

Plasmodium falciparum and Dihydrofolate Reductase I164L Mutations in Africa

The paper by Ochong et al. modifies the real-time PCR method we published previously and applies it to samples from Malawi, Zambia, and Thailand (2, 7). These authors bring up several criticisms of the original technique, and we welcome this opportunity to clarify concerns about this method. We would also like to comment on some of their conclusions.

The primary criticism of Ochong et al. of our assay of mutations of dihydrofolate reductase at position 164 (DHFR-164) is that there was nonspecific binding of the probes. We have previously reported this phenomenon (1), which is evident from the original paper describing the MGB probe (see Fig. 4 in reference 5). However, this background binding does not interfere with the assay's discriminating ability as long as proper controls (standard curves of both wild-type and mutant DNA at concentrations similar to the clinical samples) are included (1, 2). In addition, the assay's performance is dependent on the real-time PCR machine, reagents, and even the batch of probe (1). Thus, the assay's discriminating ability should be reoptimized when it is adapted to different conditions.

Overall, we find the modified assay of Ochong et al. to be a successful adaptation of our assay to a new lab. However, this adaptation was incompletely validated. For example, the authors did not determine its sensitivity and specificity by running both real-time PCR and the gold standard allele-specific PCR with the same samples. Also, the authors should address the possibility that whole-genome amplification changes the frequencies of haplotypes from that in the original sample.

There is a much stronger body of evidence supporting the emergence of the DHFR-164L mutation in Africa than is suggested in this paper. We recently confirmed the existence of the DHFR-164L mutation in Malawi by a heteroduplex tracking assay and direct sequencing (4). Furthermore, a subsequent report by Lynch et al. found the mutation in 14% of samples from southwestern Uganda collected in 2005 (6). Previously, in 2002, the mutation had been found at a prevalence of 1.25% in Kanungu in southwestern Uganda (3). This suggests that the mutation may be emerging regionally in Africa.

Finally, it is important to note that all of the reported studies of the prevalence of the DHFR-164L mutation use patients enrolled in studies and were not sampled to represent the general population. Thus, small differences in prevalence between studies and study sites are to be expected.

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Authors' Reply

We thank Dr. Alker and colleagues for raising some interesting issues regarding our article (6) and for the opportunity to clarify any misunderstandings.

We are grateful for a more robust description of controls. However, we remain a little confused about how the inclusion of standard curves of plasmid DNA in the plate (even if included in every run) prevents problems associated with cross-reacting probes for individual reactions, with variable isolate DNA concentrations. The authors are correct that the performance of real-time assays can depend on the cycler, reagents, and batch of probe. However, we observed specificity and variability problems in three real-time PCR cyclers (Bio-Rad Chromo 4, MJ Research Opticon 2, and ABI Prism 7700 system) and with multiple batches of probe. Conversely, our optimized assay was found to be accurate and precise in all three systems, irrespective of the batch of probe used.

We strongly disagree that our method was incompletely validated. As stated in our original article (see Materials and Methods in reference 6), the same Malawian samples were previously genotyped by allele-specific restriction analysis, by which the DHFR-164 mutation was also undetectable (2). We agree that whole-genome amplification (WGA) may change haplotype frequencies. However, we were assessing the allele rather than the intact haplotype. Perhaps of more concern in this context is allele dropout, which sometimes arises from an imbalanced efficiency between alleles and can result in one allele being preferentially amplified in WGA (7). However, this is unlikely to have occurred, because WGA was also necessary for eight (21%) of the Thai samples and six of these were positive for the DHFR-164 mutant allele. In the unlikely event that allele dropout did occur, this would not affect our interpretation, since only 22 (13.9%) of the Malawian cohort and 11 (26%) of the Zambian cohort had <10,000 copies of parasite DNA per microliter and were thus subjected to WGA.

The authors infer that we understated the body of evidence supporting the emergence of the DHFR-164L mutation in Africa and cite three additional articles to support this (3–5). However, the article by Juliano et al. with the associated heteroduplex tracking assay was cited in the introduction of our article. All newly emerging technologies have inherent limits of sensitivity and specificity. Currently, therefore, the most convincing data for the emergence of this allele in Africa are those described by Lynch et al., who utilized more traditional methodologies (5). That article was published after we submitted our article to *Antimicrobial Agents and Chemotherapy*, and both the additional studies (3, 5) were conducted in Uganda, not Malawi (or Zambia). Nonetheless, the regional selection hypothesis is certainly interesting and worthy of further study.

Finally, as discussed in our original article, we completely concur that there are differences between the patients in our study and those in the study by Alker et al. (see Discussion in reference 6), and neither were sampled to represent the general population.

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