Similarly, ring-specific transcripts were observed until Day 14 after ACT treatment[15], and DNA from parasites persisting at very low levels or, debatably, from dead parasites can sometimes be detected by PCR weeks after clearance.[17] Like gametocytes, these are low-level genetic signals and should be negligible (see below) in those patients whose asexual parasitaemia is patent by microscopy. This highlights the importance of genotyping only microscopy-positive infections.

B. Technical, genotyping limitations

Allelic suppression and detection limit of minority clones

Systematic investigation of technical limitations has revealed that PCR template competition during amplification (allelic suppression) contributes to imperfect detectability of individual clones.[9,18-21] The detection probability of a genotype depends on its fragment length and the ratio of dominant to minority clone, with short fragments more efficiently amplified during PCR[21]. Amplification bias and thus limited detection of minority clones has been observed in the past[19,22] but their extent and consequences were largely ignored as quantification of such effects requires systematic analysis of mixed culture strains. Reciprocal dilution series of two precisely quantified strains in increasingly discrepant proportions indicated the following results *for msp1* and *msp2*: when a minority clone fragment was longer than the dominant clone fragment, it was detectable up to a proportion of 1:5 but not in increasingly discrepant ratios. If the minority clone carried a shorter allele (the reciprocal experiment) it was still detectable in ratios 1:500 to 1:1'000.[21] Importantly, if two genotypes of the same marker

belonged to different allelic families and the families were amplified in different reactions, then template competition was removed and detection of minority clone was possible to the lowest tested ratio of 1:5'000.[21] Such excellent sensitivity in detecting minority clones has not been reached by methods other than allele-specific PCR.

***Sequential genotyping of three markers and questionable suitability of marker* glurp**

The sequential typing strategy recommended by WHO/MMV[4] has some pitfalls because it recommends that no further markers be typed if one marker indicated the presence of a NI on Day X. Consequently, if genotyping of the first marker suffered from a technical shortfall and erroneously identified a NI, no additional typing result would question that outcome. In the past many laboratories have adopted *glurp* as first marker in sequential typing because of its technical simplicity, i.e. only one nPCR was required. However, *glurp* as first marker seems problematic because among all markers *glurp* suffered from greatest amplification bias.[21] The large size differences between *glurp* fragments leads to preferential amplification of shorter fragments and loss of larger fragments. A two-fold over-representation of the clone with the shortest allele was sufficient to completely suppress detection of three larger fragments.[21] Loss of genotypes at baseline leads to an overestimation of 'NI' and consequently results in underestimated treatment failure rates. The usefulness of *glurp* is therefore questionable despite its high polymorphism. However, in trial sites where MOI is very low, most infections will be single-clone and clone competition would be largely absent. Here *glurp,* with its large genetic diversity, could be useful as third marker to increase discriminative power.

HOW CAN WE IMPROVE THE CURRENT WHO/MMV METHOD: CORRECTIVE ACTIONS TO MINIMIZE THE IMPACT OF THESE LIMITATIONS

Maximized precision of fragment sizing

Substantial efforts have been made to overcome the above limitations and improve genotyping methodology. One key strategy has been to obtain precise sizing of length-polymorphic markers by capillary electrophoresis (CE) which has become standard in many laboratories.[11,23,24] CE is vastly superior to fragment sizing by gel electrophoresis, which had a limited resolution to discriminate between similar sized alleles and where unequal loading of amounts of PCR products strongly impaired correct sizing. CE has excellent resolution and can be considered highly robust if carefully determined cut-offs eliminate stutter peaks. Some laboratories still use gels as this is technically simpler and does not require access to an automated sequencer.

New algorithms for analysis of genotyping data

Increased appreciation of the technical limitations described above has led to suggestions to change the analysis method. One suggested revision (abbreviated as "2/3 approach") demands concordant results from at least two markers to identify a NI or recrudescence. If discordant, a third marker, which could be either an established microsatellite marker[25-27] or *glurp,* should be genotyped to resolve this sample. Two new approaches to interpret genotyping data for PCR-correction were compared to the standard WHO/MMV recommendations in a small set of 44 paired samples and revealed substantial differences in outcomes.[21] A re-analysis of a larger clinical trial conducted in Rwanda by different analysis approaches using simulation models[16] suggested that these methods differ two-fold in how frequently they identify recrudescences (Jones and Hasting, personal communication).

