METHODS AND TECHNIQUES FOR CLINICAL TRIALS ON ANTIMALARIAL DRUG EFFICACY:

genotyping to identify parasite populations

Informal consultation organized by the Medicines for Malaria Venture and cosponsored by the World Health Organization

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WHO Library Cataloguing-in-Publication Data

Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations: informal consultation organized by the Medicines for Malaria Venture and cosponsored by the World Health Organization, 29–31 May 2007, Amsterdam, The Netherlands.

1.Antimalarials - therapeutic use. 2.Antimalarials - administration and dosage. 3.Antimalarials - standards. 4.Clinical trials. I.Medicines for Malaria Venture. II.World Health Organization.

ISBN 978 92 4 159630 5 (NLM classification: WC 770)

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Design & Layout by WHO Graphics
Printed in France

ABBREVIATIONS USED

bp	base pairs
csp	gene of the circumsporozoite protein
EDTA	ethylene diamine tetraacetic acid
DNA	deoxyribonucleic acid
glurp	gene of the glutamate rich protein
MR4	Malaria Research and Reference Reagent Resource Center
msp1 & *2*	gene of merozoite surface protein 1 & 2
nPCR	nested polymerase chain reaction
PCR	polymerase chain reaction
pPCR	primary polymerase chain reaction
resa	gene of the ring-infected erythrocyte surface antigen
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SSCP	single-strand conformation polymorphism
trap	gene of the thrombospondin-related adhesive protein
WHO	World Health Organization

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EXECUTIVE SUMMARY

The treatment outcomes in trials of antimalarial drug efficacy are classified on the basis of an assessment of parasitological and clinical effects. Estimates of success are calculated as the percentage of patients who show an adequate clinical and parasitological response before and after adjustment by polymerase chain reaction (PCR) for likely reinfection.

PCR adjustment of cure rates initially based on blood-slide microscopy and clinical assessment, is necessary because, particularly in areas of high malaria transmission, super-infection with additional parasites occurs frequently during the follow-up period of trials, owing to their long duration. Towards the end of the treatment period, antimalarial drug levels can fall below curative levels, allowing new infections emerging from the liver to establish themselves.

Thus, PCR-corrected cure rates have become accepted as the end-points in regulatory clinical trials and for monitoring antimalarial drugs. In the past, there was considerable variation in sampling procedures, genotyping techniques and interpretation of data. To achieve harmonization, the Medicines for Malaria Venture convened a meeting, cosponsored by the World Health Organization (WHO), of experts in the field of *Plasmodium* genotyping. The aim of the meeting (held in Amsterdam on 29–31 May 2007) was to achieve consensus on standard operating procedures that would be applied in all specialist malaria genotyping laboratories. The procedures were designed to be used in national malaria control programmes for routine monitoring of the efficacy of antimalarial drugs, by teams researching and developing antimalarial drugs in clinical trials conducted for regulatory purposes, and more generally for clinical research. The meeting agreed to the following definitions:

- A 'new infection' is a subsequent occurring parasitaemia in which all the alleles in parasites from the post-treatment sample are different from those in the admission sample, for one or more loci tested.
- In a 'recrudescence', at least one allele at each locus is common to both paired samples.

A number of recommendations were made in an effort to harmonize the approach used in trials to genotype malaria parasites. They cover six basic aspects of molecular typing: sampling scheme, methods of blood sampling and sample storage, genotyping strategy, analyses and outcome classification, quality control and genotyping of *Plasmodium vivax*.

The recommended sampling scheme for molecular genotyping in both regulatory clinical trials and antimalarial drug monitoring trials is to take blood samples for genotyping immediately before the first treatment (day 0) and on the first reappearance of parasitaemia after initial parasite clearance (day X). No blood samples taken 24 h after day 0 or day X need be analysed. Genotyping after treatment failure as early as day 7 is recommended.

The use of commercial blood filter paper collection cards is mandatory for regulatory trials. If no collection cards are available, untreated filter paper may be used, but only in public health surveillance trials. The use of untreated filter paper is likely to compromise the quality of genotyping substantially by increasing the missing data.

For genotyping in trials of drugs against *P. falciparum*, *msp1*, *msp2* and *glurp* should be used as the marker genes. Nested PCR (nPCR) should be used according to the genotyping procedures recommended by the consultation. Family-specific primers should be used for *msp1* and *msp2* nPCR. Genotyping of the three markers should be performed sequentially, starting with the highest discriminatory marker, which is either *msp2* or *glurp*; the third marker to be analysed should be *msp1*. The choice allows for potential variation in the performance of the different marker genes in different locations and will give priority to the marker showing the highest diversity. Primary end-point analysis should be performed with the three recommended markers, while exploratory end-point analysis can be conducted with more than three markers.

Each marker should be genotyped with the technique that provides optimal discriminatory power. Capillary electrophoresis is recommended to increase test sensitivity and discriminatory power, and the capacity for this technique should be expanded in developing countries, perhaps by setting up shared facilities, which would require countries to work with regional reference centres if national capacity was not yet adequate. If capillary electrophoresis is not available, family-specific PCR should be used for *msp1* and *msp2*. In order to increase discriminatory power, *msp2* restriction fragment length polymorphism (RFLP) can be used. Bands on agarose gels should be interpreted with software on digitized images or by two independent, experienced readers.

Once the analysis of one marker has defined a sample as a new infection, the analysis should be stopped. If no evidence of new infection is detected with the first marker, the second marker should be analysed. If no new infection is detected, then the third marker should be used. If the analysis of the third marker does not show new infection, the results indicate recrudescence.

If the PCR-corrected failure rate is > 10%, the following information should be reported to provide information on the probability of misclassification of a new infection as a recrudescence:

(i) presence of gametocytes on the day of failure;
(ii) mean multiplicity of infection calculated from at least 50 random samples from baseline for the respective site with the highest discriminatory marker; and
(iii) allelic frequencies of all genotypes identified in at least 50 random samples from baseline, or, if the genotyping technique used does not allow easy determination of allelic frequencies, at least the frequency of the dominant genotype.

Allelic frequencies can be used to calculate 'true' new infections that were missed because they share the same genotype as that in the paired baseline sample. Items (i) and (ii) of this extra information are required for both regulatory trials and public health surveillance; item (iii) is needed for regulatory trials only.

For quality assurance, it is recommended that all laboratories in which PCR analysis is performed aim for accreditation. The genotyping procedures recommended by the experts, which are available on the Medicines for Malaria Venture and WHO websites, should be used in combination with laboratory-specific practices. To test the reproducibility of results, 10% of all samples, or at least 10 randomly selected paired samples, should be internally controlled, from deoxyribonucleic acid (DNA) extraction, to PCR, fragment sizing and interpretation. A discordance of ≤ 10% of all samples is acceptable; if > 10% of the repeated samples are discordant, however, another randomly selected 10% of samples should be controlled. If the discordance remains > 10%, the whole genotyping analysis must be redone.

No recommendation is given for genotyping in trials of drugs against *P. vivax* because the interpretation of genotyping in the context of relapsing *P. vivax* infections requires further investigation.

1. INTRODUCTION

Standardization of end-points for the purposes of regulatory clinical trials and antimalarial drug monitoring has become widely accepted, driven to a large extent by the WHO Global Malaria Programme treatment guidelines, the most recent having been issued in 2006 (WHO, 2006). Use of PCR-corrected adequate clinical and parasitological response cure rates (or the complement, failure rates) as a primary or secondary end-point is accepted, as it improves the overall comparability of clinical trials. The updated WHO protocol for monitoring drug efficacy (WHO, 2003) recommends follow up for 28 days or longer, depending on the half-life of the drug, in all transmission areas, including Africa. Outcomes are classified as 'adequate clinical and parasitological response', 'early treatment failure', 'late clinical failure' and 'late parasitological failure' (WHO, 2005) (Appendix 1). The long follow-up periods, however, make interpretation of antimalarial drug efficacy outcomes difficult, particularly in high-transmission areas, because new *P. falciparum* infections occurring during follow-up can be wrongly interpreted as treatment failures. Therefore, the WHO protocol emphasizes that molecular genotyping must be used to distinguish between new and recrudescent infections. It is customary to report end-points as crude (unadjusted) and PCR-adjusted day 28 failure rates.

To further the standardization of clinical trial protocols, the Medicines for Malaria Venture and the National Institutes of Health in the United States of America cosponsored a consensus meeting on phase-III guidelines for new antimalarial drugs (held in Washington on 8 December 2005). The past 5 years have seen a large increase in the number of artemisinin-based combination drugs undergoing stringent development, four new such drugs being submitted to the competent regulatory authorities by the Medicines for Malaria Venture alone by 2008.

