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PCR-adjustment in Antimalarial Trials – Molecular malarkey?

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Malaria drug trials necessitate both clinical and microbiologic endpoints to establish the efficacy of antimalarials. The recurrence of parasitemia following treatment is an indicator of treatment failure. The study by Deloron et al in Benin demonstrates both substantial *in vivo* resistance to sulfadoxine-pyrimethamine (SP) and good efficacy of two new artemisinin combination therapies (ACT); artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ). Both ACTs were markedly more effective than SP. Notably, there were significantly fewer recurrent parasitemias in the AL group than the ASAQ group in the per-protocol analysis, but the difference disappeared after a process called PCR-adjustment (or, often, PCR-correction). Are the ACT regimens equivalent, or is AL better? The answer hinges on whether PCR correction truly corrects.

Distinguishing recrudescent infections from new infections during follow-up bedevils the interpretation of antimalarial efficacy data. To this end, PCR-adjustment is used to prevent reinfections from looking like drug failures. Over 100 trials have been reported in PubMed using PCR adjustment of results. This adjustment is used because clinical trials for antimalarials are typically conducted in areas with relatively high malaria prevalence. While a high prevalence is good for recruitment, trial participants risk acquiring a new infection during the follow-up period by being rebitten by an infected mosquito. Thus, recurrences of parasitemia after treatment due to reinfection might be mistaken for recrudescences (drug failures). This could lead to spuriously low cure rates and mistakenly make efficacious drugs look less effective. Though preventing both recrudescence and reinfection are clinically desirable, recrudescence alone is the primary correlate for antimalarial efficacy.

For *Plasmodium falciparum* malaria, one early solution to this problem was simply to follow patients for 14 days. It was felt that, given the 7–14 day prepatent period (time required for the parasite to mature in the liver before entering red blood cells), a reinfection would be unlikely to lead to patent malaria infection within this time period. However, Stepniewska et al showed that many recrudescences occurred later than 14 days, and that a 28 or 42 day follow-up period was needed to catch all failures [1]. Longer follow-up, however, increases the likelihood of reinfection. Accordingly, in order to measure efficacy in trials with 28 or 42 day follow-ups, a better means of accurately identifying recrudescence was needed.

“PCR- correction” was introduced in the 1990’s by Snounou, Beck, and others as a means to better distinguish reinfections from recrudescences [2]. Subsequently, a variety of genotyping methods and interpretations were introduced [3]. To formalize the laboratory protocols and interpretation of data, the WHO and Medicines for Malaria Venture (MMV) recently published a suggested standard operating procedure [4]. This method involves comparing size polymorphisms of variable surface antigens by PCR amplification from parasites before treatment and upon recurrence. Amplicon length can be assessed either by gel or capillary

electrophoresis. Because amplicons of different sequences or slightly different sizes may look identical, PCR amplification of 3 polymorphic genetic loci (*msp1*, *msp2*, and *glurp*) is recommended. If the pretreatment and recurrent infections share at least one band in all 3 loci, then the recurrence is diagnosed as a recrudescence (Figure 1, Panel A). If no bands are shared at any locus, then the recurrence is considered a reinfection (Figure 1, Panel B). Despite this standardization, multiple factors compromise the ability of PCR-correction to robustly evaluate recurrent parasitemia and diagnose recrudescences, resulting in false under- and overestimation of antimalarial clinical efficacy.

A major drawback of PCR-correction is its inability to fully capture the polyclonality of *Plasmodia* infections. Recrudescences can be misclassified as reinfections, primarily due to inhost diversity. Single individuals with malaria may harbor > 10 genetically distinct variants, and some variants ("minority variants") may be present at very low levels. PCR amplification of mixtures of alleles cannot reliably detect alleles present at <20% of the population; therefore some variants from an infected patient are cryptic and not represented in the final amplification products [5,6]. A cryptic drug-resistant minority variant present at enrollment could cause recrudescence, but only be visible in the recurrent sample. Thus recrudescences would be falsely classified as reinfections. The intensity of this effect will vary with transmission intensity. In areas where transmission intensity is high and parasite genetic diversity is great (both in the patient and in the mosquito population), minority variants are likely to be more common than in less genetically-diverse, low-transmission areas. As a result, PCR-correction can lead to overestimates of drug efficacy.

Reinfections can also be misdiagnosed as recrudescences. In any geographic region, a few variants may be much more common than others. If a patient carried a common variant at enrollment, there is a very good chance that he/she was reinfected with the same variant during follow-up. Indeed, two studies from Africa estimated that approximately 40% of PCR-adjusted recrudescences were really reinfections [7,8]. In areas of low transmission, where the average complexity of infection is low and the genetic diversity of parasites in mosquitoes is limited, the likelihood of this type of misclassification is even higher. Reinfections may also be misdiagnosed as recrudescences because of the limited ability of current techniques to detect unique alleles. PCR-adjustment protocols will detect size polymorphisms but not sequence polymorphisms. Additionally, they are unable to differentiate small size polymorphisms. Two genetically distinct variants, one present at enrollment and one at recurrence, whose PCR products are close in size may be considered the same variant leading to the misclassification of a reinfection as a recrudescence. As a result of both of these biases, misdiagnosis by PCR-correction may underestimate the efficacy of a drug regimen.