Modelling provides a new approach to validate potential algorithms for interpreting molecular data. Pharmacological models can simulate a population of patients in a drug efficacy trial, their therapeutic outcomes, and the genotyping results that would occur at Day 0 and Day X.[16] PCR-corrected failure rates were compared using several molecular correction approaches based on simulation models from Jaki and coworkers.[16] The "2/3 approach" (i.e. using *glurp* only if *msp1* and *msp2* results were discordant) provided the best fit with the simulated treatment failure rates in a comparison to other molecular correction strategies (Figure 1). Adopting the 2/3 approach instead of the current WHO sequential typing method led to higher failure rate estimates but these were closer to the theoretical true failure rate.

The current WHO/MMV method and new algorithms currently under investigation (Jones and Hastings, personal communication), all seek to define recurrent infections as either NI or drug failure. In reality there is often some uncertainty in this decision, and a method incorporating this uncertainty could lead to improved efficacy estimates. A Bayesian algorithm was developed to adjust drug efficacy for length of microsatellite PCR products and population frequency of each genotype detected in paired samples,[28] permitting to estimate the probability of misclassification and allelic suppression. This highly promising new approach incorporates the uncertainty around the classification of NI and recrudescence.

POSSIBLE USE OF ALTERNATIVE GENOTYPING TECHNIQUES

This section briefly describes developments with great potential to improve genotyping of *P. falciparum* multiple-clone infections. One suggestion is to identify regions of <500 bp that are

rich in single nucleotide polymorphisms (SNP), which can be genotyped by next generation sequencing (NGS).[29-33] SNP-based typing methods have improved ability to detect minority clones: Low-abundant *P. falciparum* clones can be detected at a ratio of 1:1000 in mixed infections.[32] In addition, NGS can also quantify the relative abundance of concurrent clones in a host.[29,31,32] Molecular inversion probes may be useful for highly multiplexed targeted sequencing[33]. NGS-based typing has not yet been validated for molecular correction in clinical trials, but its greatly improved sensitivity to detect minor clones suggests an urgent need to do so.

Another technique genotypes a large number of SNPs distributed over the entire genome (molecular barcode).[34] Individual qPCRs using allele-specific probes and high-resolution melting curve analysis are performed for all SNPs. Multi-clonal infections will yield mostly mixed signals and haplotypes for concurrent clones cannot be established for samples of high multiplicity. Barcodes could be suitable for clonal infections in low transmission areas but their use in higher transmission areas still needs to be validated.

These methods have the potential to improve detection of minority clones and overcome allelic suppression. Thus, these should be validated in clinical trials at high priority. Particularly regulatory trials would benefit from state-of-the-art techniques that overcome the technical limitations stated above. Yet, biological constraints will remain the same, such as sequestration or detection limits for minority clones. For surveillance trials, optimized protocols of established typing techniques may be easier implemented than NGS-based methods in laboratories in endemic countries.

STEPS FORWARD AND CONCLUSIONS

The community of technical experts, trial investigators, regulators and policy makers should consider rapid adoption of new consensual protocols for genotyping in regulatory malaria drug trials, with harmonized laboratory procedures and data analyses. In particular, obtaining FDA validation of PCR-correction in drug efficacy trials requires a demonstrably robust genotyping strategy generating reproducible results. While the intrinsic biological constraints from the parasite's biology cannot be resolved, all technical issues should be addressed jointly by laboratories involved in recrudescence typing. We propose to adopt the following technical procedures: (i) separate PCR reactions for each allelic family, rather than multiplex reactions (for *msp1* and *msp2*), which improves detection of minority clones; (ii) use of capillary electrophoresis by automated sequencer in combination with fluorescently-labelled primers for better accuracy of fragment sizing; (iii) markers *msp1* and *msp2* provide the minimal essential data for PCR-correction. If both markers give congruent results (either

Recrudescence or NI), no further marker needs be typed; in the case of discrepant results, one additional marker should be added (either *glurp* or new marker); if the “2/3 approach” is not possible one should classify the episode as a recrudescence, a stringent interpretation that avoids underestimation of treatment failures. During the transition phase both the “2/3” and previous WHO/MMV algorithm should be used for backwards compatibility with previous studies and to build an evidence base to document the differences between algorithms in estimated failure rates; (iv) *glurp* could still be valuable in low endemicity areas, where it can be expected that only one allele is present per sample and no allelic suppression occurs.