As the primary end-point of both clinical trials and studies of antimalarial drug efficacy is defined as the cure rate, which in turn is dependent on genotyping analyses, the Medicines for Malaria Venture convened a meeting, cosponsored by WHO (held in Amsterdam on 29–31 May 2007), to agree on molecular parameters to distinguish between reinfection and recrudescence. The main objectives of the group of experts was to reach consensus on strategies, how to use molecular tools in clinical trials of antimalarial drugs and on the methods and procedures to be used. Participants focused on reaching consensus on genotyping studies of *P. falciparum*, with the goal of publishing a document to be used as a reference by the sponsors of trials of new antimalarial drugs as well as in national malaria control programmes responsible for monitoring the efficacy of antimalarial drugs. Additionally, the meeting reviewed the current state of knowledge about the molecular tools for genotyping *P. vivax* with the aim of drawing up a research agenda for evaluating the tools to be used in assessing the efficacy of new drugs against *P. vivax* malaria.

The existing sampling procedures, sample storage methods, sample analyses and interpretation methods vary considerably, thus limiting comparisons of data from different sites and trials and rendering meta-analyses difficult (Färnert et al., 2001). This shortcoming should be limited by adoption of the consensus procedures. The discussions at the meeting and the agreed recommendations were structured around six topics: (i) sampling scheme, (ii) methods of blood sampling and sample storage, (iii) genotyping strategy, (iv) analyses and outcome classification, (v) quality control and (vi) genotyping of *P. vivax*.

2. SAMPLING SCHEME FOR GENOTYPING

2.1 Nomenclature

'**Day 0**' refers to the baseline sample collected shortly before giving the first treatment dose. Thus, day 0 is the first day of treatment. '**Day 1**' is 24 h after initiation of treatment. '**Day 7**' is the eighth day after the first day of treatment. '**Day X**' is the day on which parasitological treatment failure (WHO criteria) is first detected; it could be any day from day 7 until the end of follow-up, depending on the frequency of follow-up visits. '**Day X+1**' is 24 h after parasitological treatment failure (WHO criteria) was first detected.

2.2 Overview of current sampling strategy

Many investigators (von Seidlein et al., 2000; Adjuik et al., 2002; Gil et al., 2003; Obonyo et al., 2003; Priotto et al., 2003; Sirima et al., 2003; Adjuik et al., 2004; Happi et al., 2004; Mugittu et al., 2005; Nyachieo et al., 2005) have conducted genotyping only for recurrent infections after day 14. This is based on the fact that the prepatent period of *P. falciparum* is 6–12 days, and, therefore, genotypes that are sensitive to antimalarial drugs are unlikely to survive, multiply and initiate a new wave of parasitaemia shortly after treatment, especially with drugs with a long half-life. Molecular monitoring in previous trials showed that, already at day 7, a substantial number of genotypes (19%) were new infections (Mugittu et al., 2007); 47% of recurrent parasitaemias were genotyped as new infections at day 14; and new infections accounted for almost 60% of recurrences by day 21. This clearly shows that 1 and 2 weeks after treatment, new infections are possible. Between the time of the inoculation that resulted in the treated episode and day 0, several other inoculations can occur. For antimalarial drugs with short half-lives, the latter could reach high parasite densities and cause fever and other disease symptoms shortly after the antimalarial drug has reached subcurative levels, even before day 14.

It remains open whether the new genotypes observed are true new infections or whether they represent drug-resistant parasites that were sequestered at baseline or were at levels below the detection limit (Färnert et al., 1997). These biological limitations could be compensated partly for by sampling and genotyping on days 0 and 1 and on days X and X+1. Disappearance and reappearance of some clones within a few hours was observed in 10 of 15 symptomatic persons tested, and the resistant genotypes varied in different sample from one individual (Jafari et al., 2004). In contrast,

Färnert and Bjorkman (2005) detected the same genotypes in consecutive samples obtained every 12 h for at least 3 days after treatment of Swedish nonimmune travellers who had acquired falciparum malaria in Africa. In a more recent field trial in the United Republic of Tanzania, however, consecutive day sampling showed an increase in the number of recrudescenses in 78 recurrent episodes from 17 to 21 (Martensson et al., 2007).

2.3 Recommended sampling scheme

The data highlight the value of recommending genotyping samples of recurrent parasitaemia from day 7 onwards.

Collection of blood specimens should cause minimal discomfort to children. Most studies of antimalarial drug efficacy are conducted in children, and therefore sampling is preferably done at the same time as blood smears are collected for microscopy at or after day 7 (day 0, day 7, day 14, day 21, day 28 and any other day in case of failure). Genotyping should be performed only if asexual parasites are identified microscopically. The presence of gametocytes only is not a criterion of treatment failure.

The recommended scheme for molecular genotyping in both regulatory clinical trials and antimalarial drug monitoring trials is to perform genotyping in cases of treatment failure on blood samples collected just before treatment and on the first reappearance of asexual parasitaemia at or after day 7. These paired samples constitute those for day 0 and day X. Blood samples need not be taken 24 h after either the initial baseline sample (day 1) or on day X+1, that is 24 h after the day X sample was taken.

2.4 Rationale for sampling scheme

Genotyping after treatment failure as early as day 7 is useful. If a genotyping study is performed for a failing antimalarial drug, recrudescence will be earlier than with a new, effective antimalarial drug with which there may be proportionally more new infections. Similarly, earlier reinfection might be seen with antimalarial drugs with short half-lives. According to the WHO protocol, failures that occur before day 7 (such as all early treatment failures and any late clinical failures occurring on days 4–6) are assumed to be due to the parasite present in the baseline sample. No PCR should be performed on these samples.

Concern was raised about the practicability of sampling on consecutive days. In the case of fast-acting antimalarial drugs, such as artemisinin-based combination therapy, with which parasitaemia initially decreases dramatically (100-fold), most patients are microscopically negative on day 1, and sampling is redundant. Furthermore, it was considered that genotyping on day 1 would involve complex statistical analysis, added cost and possibly more errors. In clinical practice, it might be difficult to obtain a day X+1 sample, as the patient would already have met a study outcome and might not be available anymore. Moreover, the patient would have been given rescue treatment on day X. It was considered that more evidence is needed before 24-h sampling can be recommended. Therefore, sampling on consecutive days for both baseline and day of recurrent parasitaemia is not recommended.

3. METHODS OF BLOOD SAMPLING AND SAMPLE STORAGE

3.1 Current method of blood sampling and storage

The low rate of success in genotyping in some studies of antimalarial drug efficacy, i.e. < 90% or even < 50% (Collins et al., 2006), might be due partly to the blood sampling and storage conditions. In view of the cost and labour of molecular analyses and the importance of valid, reliable results, strategies for handling specimens for genotyping in antimalarial drug trials conducted in malaria-endemic areas deserve adequate attention.

The method used should be safe and technically easy for field workers and adapted to tropical conditions and shipment. Contamination and degradation of DNA should be prevented, and, as only small blood volumes are available, the DNA extraction technique should be robust, reliable, reproducible and easy to perform.

Capillary sampling is the method of choice. In most protocols, a blood volume of 50–200 µl is collected. Drawing venous blood means that larger volumes can be collected, but it is regarded as an invasive, complicated method. No data are available on the differences between venous and capillary blood.

In previous trials of antimalarial drugs, blood for subsequent PCR analysis was stored either by sampling on filter paper or as whole blood (Collins et al., 2006). Whole blood provides high-quality DNA and is easy to handle for DNA extraction. Storage and shipping, however, have their difficulties, as a cold chain is required. In addition, samples must be treated with anticoagulants. For this purpose, ethylene diamine tetraacetic acid (EDTA) and citrate are equivalent (Färnert et al., 1999). Another study showed better detection with EDTA than with acid citrate dextrose when blood was stored more than 2 months (Patibandla et al., 2007). Heparin should be avoided as it can inhibit PCR (Beutler, Gelbart, Kuhl, 1990; Färnert et al., 1999). For sampling small volumes of whole blood, devices such as Microvette® 100/200 (Sarstedt) or Microtainer® (Becton Dickinson) can be used. Venous blood should be drawn into plastic tubes with EDTA providing safe handling and storage, e.g. the evacuated Vacutainer® (Becton Dickinson) or Monovette® (Sarstedt). More details on storage and thawing of whole blood are given by Färnert et al. (1999).

Blood from microscopy smears (Kimura et al., 1995; Edoh et al., 1997) or rapid diagnostic tests (Veron, Carme, 2006) is not satisfactory because of low sensitivity. Moreover, blood smear samples might be cross-contaminated from other slides.