In the end, is PCR-correction a good idea? Undoubtedly, we need the tools to accurately diagnose recrudescent parasitemia as a means to monitor drug efficacy and resistance. Though it may be possible to statistically compensate for transmission rates in order to merge or compare results at different sites [7–9], the technological inability of current PCR-correction strategies to detect minority variants and distinguish alleles of close size remains problematic. Newer technologies such as heteroduplex tracking assays (HTA) or ultra-deep sequencing by massively parallel pyrosequencing could be employed for diagnosing recrudescence [8,10, 11]. These newer technologies may yield a more sophisticated and reliable test than the crude tool that is PCR-correction. Until that time, we cannot conclusively interpret the puzzling results of studies like Bejon et al. Therefore, policy makers need to look at both the raw and "PCR-corrected" data before making recommendations.

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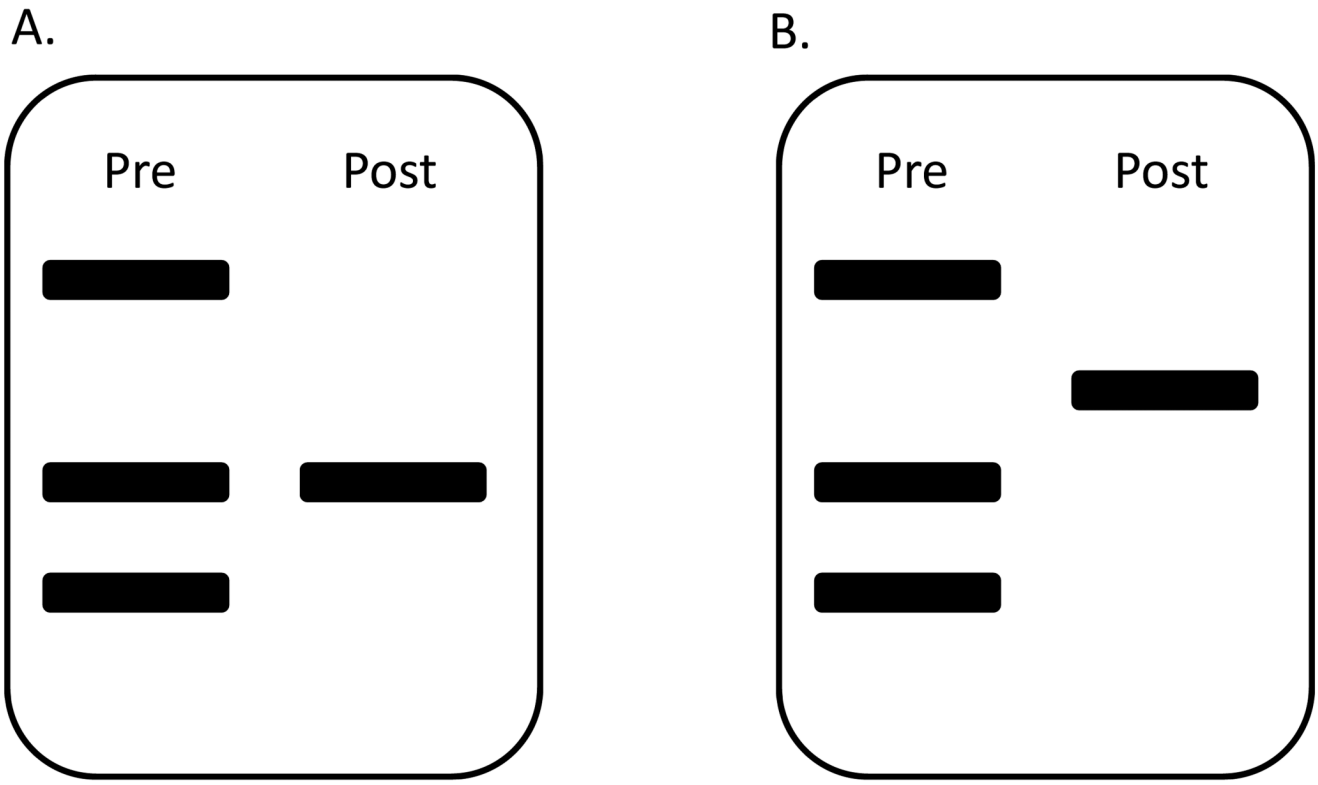

Figure 1. Representative Banding Patterns for PCR Correction

These panels show typical banding patterns from pre-treatment and post-treatment samples by the WHO/MMV guidelines for PCR correction of antimalarial clinical trials. Panels A would be interpreted as a recrudescence because of a shared band before and after treatment. Panel B shows the pattern for a reinfection with no shared alleles.