PCR-correction is necessary to obtain accurate measures of outcomes in malaria clinical trials. The basic methodology, though subject to biological and technical limitations, has consistently provided better estimates of drug efficacy in clinical trials than non-PCR-corrected outcomes. We suggest that improved understanding and technical developments since publication of the WHO/MMV consensus protocols (2008) makes it important that its recommendations should be updated. Another technical meeting should be convened, particularly to address the methodological requirements for drug registration trials that are more demanding than drug resistance monitoring studies. Regulatory trials require precise methodologies and should be implemented as state-of-the-art. The present paper will serve as a basis for discussion towards a revised consensus.

In addition to adopting the “2/3 approach”, Bayesian methodologies should be applied in future to incorporate the uncertainty inherent in genotyping. Consensus should be reached to implement strictly standardized protocols. PCR-correction should become an acceptable and mandatory endpoint in both surveillance and regulatory trials of new drugs.

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Author contributions

HPB and IF conceptualized the article and designed the typing methodology.
IF wrote the original draft.
IH validated genotyping approaches by modelling, contributed figure 1, and edited the article.
GS contributed to methodology, article writing and editing.
JM critically revised the manuscript and contributed expertise in regulatory trials of antimalarials.
All authors agreed with the submitted version.

Declaration of interests

The authors declare no conflict of interest.

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Figure legend

Figure 1: Analysis of simulated trial data for DHA-PPQ with a follow-up period of 42 days. Estimated failure rates, calculated using survival analysis, are shown , for no PCR correction the WHO/MMV method of consecutively genotyping the 3 markers *msp1*, *msp2*, *glurp*, and the "2/3 marker" method. These estimates were obtained under a range of transmission intensities (the X axis) and can be compared to the true failure rate in the simulation i.e. 0.12. The most promising data analysis algorithm seemed to be the "2/3 marker" method. Data from Jones and Hastings (personal communication) using published simulation models[16].

Abbreviations

ACT	Artemisinin Combination Therapy
EMA	European Medicines Agency
DHA-PPQ	Dihydroartemisinin - Piperaquine
FDA	Food and Drug Administration (United States of America)
FOI	Force of Infection
Glurp	*Plasmodium falciparum* gene „*glutamate-rich protein"*
LM	Light Microscopy
MMV	Medicines for Malaria Venture
msp1	*Plasmodium falciparum* gene "*merozoite surface protein 1"*
msp2	*Plasmodium falciparum* gene *"merozoite surface protein 2"*
NGS	Next Generation Sequencing
NI	New Infection
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
WHO	World Health Organization

Personal View

PCR-correction strategies for malaria drug trials: Updates and Clarifications

Ingrid Felger[1,2*], Georges Snounou[3], Ian Hastings[4], Joerg J. Moehrle[5], Hans-Peter Beck[1,2]

[1]Swiss Tropical and Public Health Institute, Socinstrasse 57, 4002 Basel, Switzerland
[2]University of Basel, Petersplatz 1, 4001 Basel, Switzerland
[3]CEA-Université Paris Sud 11-INSERM U1184, Immunology of Viral Infections and Autoimmune Diseases (IMVA), IDMIT Department, IBFJ, DRF, Fontenay-aux-Roses, France
[4]Liverpool School of Tropical Medicine, Pembroke Place, L3 5QA UK Liverpool, UK
[5] Medicines for Malaria Venture, ICC, Route de Pres-Bois 20, 1215 Geneva 15, Switzerland

***Corresponding author:**

Prof. Ingrid Felger
Swiss Tropical and Public Health Institute
Dept. Medical Parasitology and Infection Biology
Molecular Diagnostics Unit
Socinstrasse 57
P.O.Box
CH-4002 Basel
Switzerland
Tel.: +41-61-284 8117
Fax: +41-61-284 8101
email: ingrid.felger@unibas.ch

Summary

Malaria drug trials conducted in endemic areas face a major challenge in their analysis: parasitaemia in blood samples collected after treatment may indicate a drug failure or originate from a new infection (NI) acquired after treatment. It is therefore vital to reliably distinguish drug failures from NI to obtain accurate estimates of drug failure rates. This is achieved in *Plasmodium falciparum* by comparing parasite genotypes obtained at time of treatment (the baseline) and on the day of recurring parasitaemia. Such 'PCR-correction' is required, even for new effective drugs, to obtain accurate failure rates. Despite its routine use in surveillance of drug resistance and in clinical drug trials, limitations inherent to the molecular genotyping methods have led some commentators to question the validity of current PCR-correction strategies. We describe and discuss recent developments in these genotyping strategies with particular focus on method validation and limitations. Our aim is to update scientists from public and private bodies working towards the development, deployment, and surveillance of new malaria drugs. We aim to promote discussion around these issues and argue for the adoption of improved standardized PCR-correction methodologies.