Untreated filter papers are commonly used for genotyping in drug trials. Filter paper allows easy shipping and archiving at room temperature, but it must be kept dry to avoid fungal growth and DNA degradation. Furthermore, the amount of blood is generally small and not defined. The quality of DNA also depends on the filter paper used. The success rate of PCR performed with blood spots on untreated filter paper was as low as 60% in some studies, largely due to unsatisfactory storage conditions and long storage until the DNA was eluted from the filter paper. The consensus meeting recommended that untreated filter paper should be avoided, in particular in trials for regulatory purposes, as low PCR success rates can result in outcomes that do not reflect the true efficacy of an antimalarial drug.

Increasingly, commercially available treated filter paper collection cards have been used in antimalarial drug efficacy trials in vivo. These cards overcome the major disadvantages of untreated filter paper because they prevent DNA degradation, inhibit microbial growth and render a blood sample noncontagious (for instance, for human immunodeficiency virus and hepatitis B virus).

Few comparisons of the use of different filter paper types for PCR detection of *Plasmodium* parasites have been reported (Färnert et al., 1999; Zhong et al., 2001; Coleman et al., 2006). The sensitivity of detection achieved with different filter papers can depend on either storage conditions or the method of DNA extraction (Cox-Singh et al., 1997; Bereczky et al., 2005). The newer blood collection cards provide safer storage and better DNA quality. The sensitivity of detection with IsoCode™Stix was greater than that with 903® paper (Coleman et al., 2006). PCR detection of *Plasmodium* was better from FTA® cards than from IsoCode™Stix, but none were as sensitive as frozen whole blood (Zhong et al., 2001). In another study, the results obtained with IsoCode™Stix were similar to those with whole blood when assessed without storage (Henning, Felger, Beck, 1999). A detailed assessment of various filter paper devices for optimal detection of *Plasmodium* parasites is needed.

3.2 Recommendations

3.2.1 Collection cards

At the time of the meeting, three blood collection cards made of special filter paper were commercially available: FTA® Whatman cards, Generation® Capture Cards (Gentra/Qiagen) and FP705™ DNA collection paper (FITZCO). Use of these specialized cards is mandatory for regulatory trials.

FTA® Whatman cards contain denaturants, chelating agent buffers and free radical traps that lyse cells, denature proteins and protect nucleic acids from nucleases, oxidation and ultraviolet damage. They rapidly inactivate organisms, including blood-borne pathogens, and prevent the growth of bacteria and other microorganisms. The cards can be stored at room temperature, and nucleic acids collected on these cards are stable for years at room temperature.

Generation® Capture Cards and FP705™ DNA collection paper are suitable for collection, transport and storage of low-volume samples of whole blood, bone marrow, buccal cells, saliva and cultured cells. Biological samples can be either fresh or frozen. Dried samples can be stored at room temperature for at least 9 months and probably longer; for long-term storage, however, the cards should be placed at –20 °C. They are apparently not coated with material that prevents bacterial or fungal growth and thus must be dried properly and kept with desiccants.

Collection cards should be loaded according to the supplier's instructions. As many drops of blood as can be obtained by pricking the patient's finger should be applied directly from the finger onto the circled areas of the collection card. One circle holds about 125 µl of whole blood, roughly corresponding to three drops of blood. Blood samples on collection cards should dry in about 1 h at room temperature; accelerating the drying step with heat might fix PCR inhibitors onto the matrix. Samples must be fully dried before storage and be stored dry, clearly labelled, out of direct sunlight, at room temperature, individually packed in sealed plastic bags with a desiccant. DNA can be prepared from these cards by following the manufacturer's instructions, punching a small (3 mm) disc out of the blood spot, washing it and adding it to the PCR mixture.

3.2.2 Filter paper

The use of untreated filter papers for blood collection should be avoided and is an option only for public health surveillance where no collection cards are available. Untreated filter paper, such as Whatman 3MM® (chromatography paper) or Whatman 903®, is also available as small cards. Individual samples must be stored in separate bags to avoid cross-contamination. Filter paper must be kept dry to avoid fungal growth and DNA degradation, and initial drying of untreated filter paper, either in sunlight or with a hairdryer, and dry storage are of critical importance. Filter paper should be dried rapidly in sunlight, so that the ultraviolet radiation does not degrade the DNA. The quality of the DNA depends on the filter paper used and the storage conditions, humidity being the main problem. Storage of blood spotted on paper at 30 °C and 60% humidity resulted in a significant loss of PCR sensitivity. After thorough drying, untreated filter paper is best stored at 4 °C or at 20 °C with silica gel in sealed plastic bags. When air conditioning is

not available, filter papers should be kept at 4 °C or frozen, but storage with silica gel is essential to avoid condensation. DNA extraction from blood archived on filter paper can be improved by using Tris-EDTA buffer-based extraction (Bereczky et al., 2005). The methods of sampling, storage and transit should be described in every trial report.

The consensus meeting did not recommend any specific procedure for ribonucleic acid (RNA) sampling (e.g. for genotyping gametocytes) because no validated information was available on the performance of treated filter collection cards for RNA sampling.

3.3 Rationale for recommendations

Specialized filter-paper collection cards are practical, protect DNA and allow easy DNA extraction. Their use has resulted in reproducibly higher success rates than with untreated filter paper. A minor disadvantage of these cards is the nonstandardized blood volume that is subject to DNA extraction. A fixed quantity per spot (125 µl) is claimed by the manufacturers, but the number of erythrocytes can vary, e.g. in anaemic blood. Precise quantification of the blood volume per 3-mm disc on a blood-loaded collection card was, however, not considered important. While the cost of such cards is considerably higher than for filter paper, the higher quality of the data set and the fewer unsuccessful PCRs were considered to justify the use of cards.

4. GENOTYPING STRATEGY

4.1 Definitions of 'recrudescence' and 'new infection'

The definitions of 'new infections' and 'recrudescence' were shown to have a significant effect on genotyping outcomes (Collins et al., 2006). Various definitions were used in previous trials. In order to harmonize the interpretation of genotyping results, consensus definitions were agreed upon, as explained in more detail in Appendix 2.

- A 'new infection' is a subsequent occurring parasitaemia in which all the alleles in parasites from the post-treatment sample are different from those in the admission sample, for one or more loci tested.
- In a 'recrudescence', at least one allele at each locus is common to both paired samples.

These definitions have the consequence that 'recrudescence' and 'new infection' are mutually exclusive for the analysis of trial outcomes. Any day-X sample can be either a recrudescence or a new infection. The presence of one or more new alleles in the day-X sample is not sufficient to indicate a new infection while the day-0 and day-X samples have one or more alleles in common. Thus, a sample that shows one or more new bands in the presence of at least one matching band (indicating recrudescence) remains a recrudescence.

4.2 Currently used genetic markers

Markers for genotyping are single-copy genes that are stable throughout the life cycle. They must have high allelic diversity and allow alleles to be easily distinguished. Genes with extensive size polymorphism meet these criteria. Diversity is due mainly to intragenic repeats that vary between different alleles in the copy number and the length of the repeat unit.

4.2.1 *msp1*, *msp2* and *glurp*

The antigen genes *msp1*, *msp2* and *glurp* are the most commonly used molecular markers (Snounou, Beck, 1998). They have been widely used and have given useful results. Of 116 clinical trials of antimalarial drugs conducted in endemic areas in 1997–2007, *msp2* was used in 97%, *msp1* in 78% and *glurp* in 53%. *msp2* alone was used in 19% of the studies, *msp1* and *msp2* were used in 25% and *msp1* and *msp2* & *glurp* were used in 53% (G. Snounou, personal communication).

The main reservation for the use of polymorphic antigen genes, such as *msp1*, *msp2* and *glurp*, as molecular markers is the possibility that para-

sites carrying a particular allelic variant might be selected for or against by naturally acquired immunity. The levels and specificity of these immune responses can vary among individuals. None of the clinical trials in which these markers have been used over the past 10 years, however, provided any evidence that such selection occurs in practice. The PCR-corrected results obtained were generally in accordance with expectations, thus showing high levels of recrudescence when the investigated antimalarial drug was known to be failing and high levels of new infections when the comparison drug was thought to be highly efficient.

The discriminatory powers of *msp1* versus *msp2* versus *glurp* and combinations of these were assessed. *msp1* showed less discriminatory power than the other two markers, but *msp2* and *glurp* performed equally well. Variation in the performance of the different marker genes was observed in different geographical locations, but the general pattern remained constant: *msp2* and *glurp* generally perform best. Deciding which of the two markers to use for sequential genotyping depends on the study site. Many laboratories prefer to start with *msp2*, because *glurp* PCR is prone to 'artefact bands', which are weak bands that sometimes occur in high template concentrations and are probably generated by staggered annealing of partially extended primers to the repeat region of the template.

4.2.2 Other markers

A number of trinucleotide repeat microsatellites were found suitable for recrudescence typing (Anderson et al., 1999; Nyachieo et al., 2005; Greenhouse et al., 2006; Mwangi, Omar, Ranford-Cartwright, 2006). Additional markers, such as *csp*, *resa* and *trap*, and have been used in the past (Fandeur, Mercereau-Puijalon, Bonnemains, 1996; Escalante et al., 2002).

4.3 Overview of currently used genotyping techniques

4.3.1 Nested polymerase chain reaction

nPCR is essential for detecting minor parasite populations. Amplification with family-specific primers in nPCR improves sensitivity and the discriminatory power of different alleles, in particular for minority clones. Minority clones are parasite populations of low density in a blood sample that concurrently harbour additional parasites clones, some with densities higher than that of the minority clone. The nPCR protocol is more robust than that for primary PCR (pPCR) and accepts low-quality DNA. The detection limit for *msp1*, *msp2* and *glurp* is at least 50–100 parasites per PCR reaction for field samples, where the DNA template is not always optimal (G. Snounou, personal communication). To reduce costs, pPCR can be multiplexed. Template preparation (procedure for DNA extraction) is regarded as the most critical step for successful amplification.

4.3.2 Polymerase chain reaction–restriction fragment length polymorphism

msp2 nPCR products amplified with generic or family-specific primers are digested with the restriction enzyme *Hinf I*, and the restriction fragments are separated on high-resolution agarose or polyacrylamide gels. The polymorphic fragments are thus reduced to fragments of 100–300 base pairs (bp). As the resolution of small fragments is better, differences as small as 10 bp can be differentiated. In addition, mutations at the recognition site of restriction enzymes cause loss or the appearance of new restriction sites. In areas of very high multiplicity of infection, however, the banding patterns become complex and difficult to analyse.

4.3.3 Nested polymerase chain reaction and fragment sizing by capillary electrophoresis

This method is standard for determining the size of microsatellites and has also been used for *msp2* fragments (Jafari et al., 2004; Falk et al., 2006). *msp2* is amplified by duplex nPCR with two fluorescence-labelled family-specific reverse primers. Before denaturation and loading onto an automated sequencer for fragment sizing, a fluorescence-labelled size standard is added. The fragment size is calculated in relation to the size standard fragments by dedicated software. The advantages of this method are: the best possible resolution of the fluorescence-labelled fragments by capillary electrophoresis, high throughput in a 96-capilllary automated sequencer and automated read-out. The limitations are inherent to the nature of the marker gene, as alleles that differ only in sequence are identical in length.

4.3.4 Other genotyping techniques

Southern blotting of PCR products and subsequent hybridization with labelled probes, each specific for an allelic family, has been used in some laboratories to increase the resolution of genotyping. The disadvantages of this method are the cost of the labelled probes, the membranes, the need for a dark room and the time it takes to process samples. The method has been used only very rarely for genotyping in the context of drug trials (Adjuik et al., 2002).

Microsatellite typing involves PCR amplification of one or several microsatellites (2–6 bp long tandem repeats), followed by fragment sizing by capillary electrophoresis with an automated sequencer and dedicated software (Greenhouse et al., 2006) or a 'lab on a chip' device (Nyachieo et al., 2005). The main problems are 'stutter bands', which cause false-positive alleles due to short trinucleotide repeats (Greenhouse et al., 2006).

Single-strand conformation polymorphism (SSCP) has been used in areas of low malaria transmission for typing *P. falciparum* and *P. vivax* (Ohrt et al., 1999; Taylor et al., 2000). PCR–RFLP–SSCP of the *msp2* gene followed

by silver staining has also been tried (Kain, Craig, Ohrt, 1996). The limitations of this rarely used method are the requirement for optimal fragment size and problems with reproducibility.

In heteroduplex tracking assays, ^{35}S-radiolabelled probes are hybridized to amplicons from either *msp1* or *msp2* to form heteroduplexes, which migrate on polyacrylamide gels differently according to the degree of sequence homology (Ngrenngarmlert et al., 2005; Kwiek et al., 2007). The method is quantitative and sensitive to minority variants. Its disadvantages are laborious probe generation and its reliance on radioisotopes.

4.4 Sequential analysis

As the extent of diversity among molecular markers differs, they should be genotyped and analysed in sequence. Genotyping is initiated with the most diverse marker, and the experimental work is stopped once a marker classifies the paired samples as a new infection (Appendix 3). This strategy reduces costs.

4.5 Optimization of discriminatory power and standardization of fragment sizing

The discriminatory power of a polymorphic marker depends on separation of the amplified fragment by electrophoresis. The amplified fragments range in size from 150 bp for *msp1* to 1200 bp for *glurp*. Several options are available for increasing the discriminatory power of genotyping:

(i) improving gel electrophoresis: In standard agarose gels, minor size differences cannot be determined for large fragments. Standard agarose gels can be replaced by polyacrylamide gels (in particular for small fragments) or by high-resolution agarose for fragments up to 800 bp. These alternatives increase electrophoretic resolution substantially.

(ii) improving discrimination of similar-sized fragments: Several techniques, such as PCR–RFLP and fragment sizing by capillary electrophoresis, are available (Felger et al., 1999; Jafari et al., 2004; Falk et al., 2006).

(iii) improving sizing and interpretation of PCR fragments: For ethidium bromide-stained gels, the dedicated software for sizing fragments usually included in digital gel documentation systems should be used. Alternatively, commercial software exists for comparing bands in gels. Any gel analysis software can be used to ensure unbiased comparison of paired samples and for setting cut-off intensities for spurious bands.

4.6 Effect of transmission intensity

The main factors influencing genotyping outcome are the endemicity of malaria at the trial site, reflected in the multiplicity of infection, and the diversity of molecular markers.

In areas of low and moderate transmission intensity (corresponding to a low mean multiplicity of infection), only a few new infections are to be expected during the trial period. Genotyping samples of low complexity is straightforward; however, the marker genes have few alleles. Restricted diversity thus compromises the discriminatory power of a marker. Also, some alleles reach high allelic frequencies (> 0.1), thus increasing the chance that new infections will have the same genotype as an infection at baseline. As a practical consequence, in low transmission areas, the discriminatory power of marker genes should be optimized by maximizing the number of distinct alleles for genotyping. This can be achieved by adding restriction digests, using family-specific primers or by high-resolution fragment sizing (capillary electrophoresis).

With increasing transmission intensity (corresponding to a high mean multiplicity of infection), the number of possible new infections during the trial period increases. Also, the diversity of molecular markers is greater, and, as a consequence, the allelic frequencies decrease. With a large number of concurrent alleles, genotyping becomes increasingly difficult (similar sized alleles might end up in the same size bin). Alleles often differ by only 3 bp, which cannot be determined by standard agarose gel electrophoresis. In these settings, it is essential to optimize the resolution of fragment sizing (capillary electrophoresis).

4.7 Standard protocols

Marker genes for genotyping, PCR primer sequences, documentation and reporting should follow the recommended genotyping procedures agreed upon by the experts, which are available as a separate document from the Medicines for Malaria Venture and WHO websites. These recommended procedures should be used as the basis for laboratory-specific standard operating procedures but should be completed with the materials, reagents and reaction conditions specific to the laboratory concerned. Positive DNA controls should be prepared by a central laboratory and provided on collection cards through the Medicines for Malaria Venture and WHO at no cost. Details of positive controls are given in section 6 on quality assurance.

4.8 Genotyping of gametocytes at day X

An allele detected on day X might be due to circulating gametocytes that have the same genotype as a parasite clone found on day 0. Especially in trials of antifolates, it is likely that most of the parasitaemia at day 7 to day 14 is due to gametocytes (H. Babiker, personal communication). This can lead to misclassification of a new infection as a recrudescence, and this possibility should be considered in particular if, on day X, gametocytes are detected by thick-smear microscopy. The possibility of genotyping gametocytes from RNA collected on day X was discussed, but no recommendation for

genotyping gametocytes was made because of its technical difficulty (starting from storage of RNA) and because the methods are still under development (Menegon et al., 2000; Nwakanma et al., 2008).

4.9 Recommendations

Samples for PCR should be prepared according to the recommended genotyping procedures (to be downloaded from the Medicines for Malaria Venture and WHO websites) and the manufacturers' instructions supplied with filter paper collection cards.

For genotyping *P. falciparum* in antimalarial drug trials, *msp1*, *msp2* and *glurp* should be used as the marker genes. nPCR should be used according to recommended genotyping procedures. Family-specific primers should be used for *msp1* and *msp2* nPCR.

The three markers should be genotyped sequentially, from the highest to the lowest discrimination power. It is recommended that the procedure start with the highest discriminatory marker, which is either *msp2* or *glurp*. The choice allows for potential variation in the performance of the different marker genes in different locations. The third marker to be analysed should be *msp1*.