INTRODUCTION

Cure rates in clinical trials of antimalarial drugs are based on clinical assessments and microscopy conducted during several weeks of post-treatment follow-up. These rates may be corrected after genotyping parasites in patient blood samples to distinguish recrudescent infections (i.e. those containing parasites that survived drug treatment) from new infections (NI) acquired after treatment. In the past years recrudescence typing and PCR-correction have become an integral part of malaria drug efficacy trials. The 'PCR corrected efficacy' excludes all NI from treatment failures and is essential when trials are conducted in high transmission areas with frequent NI because, in those areas, even a perfectly efficacious drug would have a high apparent failure rate as, without correction, NIs would be mistaken for drug failures.

PCR-corrected cure rates and adequate clinical and parasitological response (considered as primary endpoints by European Medicines Agency and World Health Organization (WHO) are now routinely reported as primary endpoints in regulatory trials of new drugs, yet the United States Food and Drug Administration (FDA) requires uncorrected cure rates as primary endpoints and FDA's assessment of PCR-corrected rates is still pending. Nonetheless, there is wide consensus that the best available genotyping methodology, following a critical and systematic validation, would be highly relevant for regulatory trials of antimalarial drugs, because this enables more precise efficacy estimates.

PCR-adjusted outcomes are also accepted as end-points for monitoring drug resistance. The WHO widely implements 'PCR-correction' in surveillance of drug efficacy in malaria endemic areas and recommends changing first-line antimalarial therapy if the PCR-corrected failure rate exceeds 10%. Without 'PCR-correction' this threshold would be reached in high transmission areas even with highly efficient drugs because NIs would be mistaken as drug failures. Genotyping is performed on archived blood samples at a later point in time and does not inform or influence treatment of recurrent parasitemia. Treatment is always given, either per protocol in clinical trials or according to local treatment guidelines in drug efficacy monitoring, for all parasitemic episodes. Many laboratories in endemic countries already have the necessary equipment for genotyping so that molecular analyses can be routinely performed within country to monitor the effectiveness of their first-line antimalarials.

PRINCIPLE OF PCR-CORRECTION

High frequency of multi-clonal infections is a hallmark of *Plasmodium falciparum* epidemiology, whereby genetically distinct parasite clones persist concurrently and over long periods of time.[1] The number of concurrent infections in a host (multiplicity of infection)

depends on transmission intensity, acquired immunity and other host factors. Patients in high-transmission areas harbour a mean of about five parasite clones, and one or two clones in regions of intermediate or low transmission.[2,3] Highly length-polymorphic *P. falciparum* genes can differentiate these co-infecting clones.

Three molecular markers are routinely genotyped to distinguish NI from recrudescent infections in clinical trials of antimalarial drugs, the *merozoite surface proteins 1* (*msp1)* and *2* (*msp2)* and *glutamate-rich protein* (*glurp)*. In 2007 a group of experts, convened by WHO and the Medicines for Malaria Venture (MMV), released recommendations for genotyping protocols, and a consensus for the analysis of genotyping data and outcome classification was presented.[4] Any microscopy-positive parasitaemia recurring seven or more days after treatment (Day X) is genotyped and compared to the baseline sample (Day 0). Recrudescence is defined by a genotype that had already been detected in the blood sample taken prior to treatment (i.e. alleles are shared at Day 0 and Day X at all three loci). A NI is defined by the absence of a shared allele between Days 0 and X at any of the three loci. Molecular correction allows statistical estimation of drug efficacy via survival analysis or the WHO 'per protocol' method to censor new infections.[5]

Inherent limitations of currently used genotyping techniques and reluctance of laboratories to adopt more precise methods have prompted calls for caution in adopting PCR-correction as endpoint.[6,7] Two technical problems were singled out as major draw-backs: first, the difficulty in detecting all parasite clones present in a blood sample, notably so-called minority clones, and second, the limited discriminatory power of gel-electrophoresis to distinguish PCR fragments similar in size.[6]

LIMITATIONS INHERENT IN THE CURRENT, WHO/MMV-RECOMMENDED METHOD OF PCR-CORRECTION

In this section, we review limitations of 'PCR-correction' and summarize the impact of parasite biology on clone detectability. These limitations are often insufficiently acknowledged by users and in publications.