Each marker should be genotyped with the technique that provides optimal discriminatory power. To increase test sensitivity and discriminatory power, capillary electrophoresis is recommended. The capacity for using this technique should be expanded in developing countries, perhaps by setting up shared facilities, thus requiring countries to work with regional reference centres if national capacity is not yet adequate. If capillary electrophoresis is not available, family-specific PCR should be used for *msp1* and *msp2*. To increase discriminatory power, *msp2*-RFLP can be used (optional).

Bands on agarose gels should be interpreted with software on digitized images. If a digital gel documentation system is not available, paired samples should be analysed by two independent researchers after side-by-side runs on the same gel. The means of visualization should be reported in any publication.

Primary end-point analysis should be performed with the three recommended markers. Exploratory end-point analyses could be performed with more than these three markers. Once the analysis of one marker has shown a new infection, the analysis should be stopped. If no evidence of new infection is detected with the first marker, the second marker should be analysed. If no new infection is detected, then the third marker should be used. If the analysis of the third marker does not show new infection, the results indicate recrudescence.

4.10 Rationale for genotyping strategy

nPCR is recommended. Although it is costly, it increases sensitivity. Family-specific nested primers are recommended because they add discriminatory power and may support amplification of minor clones, provided they belong to another allelic family than the dominant clone.

Sequential processing of marker genes is recommended to save costs and avoid unnecessary experiments. *msp2* and *glurp* perform equally well in most areas. *msp2* was found to be more diverse in some laboratories (Cattamanchi et al., 2003) and less prone to generation of artefact bands, but *glurp* is more convenient because it requires only one nPCR. The primarily typed marker gene can be defined by each laboratory. The choice of *msp2* or *glurp* depends on the location.

Poor resolution of fragments results in apparent little diversity of the marker gene and thus increases the number of false recrudescences. Therefore, every effort should be made to optimize the discriminatory power of the genotyping system.

Particularly in areas of high multiplicity of infection, it is difficult or even impossible to analyse the many PCR fragments present per sample, if the fragments differ by only three or six nucleotides. Separation of PCR products by capillary electrophoresis in an automated sequencer combined with sizing of fragments provides optimal resolution. Three to four different fluorescent dyes can be used in each sample, in addition to the fluorescence-labelled size standard, making it possible to perform multiplex PCR (e.g. for family-specific primers or for multiple genes) and analyse the differentially labelled fragments in a single well. As optimal resolution of the sizes of alleles is critical for identification of recrudescences or new infections, this method is recommended, and efforts should be made to establish the technique. Although fluorescence-labelled primers and capillary electrophoresis mean higher costs, the resulting gain in precision by an automated read-out and reduced labour costs justify its adoption. A further advantage of capillary electrophoresis is the automatic determination of allelic frequencies, as the absolute size of each fragment is recorded.

If PCR fragments are sized by gel electrophoresis, digitized images of the stained gels should be analysed with dedicated software for fragment sizing. This provides a digital output, increases the precision of sizing and leads to impartial measurements and higher resolution of individual genotypes.

An alternative for increasing the diversity of marker genes is PCR–RFLP. This technique has been used for longitudinal tracking of individual alleles in trials of antimalarial drugs and other intervention studies (Irion et al., 1998; Felger et al., 2003; Mugittu et al., 2006). Distinguishing multiple allelic restriction patterns becomes increasingly difficult when the multiplicity of infection is > 5.

5. ANALYSIS AND OUTCOME CLASSIFICATION

Classification of treatment success is based on an assessment of the parasitological and clinical outcome of antimalarial treatment, according to the latest WHO recommendations (WHO, 2005) (Appendix 1).

Two estimates of treatment failure rates are reported for a 28-day surveillance, as the primary outcome of treatment efficacy trials: failure unadjusted for reinfection by genotyping and failure adjusted for reinfection by genotyping.

Analysis by genotyping should be performed as indicated in the flow chart shown in Appendix 3. By adopting the most stringent definitions of new infection and recrudescence (Appendix 2), only mutually exclusive results with respect to trial outcomes can be obtained in a successful PCR.

5.1 Limitations of genotyping

New infections can be misclassified as recrudescences if alleles detected on the day of recurrent parasitaemia by chance match a pretreatment allele. Methods to minimize such misclassification are recommended in section 4.5 on optimization of discriminatory power and standardization of fragment sizing.

The presence of rare gametocytes persisting from the initial infection, resulting in a false classification of recrudescence, is discussed in section 4.8 on genotyping of gametocytes at day X.

For further information on limitations of genotyping, see Snounou and Beck (1998).

5.2 Considerations for the interpretation of PCR-corrected failure rates under specific epidemiological conditions

The ability of the genotyping strategy to differentiate recrudescences from new infections with a good degree of accuracy depends on the following assumptions: (i) the genetic markers have high diversity; (ii) the frequency of each allelic variant present in the parasite population under study is unbiased, i.e. the most frequent allelic variant is present in < 20% of the parasites found in the areas in which the study was undertaken; and (iii) the average multiplicity of infection in the patients, particularly on day X, is not excessively high (multiplicity of infection < 4).

In some clinical study sites in geographical areas where extreme epidemiological conditions prevail, one or more of these assumptions might not be fully met.

If the genetic diversity of the parasite population is extremely limited, such that each of the markers used for genotyping has only a few variants, any new infection is likely to be due to parasites with genotypes similar to those in the baseline sample; thus, the new infection will be falsely classified as a recrudescence. This is a relatively rare situation in areas in which trials of antimalarial drugs are conducted, as such low genetic diversity is usually associated with isolated areas with low transmission intensity or with the introduction of a limited parasite population in a nonendemic area. In such areas, PCR genotyping corrections are superfluous. A particular allelic variant will probably dominate the parasite population for all the genetic markers used for genotyping. Thus, the chance of being newly infected with parasites with the same genotype as those present in the baseline sample increases with the frequency of the dominant marker, as does the probability of misclassifying these cases as recrudescences.

Generally, a situation with a dominant parasite genotype is not encountered in most endemic areas in Africa, although it can occur in areas on the fringe of the main transmission regions, especially in geographical regions of highly seasonal transmission, or in areas of lower transmission intensity. In order to correct for the biased distribution of a particular parasite genotype, the probability of reinfection with the same genotype could be calculated, after establishing the allelic frequency distribution in the parasite population at the time of the trial.

A high multiplicity of infection is usually associated with high transmission intensity. This is a common situation in clinical trials, which tend to be carried out in areas where malaria is a serious problem and patient recruitment is therefore easier. In such extreme transmission situations, with very high mean multiplicity of infection values (> 4), the probability of being newly infected with one of the genotypes present in the day-0 sample can reach unacceptable levels, because many of the new infections will be falsely classified as recrudescences.

As the transmission intensity increases, the genetic diversity of the parasite population tends also to increase and the frequencies of the dominant allelic types to decrease, thus minimizing this risk of new infections by the same

genotype. Nonetheless, there are only limited options to minimize overestimation of recrudescences. In order to reduce the number of new infections by mosquito bites, other measures for inhibiting transmission should be considered, e.g. providing bednets to patients in order to reduce transmission after treatment.

These points were not taken into consideration in most of the trials of antimalarial drug efficacy in which PCR genotyping was used to correct the outcome. Nonetheless, the conclusions derived from these studies were consistent with expectations. The meeting therefore decided that more refined analyses of parasite genetic diversity should not be a prerequisite for PCR genotyping in antimalarial drug trials under most circumstances. If the PCR-corrected failure rate is found to exceed 10%, however, especially if an otherwise highly efficient antimalarial drug or antimalarial drug combination is used, it becomes important to review the genotyping data to determine if the high rate of failure is due to the prevailing epidemiological conditions or to the appearance of drug-resistant parasites (Greenhouse et al., 2007).

5.3 Missing data

The reasons for missing data must be indicated; for example, 'sample not taken', 'missing follow-up sample' or 'no PCR result' ('PCR negative'). Missing values reduce the sensitivity of a trial, and losses in comparative trials lead to loss of power (Guthmann et al., 2006). Therefore, all efforts should be made to minimize missing data.

5.4 Recommendations

5.4.1 Provision of extra data if PCR-corrected failure rates > 10%

If the PCR-corrected failure rate in a clinical trial of an otherwise efficient antimalarial drug or drug combination exceeds 10%, information should be provided to facilitate interpretation of the results and to allow comparison of the data. The following information should allow assessment of the probability of misclassification of a new infection as a recrudescence because by chance it carries the same genotype as an infection present at baseline:

(i) mean multiplicity of infection determined from at least 50 randomly chosen admission samples (day 0) for the respective site with the most discriminatory marker. Samples that have previously been analysed to determine PCR corrected failure rates (day 0 from paired samples) can be used. Multiplicity of infection is a rough but accepted and easily obtained measure of transmission intensity.