A. Biological and epidemiological limitations

Biological constraints resulting in undetected clones

Parasite clones occasionally remain undetected by PCR despite their presence in the host, an observation denoted as 'imperfect clone detectability'. Imperfect detectability is attributed

to sequestration of late *P. falciparum* stages, a biological characteristic of this species, and to naturally acquired immunity leading to fluctuations of parasite densities around the PCR detection limit. For example, clone detectability in a host has been estimated at 79% based on samples collected 24 hours apart.[8] Daily samples of infected children over 14 days showed a 48-hour periodicity for some clones.[9] Thus, the near-complete sequestration of late stages prevents the detection of some clones. One way to improve detectability is by taking blood samples on consecutive days. The effect of analyzing consecutive samples (Day 0 + Day 1 and Day X + Day X+1) was evaluated[10] and shown to significantly increase recrudescences. Nevertheless, the WHO/MMV consultation of experts did not recommend including extra samples[4] as these would be redundant with fast-acting drugs, as most patients would be negative by light microscopy on Day 1 and equally at Day X+1 after receiving rescue treatment on Day X.

Effects of long follow-up periods and local transmission intensity: the need to genotype several markers

The longer the follow-up period, the more likely NIs are acquired. Thus, PCR-correction becomes increasingly important. For drugs with a long half-life, follow-up periods may last for 63 days. NI accumulating during that period, may contain, purely by chance, an allele identical to that present at baseline. This mimics a recrudescence. To counter this effect, typing of more markers is crucial.

Even when three markers were analysed, extremes in both low and high transmission cause problems for PCR-correction. In intermediate or high malaria transmission areas, a genotype can reach 16% allelic frequency,[3] whereas in low transmission the parasite population is genetically less diverse and the frequency of the most abundant allele may reach 25%. Consequently, independent infections can share the same genotype by chance. In the context of a drug trial, NIs carrying by chance the same genotype might therefore be misclassified as recrudescence leading to overestimation of drug failure.[11,12] To prevent this error caused by limited genetic diversity in molecular markers, the WHO recommendations suggested typing of three markers to confirm a recrudescence.[4] In areas of known low endemicity or when PCR-corrected failure rates exceed 10%, baseline frequencies should be determined from a representative set of admission samples to statistically calculate the likelihood of misclassifications. Despite a multi-marker strategy, genotyping methods will suffer from lack of discriminatory power in areas with almost clonal parasite population structure. In the past, conducting trials under diverse transmission intensity has been considered important, but from a genotyping perspective, sites with moderate transmission would be ideal.

The dangers of low-level genetic signals post-treatment: gametocytes, dying and dead asexual parasites

Early gametocyte stages are susceptible to (some) antimalarials, but only 8-aminoquinolines (*i.e.* primaquine, tafenoquine[13]) are effective against circulating stage V gametocytes. Most current drugs (artemisinin combination therapies; ACTs) are given without 8-aminoquinolines (although this may change), so mature gametocytes may persist for few weeks after asexual forms have been cleared.[14,15] Gametocytes are detectable by PCR and could lead to incorrect classification as treatment failures. The WHO/MMV consultation recognised this threat and recommended that genotyping should only be performed if asexual parasites had been observed by microscopy. The persistence of gametocytes alone was not considered a criterion for treatment failure.[4] Unpublished results from simulations based on published models[16] support this recommendation by suggesting that genetic signals from gametocytes will have a negligible impact on PCR-correction provided only patients are genotyped that are microscopy-positive at Day X. Similarly, ring-specific transcripts were observed until Day 14 after ACT treatment[15], and DNA from parasites persisting at very low levels or, debatably, from dead parasites can sometimes be detected by PCR weeks after clearance.[17] Like gametocytes, these are low-level genetic signals and should be negligible (see below) in those patients whose asexual parasitaemia is patent by microscopy. This highlights the importance of genotyping only microscopy-positive infections.

B. Technical, genotyping limitations

Allelic suppression and detection limit of minority clones

Systematic investigation of technical limitations has revealed that PCR template competition during amplification (allelic suppression) contributes to imperfect detectability of individual clones.[9,18-21] The detection probability of a genotype depends on its fragment length and the ratio of dominant to minority clone, with short fragments more efficiently amplified during PCR[21]. Amplification bias and thus limited detection of minority clones has been observed in the past[19,22] but their extent and consequences were largely ignored as quantification of such effects requires systematic analysis of mixed culture strains. Reciprocal dilution series of two precisely quantified strains in increasingly discrepant proportions indicated the following results *for msp1* and *msp2*: when a minority clone fragment was longer than the dominant clone fragment, it was detectable up to a proportion of 1:5 but not in increasingly discrepant ratios. If the minority clone carried a shorter allele (the reciprocal experiment) it was still detectable in ratios 1:500 to 1:1'000.[21] Importantly, if two genotypes of the same marker

belonged to different allelic families and the families were amplified in different reactions, then template competition was removed and detection of minority clone was possible to the lowest tested ratio of 1:5'000.[21] Such excellent sensitivity in detecting minority clones has not been reached by methods other than allele-specific PCR.

***Sequential genotyping of three markers and questionable suitability of marker* glurp**

The sequential typing strategy recommended by WHO/MMV[4] has some pitfalls because it recommends that no further markers be typed if one marker indicated the presence of a NI on Day X. Consequently, if genotyping of the first marker suffered from a technical shortfall and erroneously identified a NI, no additional typing result would question that outcome. In the past many laboratories have adopted *glurp* as first marker in sequential typing because of its technical simplicity, i.e. only one nPCR was required. However, *glurp* as first marker seems problematic because among all markers *glurp* suffered from greatest amplification bias.[21] The large size differences between *glurp* fragments leads to preferential amplification of shorter fragments and loss of larger fragments. A two-fold over-representation of the clone with the shortest allele was sufficient to completely suppress detection of three larger fragments.[21] Loss of genotypes at baseline leads to an overestimation of 'NI' and consequently results in underestimated treatment failure rates. The usefulness of *glurp* is therefore questionable despite its high polymorphism. However, in trial sites where MOI is very low, most infections will be single-clone and clone competition would be largely absent. Here *glurp,* with its large genetic diversity, could be useful as third marker to increase discriminative power.

HOW CAN WE IMPROVE THE CURRENT WHO/MMV METHOD: CORRECTIVE ACTIONS TO MINIMIZE THE IMPACT OF THESE LIMITATIONS

Maximized precision of fragment sizing

Substantial efforts have been made to overcome the above limitations and improve genotyping methodology. One key strategy has been to obtain precise sizing of length-polymorphic markers by capillary electrophoresis (CE) which has become standard in many laboratories.[11,23,24] CE is vastly superior to fragment sizing by gel electrophoresis, which had a limited resolution to discriminate between similar sized alleles and where unequal loading of amounts of PCR products strongly impaired correct sizing. CE has excellent resolution and can be considered highly robust if carefully determined cut-offs eliminate stutter peaks. Some laboratories still use gels as this is technically simpler and does not require access to an automated sequencer.

New algorithms for analysis of genotyping data

Increased appreciation of the technical limitations described above has led to suggestions to change the analysis method. One suggested revision (abbreviated as "2/3 approach") demands concordant results from at least two markers to identify a NI or recrudescence. If discordant, a third marker, which could be either an established microsatellite marker[25-27] or *glurp,* should be genotyped to resolve this sample. Two new approaches to interpret genotyping data for PCR-correction were compared to the standard WHO/MMV recommendations in a small set of 44 paired samples and revealed substantial differences in outcomes.[21] A re-analysis of a larger clinical trial conducted in Rwanda by different analysis approaches using simulation models[16] suggested that these methods differ two-fold in how frequently they identify recrudescences (Jones and Hasting, personal communication).

Modelling provides a new approach to validate potential algorithms for interpreting molecular data. Pharmacological models can simulate a population of patients in a drug efficacy trial, their therapeutic outcomes, and the genotyping results that would occur at Day 0 and Day X.[16] PCR-corrected failure rates were compared using several molecular correction approaches based on simulation models from Jaki and coworkers.[16] The "2/3 approach" (i.e. using *glurp* only if *msp1* and *msp2* results were discordant) provided the best fit with the simulated treatment failure rates in a comparison to other molecular correction strategies (Figure 1). Adopting the 2/3 approach instead of the current WHO sequential typing method led to higher failure rate estimates but these were closer to the theoretical true failure rate.