(ii) allelic frequency of the dominant genotype that serves as an indicator of those 'true' new infections that were missed because they had the same genotype as the paired baseline sample. If the genotyping technique

used can provide the frequencies of all genotypes (e.g. when sizing PCR fragments by capillary electrophoresis), these should be reported.

(iii) presence of gametocytes on the day of failure.

Determination of the mean multiplicity of infection and the allelic frequencies of all or of at least the most frequent allele(s) is mandatory for regulatory trials if the failure rate is > 10%, whereas for surveillance determination of multiplicity of infection is sufficient.

The average multiplicity of infection for the most diverse genetic marker is obtained by simply counting the number of bands in all samples and dividing this sum by the total number of PCR-positive samples. The allelic frequency of the dominant genotype is determined from the same gels and is obtained by counting the number of times a particular allelic variant (a band of a defined size on the gel) is observed, divided by the total number of bands observed in all the samples.

The main difficulty is in identifying a band of a defined size in different gels. Therefore, the amplified products of the 50 or more day-0 samples should be run side-by-side on a small number of gels. The gel pictures can be enlarged to an A4 format to increase the accuracy of migration distance measurements. If a gel documentation system with corresponding software is not available for sizing individual fragments, bins should be defined on the enlarged gel picture; a ruler placed in line with two markers of relative molecular mass should be used to define areas for individual bins of about 20 bp. Irrespective of the method of size determination (visually or with gel documentation software), the main limiting factor is the quality of the gels used to analyse the amplification products. Use of agarose with the most appropriate resolution for the size range of the allelic variants in question is of paramount importance. Ultimately, capillary electrophoresis will provide the best results, but the cost of the equipment makes it unlikely that it will be universally adopted (see section 4.5).

In order to detect the presence of gametocytes on the day of failure, at least 2000 white blood cells should be counted.

5.4.2 Interpretation of results

All PCRs of both pre- and post-treatment samples that do not give an amplification product should be repeated twice; if still no amplification product is obtained, genotyping should be carried forward to the next marker. If all three markers give no amplification product, PCR for *Plasmodium* species should be performed (according to recommended genotyping procedures)[1]. If no other *Plasmodium* species is identified, the blood film should be re-checked microscopically. If microscopy shows the presence of *Plasmodium*,

[1] For samples from South East Asia: *P. knowlesi* should be included in *Plasmodium* species PCR.

the PCR should be considered uninterpretable, and the patient should be considered as 'failure', 'excluded' or 'censored', according to the method of analysis dictated by the protocol.

If another *Plasmodium* species is identified in the day-X sample (in the absence of *P. falciparum*), the parasitaemia is regarded not as a recrudescence but as a new infection, and the patient will be considered as 'failure', 'excluded' or 'censored' according to the method of analysis dictated by the protocol.

DNA extraction from a Giemsa-stained blood smear can be attempted, but DNA preparation is not very reliable, and the sensitivity of PCR detection is much lower than with filter paper samples (Kimura et al., 1995; Edoh et al., 1997; Scopel et al., 2004).

If only two of the three loci can be amplified in paired samples and the PCR fragments in the paired samples are completely different in at least one locus, then the day-X sample is considered a new infection. If the paired samples have at least one identical band and this is found at both loci amplified, the day-X sample is considered a recrudescence. If only one locus can be amplified and this marker indicates recrudescence, with shared bands, the sample is defined as recrudescence.

5.5 Rationale for recommendations on outcome analysis

As samples in clinical trials of antimalarial drug efficacy at sites with high malaria transmission can be difficult to analyse because of frequent super-infections during the trial period, reporting of extra information, in addition to genotyping-adjusted trial outcomes, is strongly recommended. While multiplicity of infection and the presence of gametocytes give a straightforward indication of potential misclassification of new infections as recrudescences, allelic frequencies provide additional information about the resolution of the genotyping technique used and a rough estimate of the minimum error due to missed new infections. The additional information is useful for inter-trial comparisons of the performance of genotyping.

The probability of a new infection with a parasite with a genotype that is already present can be estimated by statistical methods. These would take into account the frequencies of alleles in the baseline sample and the probability of reinfection with the same genotype, and could thus 'correct' for

misclassification. The expert meeting did not advocate any specific procedure for mathematical correction of rates of new infection, because the usefulness of the various approaches remains to be validated[2].

An estimate of expected heterozygosity or a similar index provides a measure of the discriminatory power of a particular genotyping method. In addition, it permits a more accurate correction of a clinical trial outcome than the currently used, simple PCR adjustment. Mathematical estimation of true new infections can compensate for the shortcomings and limitations of genotyping in a clinical trial.

A precondition for estimating the true rate of new infections is a precise determination of the allelic frequencies of all the genotypes of the most polymorphic marker gene. Depending on the genotyping technique, this can easily be achieved (if capillary electrophoresis is available), but it can involve considerable effort if automated fragment sizing is not used. The effort is justified, however, for antimalarial drug trials for regulatory purposes if the failure rate exceeds 10% and genotyping adjustment is complicated by environmental factors such as high transmission intensity or little genetic diversity of molecular markers.

The experts considered it essential to generate additional information to allow interpretation of trial outcomes, particularly when the observed failure rate with an otherwise effective drug is high, thus highlighting the limitations of genotyping. While multiplicity of infection and the presence of gametocytes can be observed easily in any trial, determination of allelic frequency requires more investment and equipment. This will remain a challenge for many national malaria control programmes in endemic countries. Therefore, information on allelic frequencies in cases of > 10% failure rates in surveillance trials was considered the preferred but not the mandatory genotyping result.

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[2] One equation for estimating the number of true recrudescent infections was presented by Greenhouse et al. (2007). Other authors have proposed similar approaches (Brockman et al., 1999; Kwiek et al., 2007). The Nei index describes the expected heterozygocity at one locus (Nei, Chesser, 1983) and is another indicator of the probability that two infections share the same allele by chance.

6. QUALITY ASSURANCE

Unequivocal standards and minimal criteria have been designed to help generate accurate, reliable molecular diagnostic laboratory testing results. For detailed information, see the e-publication of the American College of Medical Genetics (2007).

6.1 Essential preventive measures

The conduct of PCR on an epidemiological scale is prone to contamination. To avoid this risk, a number of measures must be adhered to:

- 'Contamination controls' are negative controls, comprising uninfected human blood for DNA extraction control and PCR control or water for PCR control. For each 96-well microtitre plate, at least three randomly distributed negative controls are appropriate. Because family-specific PCRs are carried out for *msp1* and *msp2*, all samples harbouring members of only one family will provide additional contamination controls for the PCR mix of the alternative family. For *glurp*, which is amplified with a generic primer set, six controls should be used.
- As soon as contamination is identified, e.g. by detection of a PCR product in a negative control, the reporting of any results should be stopped until the source of contamination has been identified and eliminated.
- The sample preparation steps must be clearly separated spatially from the amplification and post-amplification procedures. A PCR product-free room should be used for DNA preparation, and the master mix for PCR should be prepared in a dedicated template-free space and, if possible, in a flow hood that can be ultraviolet-irradiated after usage.
- It is recommended that a dedicated laboratory coat be worn for pre-PCR procedures. Use of gloves for PCR is controversial, as it does not prevent contamination but gives the illusion of safe handling.
- The working areas for all pre-PCR steps must be cleaned after each experiment with water-diluted dish-washing liquid, followed by drying with paper towels, with frequent use of 1-mol/l hydrochloric acid or a commercial cleansing solution that is active against DNA contamination, in particular for cleaning contaminated working spaces.
- Materials should not be transported from post-PCR areas into locations dedicated for DNA and master mix preparation.
- Equipment (e.g. pipettes) should be tested and calibrated at predetermined intervals.

- A template-free set of pipettes should be used for preparing master mixes. In addition, tips with aerosol barriers (filter tips) are recommended for pipetting templates, e.g. when pipetting an aliquot of pPCR into nPCR.
- All pipette tips and disposables should be collected in plastic bags, which should be cleared away after each experiment and autoclaved before disposal into general waste.
- Manuals providing laboratory-specific standard operating procedures, with details of all laboratory procedures and policies, must be compiled and followed. All changes to protocols must be signed and dated.
- All laboratories should have documented quality assurance and quality control procedures to ensure optimal performance of all methods, reagents and equipment.
- Labelling of blood collection cards must contain at least two identifiers, such as the sampling code of the patient, the patient's study identification number or other unique identifier, in addition to the date of blood collection, the day of follow-up and, when appropriate, the time of collection.
- The sample intake information of the laboratory should consist of a record of the arrival date and the quantity and the qualitative condition of the sample (e.g. insufficient quantity, exposure to extreme temperatures, inappropriate labelling or packaging).
- The laboratory should retain the original patient sample until all testing of the specimen is completed and the report has been signed off.
- Report forms must be designed and used. The results should be clearly presented (with cut-offs if appropriate), followed by an interpretive statement to explain the result in the context of the question asked (recrudescence, new infection, missing data).