The current WHO/MMV method and new algorithms currently under investigation (Jones and Hastings, personal communication), all seek to define recurrent infections as either NI or drug failure. In reality there is often some uncertainty in this decision, and a method incorporating this uncertainty could lead to improved efficacy estimates. A Bayesian algorithm was developed to adjust drug efficacy for length of microsatellite PCR products and population frequency of each genotype detected in paired samples,[28] permitting to estimate the probability of misclassification and allelic suppression. This highly promising new approach incorporates the uncertainty around the classification of NI and recrudescence.

POSSIBLE USE OF ALTERNATIVE GENOTYPING TECHNIQUES

This section briefly describes developments with great potential to improve genotyping of *P. falciparum* multiple-clone infections. One suggestion is to identify regions of <500 bp that are

rich in single nucleotide polymorphisms (SNP), which can be genotyped by next generation sequencing (NGS).[29-33] SNP-based typing methods have improved ability to detect minority clones: Low-abundant *P. falciparum* clones can be detected at a ratio of 1:1000 in mixed infections.[32] In addition, NGS can also quantify the relative abundance of concurrent clones in a host.[29,31,32] Molecular inversion probes may be useful for highly multiplexed targeted sequencing[33]. NGS-based typing has not yet been validated for molecular correction in clinical trials, but its greatly improved sensitivity to detect minor clones suggests an urgent need to do so.

Another technique genotypes a large number of SNPs distributed over the entire genome (molecular barcode).[34] Individual qPCRs using allele-specific probes and high-resolution melting curve analysis are performed for all SNPs. Multi-clonal infections will yield mostly mixed signals and haplotypes for concurrent clones cannot be established for samples of high multiplicity. Barcodes could be suitable for clonal infections in low transmission areas but their use in higher transmission areas still needs to be validated.

These methods have the potential to improve detection of minority clones and overcome allelic suppression. Thus, these should be validated in clinical trials at high priority. Particularly regulatory trials would benefit from state-of-the-art techniques that overcome the technical limitations stated above. Yet, biological constraints will remain the same, such as sequestration or detection limits for minority clones. For surveillance trials, optimized protocols of established typing techniques may be easier implemented than NGS-based methods in laboratories in endemic countries.

STEPS FORWARD AND CONCLUSIONS

The community of technical experts, trial investigators, regulators and policy makers should consider rapid adoption of new consensual protocols for genotyping in regulatory malaria drug trials, with harmonized laboratory procedures and data analyses. In particular, obtaining FDA validation of PCR-correction in drug efficacy trials requires a demonstrably robust genotyping strategy generating reproducible results. While the intrinsic biological constraints from the parasite's biology cannot be resolved, all technical issues should be addressed jointly by laboratories involved in recrudescence typing. We propose to adopt the following technical procedures: (i) separate PCR reactions for each allelic family, rather than multiplex reactions (for *msp1* and *msp2*), which improves detection of minority clones; (ii) use of capillary electrophoresis by automated sequencer in combination with fluorescently-labelled primers for better accuracy of fragment sizing; (iii) markers *msp1* and *msp2* provide the minimal essential data for PCR-correction. If both markers give congruent results (either

Recrudescence or NI), no further marker needs be typed; in the case of discrepant results, one additional marker should be added (either *glurp* or new marker); if the “2/3 approach” is not possible one should classify the episode as a recrudescence, a stringent interpretation that avoids underestimation of treatment failures. During the transition phase both the “2/3” and previous WHO/MMV algorithm should be used for backwards compatibility with previous studies and to build an evidence base to document the differences between algorithms in estimated failure rates; (iv) *glurp* could still be valuable in low endemicity areas, where it can be expected that only one allele is present per sample and no allelic suppression occurs.

PCR-correction is necessary to obtain accurate measures of outcomes in malaria clinical trials. The basic methodology, though subject to biological and technical limitations, has consistently provided better estimates of drug efficacy in clinical trials than non-PCR-corrected outcomes. We suggest that improved understanding and technical developments since publication of the WHO/MMV consensus protocols (2008) makes it important that its recommendations should be updated. Another technical meeting should be convened, particularly to address the methodological requirements for drug registration trials that are more demanding than drug resistance monitoring studies. Regulatory trials require precise methodologies and should be implemented as state-of-the-art. The present paper will serve as a basis for discussion towards a revised consensus.

In addition to adopting the “2/3 approach”, Bayesian methodologies should be applied in future to incorporate the uncertainty inherent in genotyping. Consensus should be reached to implement strictly standardized protocols. PCR-correction should become an acceptable and mandatory endpoint in both surveillance and regulatory trials of new drugs.