6.2 Positive controls

Positive controls for genotyping in trials of *P. falciparum* antimalarial drugs are provided by the Medicines for Malaria Venture and WHO. As the recommended genotyping procedures recommend use of a number of family-specific nPCRs, three *P. falciparum* in vitro culture strains are provided, which are the laboratory strains FC27, 3D7 and Ro33. These correspond to the three allelic families of *msp1* and the two allelic families of *msp2*, as follows: for the culture strain FC27, the *msp1* allelic family is Mad20 and

the *msp2* family is FC27. For culture strain 3D7, the *msp1* allelic family is K1 and the *msp2* family is 3D7, while for the culture strain Ro33, the *msp1* allelic family is Ro33.

Glurp genotyping does not involve family-specific nPCR.

To ensure the sensitivity of the PCR assay, a low template concentration should be used (200 parasites per PCR). Accordingly, the concentration of the positive controls provided by the Medicines for Malaria Venture and WHO will be such that a 3-mm filter disc will contain approximately 200 parasites of each of the three strains. In addition, a positive control of higher density will be provided, which will introduce about 2000 parasite genomes per strain into the pPCR with each filter disc. The high-density positive controls can be used to optimize suboptimal procedures. A detection limit of 50–100 parasites per PCR is generally achieved in PCRs for *msp1*, *msp2* and *glurp*. The in-vitro culture strains can also be obtained from the Malaria Research and Reference Reagent Resource Center (MR4).

As no cultured strains are available for *P. vivax*, *P. ovale* and *P. malariae*, the 18S rRNA genes of these species have been cloned into plasmids. Collection cards holding plasmid DNAs of all four species will be distributed by the Medicines for Malaria Venture and WHO. These plasmids have also been deposited at the MR4. The detection limit of species PCR based on 18S rRNA genes is about 10 parasites per PCR reaction.

6.3 Recommendations

The overriding recommendation is to use laboratory specific practices and recommended genotyping procedures that can be downloaded from the Medicines for Malaria Venture and WHO websites.

It is recommended that all laboratories performing PCR analysis should aim for accreditation.

To test the reproducibility of results, 10% of all samples or at least 10 randomly selected paired samples should be internally controlled, from DNA extraction, PCR and fragment sizing to interpretation.

Less than 10% discordant samples is acceptable. The limit of acceptability is that, within the repeated 10% of all samples or a minimum of 10 samples, there is only one discrepant result. The same cut-off applies for up to 19 repeated samples. From 20 repeated samples, two discrepancies are acceptable. From 30 onwards, three discrepancies can be accepted.

The term 'discrepancy' refers to the outcome (recrudescence versus new infection), and not to discordance in individual bands. This is based on the fact that some minority clones with densities around the detection limit will be amplified in one reaction but not in a repeat because the parasite was not present in the blood volume on the second disc. Fluctuations within minority clones are due to chance. If > 10% of the repeated samples are discordant, another randomly selected 10% of samples must be tested; if the result is still > 10% discordant, the whole analysis must be re-run.

7. GENOTYPING *PLASMODIUM VIVAX*

7.1 Relapse from liver

In infections with *P. vivax* and *P. ovale*, some parasites, the so-called hyponozoites, can remain dormant in the liver for several months or up to several years after an infective bite. In Asia, the relapse rates after hypnozoite re-activation vary considerably, ranging from 20% in India to 60% in Thailand. Most relapses resulting from such re-activations are not due to the parasites that were detected at the initial presentation. For example, in a study in which primary and relapse parasites were genotyped, more than half the parasites that caused the relapse did not match the genotype at baseline (Chen et al., 2007; Imwong et al., 2007). It is possible that febrile episodes, including vivax malaria, could trigger a *P. vivax* relapse. This poses a major obstacle for PCR-adjustment in *P. vivax* antimalarial drug trials, because true relapses caused by re-activated hypnozoites can never be excluded as a cause of recurrent infection.

7.2 Radical cure

To prevent vivax relapses, patients are treated with primaquine, which kills the hypnozoites. Primaquine is the only antimalarial drug that acts on hypnozoites, but it is not 100% effective, and the optimal dose remains unclear. In addition, G6PD deficiency should ideally be tested before administration to avoid oxidant haemolysis.

7.3 Molecular markers for *P. vivax* genotyping

The most commonly used molecular markers for *P. vivax* genotyping, *Pvcsp*, *Pvmsp1* and *Pvmsp3*, all show extensive size polymorphism (Rayner et al., 2004; Imwong et al., 2005; Kim et al., 2006; Zakeri, Barjesteh, Djadid, 2006). As in *P. falciparum* trials, sequential analysis of several markers should be used in *P. vivax* trials. The current method for genotyping to determine recrudescence or new infection in trials of *P. vivax* antimalarial drugs involves the two marker genes *Pvcsp* and *Pvmsp1* (Imwong et al., 2005). Use of *Pvcsp* as a marker involves a restriction digest, and RFLP patterns are used to identify the different genotypes. Two regions in *Pvmsp3* have been used in *P. vivax* genotyping studies, *Pvmsp3a* (Bruce et al., 1999; Zakeri, Barjesteh, Djadid, 2006) and *Pvmsp3b* (Rayner et al., 2004). *Pvmsp3a* typing involves restriction digest of the PCR product. In addition, *Pvmsp4* has been found to show some length polymorphism (Martinez et al., 2005). The polymorphic genes that display single nucleotide polymorphism are *Pvama1* and the Duffy binding protein, the latter being

more diverse than *Pvama1*. Some microsatellites (2–6 bp tandem repeats) show extensive size polymorphism and have been used for genotyping *P. vivax* (Gomez et al., 2003; Carnevale et al., 2004; Imwong et al., 2006; Alam, Agarwal, Sharma, 2007).

7.4 Potential role of genotyping in *P. vivax* clinical trials

The Indonesian experience is the basis for the suggestion that failures of chloroquine therapy until day 16 are almost always due to recrudescence, while failures between days 17 and 28 may be due to either recrudescence or relapse (Baird et al., 1997). Failures after day 28 are more likely to be due to relapses, but late recrudescence could also occur with low levels of chloroquine resistance (although this has not been studied). Measurement of chloroquine and desethylchloroquine in blood can help to distinguish between chloroquine-sensitive and chloroquine-resistant isolates. Genetic characterization of pre-treatment and post-treatment isolates by PCR can be used as an additional laboratory technique to distinguish between recrudescence, relapse and new infection, but, because of the difficulty in identifying relapses, it is considerably less precise than in *P. falciparum*.

7.5 Recommendation

No recommendation was given for genotyping *P. vivax* in antimalarial drug trials because the interpretation of genotyping in the context of relapsing *P. vivax* infections is uncertain.

7.6 Rationale for not giving a recommendation on *P. vivax* genotyping

Genotyping of *P. vivax* in an antimalarial drug trial is confounded by the occurrence of relapses, which can be due to the same genotype as at baseline or to different genotypes, which will be genotyped as a 'new infections'. Relapses due to parasites present at baseline, appearing after the levels of antimalarial drugs are no longer suppressive, will be falsely determined as recrudescences, thus leading to underestimatation of antimalarial drug efficacy. Therefore, the minimum inhibitory concentrations of the antimalarial drug used should be investigated in vitro, and the blood concentrations of the antimalarial drug should be determined at various times, including the time of failure. Studies of the pharmacokinetics of the drug in the treated population should be considered.

8. CONCLUDING REMARKS

Genotyping in antimalarial drug trials is recommended by WHO because it greatly improves the accuracy of estimates of drug efficacy. Nevertheless, the limitations of the technique must be considered. For instance, when clinical studies are carried out in areas of extreme epidemiological conditions, interpretation of PCR-corrected failure rates is likely to be impeded because of little or no diversity in the marker genes (e.g. a clonal population structure, such as in an epidemic) or because of large numbers of superinfections in areas of highest malaria transmission.

The procedures recommended for surveillance in national malaria control programmes have been chosen to reflect limited resources available for public health monitoring. In contrast, clinical trials carried out for regulatory purposes require higher standards so that maximum information can be obtained from a trial. The main differences between these two applications of genotyping are:

- Use of treated filter-paper cards for collecting blood samples is mandatory for clinical trials, but untreated filter papers may be used for resource-constrained public health surveillance.
- Determination of the mean multiplicity of infection and the allelic frequencies of all or at least the most frequent allele(s) is mandatory for regulatory trials if the failure rates is > 10%, whereas determination of multiplicity of infection is sufficient for surveillance.