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Author contributions

HPB and IF conceptualized the article and designed the typing methodology.
IF wrote the original draft.
IH validated genotyping approaches by modelling, contributed figure 1, and edited the article.
GS contributed to methodology, article writing and editing.
JM critically revised the manuscript and contributed expertise in regulatory trials of antimalarials.
All authors agreed with the submitted version.

Declaration of interests

The authors declare no conflict of interest.

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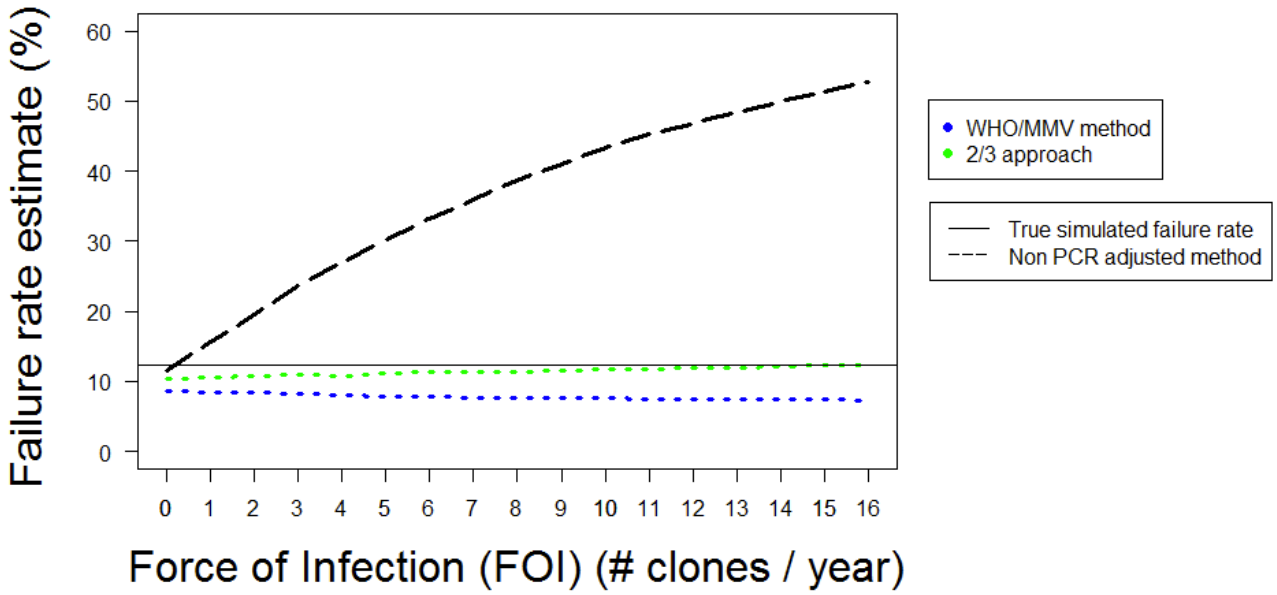
Figure legend

Figure 1: Analysis of simulated trial data for DHA-PPQ with a follow-up period of 42 days. Estimated failure rates, calculated using survival analysis, are shown , for no PCR correction the WHO/MMV method of consecutively genotyping the 3 markers *msp1*, *msp2*, *glurp*, and the "2/3 marker" method. These estimates were obtained under a range of transmission intensities (the X axis) and can be compared to the true failure rate in the simulation i.e. 0.12. The most promising data analysis algorithm seemed to be the "2/3 marker" method. Data from Jones and Hastings (personal communication) using published simulation models[16].

Abbreviations

ACT	Artemisinin Combination Therapy
EMA	European Medicines Agency
DHA-PPQ	Dihydroartemisinin - Piperaquine
FDA	Food and Drug Administration (United States of America)
FOI	Force of Infection
Glurp	*Plasmodium falciparum* gene „*glutamate-rich protein"*
LM	Light Microscopy
MMV	Medicines for Malaria Venture
msp1	*Plasmodium falciparum* gene "*merozoite surface protein 1"*
msp2	*Plasmodium falciparum* gene *"merozoite surface protein 2"*
NGS	Next Generation Sequencing
NI	New Infection
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
WHO	World Health Organization

Figure 1


Failure rate estimate (%)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Force of Infection (FOI) (# clones / year)
WHO/MMV method
2/3 approach
True simulated failure rate
Non PCR adjusted method

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