A major factor in overestimation of failure rates is limited resolution of the marker gene. This can be due to technical limitations, but, with an optimal genotyping protocol, it can be due to the high frequencies of some alleles, which increases the probability of re-infection with the same genotype as at baseline. Determination of all allelic frequencies of the most diverse marker gene permits mathematical estimation of 'true' failure rates. Thus, one shortcoming of the genotyping technique can be compensated. This requires no extra work if capillary electrophoresis is used, as allelic frequencies are easy to determine. When standard agarose gels are used for fragment sizing, however, precise allelic frequencies are difficult to establish. Capillary electrophoresis capacity should thus become available in malaria-endemic countries, so that information on allelic frequencies can be obtained in antimalarial drug surveillance programmes and for further epidemiological research.

A number of issues in molecular monitoring in antimalarial drug trials remain open, as identified at the consensus meeting. With respect to the sampling scheme, more research is required to define the importance of additional sampling 24 h after the baseline sample is taken (additional sampling at day 0+1) and 24 h after a sample is collected from a patient with recurrent parasitaemia (additional sampling at day X+1). The issue is the gain obtained by genotyping more than one sample: How many additional recrudesences are identified by testing additional samples?

The diversity and discriminatory power of each marker gene should be clearly determined at various study sites. This could be achieved in an inter-laboratory genotyping comparison based on the recommended genotyping procedures proposed. Moreover, new techniques should be investigated, such as the potential of single-nucleotide polymorphism-based genotyping techniques.

As recommended in the case of > 10% failure rates, additional information should be provided to facilitate interpretation of clinical trial results. The allelic frequencies of the marker genes used are needed in order to estimate true failure rates. The appropriate algorithm for estimating true failure rates depends on the resolution of the typing system, the intensity of transmission and the extent of multiple infections at baseline. Further mathematical analysis is needed to determine how best to estimate failure rates in different epidemiological settings. Data collected during routine genotyping conducted with the current method and markers will contribute to the design of robust models for future recommendations.

While establishing allelic frequencies is straightforward with automated fragment sizing by capillary electrophoresis, it is difficult with gels. Moreover, it is not known whether allelic frequencies determined from symptomatic baseline samples are the same as those determined in cross-sectional samples. The degree of clustering of particular alleles in time and space is also not yet known.

A subject of research agenda is the presence of gametocytes from previous infections that might be sub-patent by microscopy. In order to avoid that persistent gametocytes contribute incorrectly to treatment failure, gametocyte-specific transcripts, such as polymorphic Pfg377, might be used as additional genotyping markers. Furthermore, when the effects of drug treatment on transmission are investigated, detection of Pfg377 by RT-PCR can allow study of the parasite clones actually transmitted.

Laboratories in which genotyping analyses are performed must have robust quality systems. The recommended genotyping procedures available from the Medicines for Malaria Venture and WHO websites can form the basis for laboratory-specific standard operating procedures. Local processes must be in place to ensure adherence to standard operating procedures and work practices. The standard operating procedures and the quality assurance procedures should be reviewed regularly. The quality of genotyping can be improved significantly by establishing inter-laboratory comparisons and external quality control by exchange of samples. Procedures for handling discordant results and measures of repeatability remain to be agreed upon.

Genotyping of *P. vivax* is the main unresolved item on the research agenda. *P. vivax* molecular markers and their discriminatory powers should be investigated. Sizing of polymorphic markers of various lengths, including microsatellites, by capillary electrophoresis will reveal maximum diversity. A genotyping technique based on single-nucleotide polymorphism could be used. The highly polymorphic Duffy binding protein of *P. vivax* could serve as a marker gene.

The problems created by the presence of *P. vivax* hypnozoites remain to be addressed. This is particularly critical in trials of the efficacy of novel treatments against hypnozoites. A sample taken at the time when clinical symptoms become apparent provides no information about the genotypes of the hypnozoites already present. Therefore, true new infections appearing during the trial follow-up cannot be distinguished from hypnozoites that were not elimated by the drug.

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APPENDIX 1. CLASSIFICATION OF TREATMENT OUTCOMES (WHO, 2005)

Early treatment failure:

- danger signs or severe malaria on day 1, 2 or 3 in the presence of parasitaemia;
- parasite count on day 2 higher then that on day 0, irrespective of axillary temperature;
- parasitaemia on day 3 with axillary temperature ≥ 37.5 °C;
- parasite count on day 3 ≥ 25% of that on day 0.

Late treatment failure:

Late clinical failure:

- danger signs or severe malaria on any day between day 4 and day 28 in the presence of parasitaemia, without previously meeting any of the criteria of early treatment failure;
- presence of parasitaemia and axillary temperature ≥ 37.5 °C (or history of fever) on any day between day 4 and day 28, without previously meeting any of the criteria of early treatment failure.

Late parasitological failure:

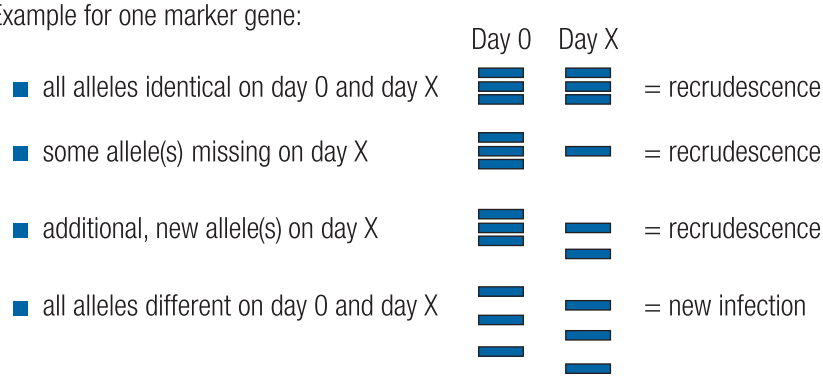
- presence of parasitaemia on any day between day 7 and day 28 and axillary temperature < 37.5 °C, without previously meeting any of the criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response:

- absence of parasitaemia on day 28, irrespective of axillary temperature, without previously meeting any of the criteria of early treatment failure or late clinical failure or late parasitological failure.

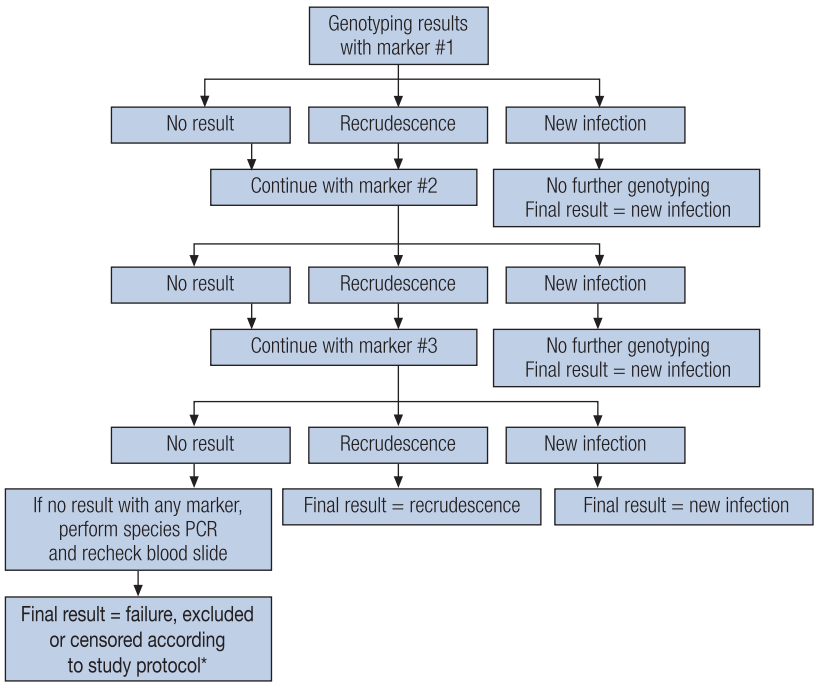
APPENDIX 2.
CONSENSUS DEFINITIONS OF 'NEW INFECTION' AND 'RECRUDESCENCE'

Example for one marker gene:



As soon as new infection is identified with one marker, the overall outcome is a new infection. A new infection identified with any marker will always override the results of genotyping any other marker(s).

APPENDIX 3.
INTERPRETATION OF GENOTYPING RESULTS WITH THREE MARKER GENES



* If another *Plasmodium* species is identified in the day X sample (in the absence of *P. falciparum*), a parasitaemia is regarded as being not a recrudescence but a new infection, and the patient will be considered as 'failure', 'excluded' or 'censored', according to the method of analysis dictated by the protocol.

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ISBN 978 92 4 159630 5