Artemisinin Resistance: The More We Know, the More Complicated It Appears

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(See the major articles by Takala-Harrison et al on pages 670–9 and Taylor et al on pages 680–8.)

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Plasmodium falciparum, one of the most deadly human malaria parasites, consistently has evolved resistance to antimalarials that have been intensively used. The genetic basis of resistance to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) has been most completely studied. The genes associated with the resistance to each drug are different, but in each case, a few single-nucleotide polymorphisms (SNPs) in 1 or 2 genes were shown to be responsible [1–4]. These SNPs were then used as molecular markers to trace retrospectively the path of the resistant parasites. For both CQ and SP, these parasites spread from very few points of origin that included the northwest region of Cambodia in Southeast Asia [5]. Studies showed that parasites with the molecular markers were present far from this origin long before intensive drug use increased their prevalence, resulting in high levels of treatment failure [6, 7]. Even more remarkable, each resistant population was introduced from Southeast Asia into East Africa by individuals carrying the resistant strain and then spread throughout the continent [8–10].

As the proportion of resistant parasites increased, treatment efficacy of each drug diminished, and the World Health Organization recommended that artemisinin combination therapies (ACTs) should replace CQ and SP for treatment of falciparum malaria [11]. These antimalarials have 2 components: artemisinin, which has a very short elimination half-life, and a partner drug with a much longer elimination half-life. The rationale is that artemisinin reduces rapidly the parasite burden and then, over a longer period, the partner drug eliminates any parasites that remain [12].

Unfortunately, in 2009, decreased susceptibility of P. falciparum parasites to artemisinins was documented by noting that, in many patients from northwestern Cambodia, clearance of parasites in the first few days after artemisinin treatment was extremely slow [13]. This observation was quantified and verified [14], and since all current ACTs depend on the artemisinin component, the specter of parasite resistance has been raised anew. By analogy to the so-called old antimalarials, it has been assumed that the resistant parasites would originate rarely and that the new areas of slow parasite clearance would reflect spreading from that focal source. Despite the difficulties of measuring the slow clearance in the field, this assumption has been supported, as slow clearance has been observed in a widening area of the Mekong region [15, 16]. The major effort, therefore, has been to contain and eliminate those parasites before they could spread, as occurred before, from the Mekong source area to the rest of Asia and to Africa, thereby prolonging the useful therapeutic life of the ACTs [17].

Two studies in this issue of The Journal of Infectious Diseases challenge this assumption but from very different points of view. In December 2013, an international team based in the Pasteur Institute and the National Institutes of Health identified a molecular marker in the kelch 13 (K13) gene (PF3D7_1343700) that is associated in Cambodian parasites with the slow clearance phenotype [18]. Both of the current studies capitalized on that breakthrough. Takala-Harrison et al showed that, as feared, some slow-clearing parasites that carry the K13 alleles from Cambodia have spread to Thailand and Vietnam [19]. However, they also found 2 locations in Myanmar with mutant parasites that are not invaders but have evolved resistance independently. In a very different kind of study, Taylor et al used a novel and very efficient strategy to search for genetic changes in the K13 gene in 1100 P. falciparum parasites in isolates from 14 African sites. They demonstrated that suspect novel
mutant alleles of K13 were commonly seen at low frequency in all 14 sites that they surveyed [20].

How do these observations challenge expectations? Even the initial observations of K13 failed to conform to the simple SP and CQ pattern—spread of a very few resistance-associated alleles from rare foci [18]. Rather, they demonstrated that 17 individual isolates from the Cambodian epicenter each carried one of 17 different SNPs within a 700-bp paddle region of the K13 gene. Before the K13 article was published, Takala-Harrison and colleagues from the University of Maryland performed a classic genome-wide association study comparing the SNPs observed in the genomes of parasites that cleared slowly with SNPs in fast-clearing parasites. They identified several associated DNA regions, including the section of chromosome 13 that contains the K13 gene, but not the gene itself [21].

If K13 is really a key determinant of susceptibility to artemisinin, why was it not strongly associated in that study? In the current study by Takala-Harrison et al, the same analyses used a larger and more geographically diverse panel of parasites, and more importantly, the authors redefined their search algorithm. Instead of requiring a single SNP to be associated with slow clearance, any slow-clearing isolate with a SNP within the 700-bp paddle region of K13 was characterized as “non–wild type”; the resistance marker was dichotomized. With that definition, several different analytical approaches showed that the probability that parasites with a single novel SNP within the paddle region of K13 were associated strongly with slow clearance with a probability that exceeded by several orders of magnitude that of any other part of the genome. This whole-genome analysis also allowed the group to examine the whole genomes of slow parasites that carried the various SNPs in the K13 paddle region. Parasites from Vietnam and Thailand shared with Cambodian parasites long sections of DNA surrounding the K13 gene, as expected from closely related parasites. In contrast, 2 isolates from Myanmar each carried an exact SNP commonly observed in the K13 gene of Cambodian isolates. The surprise was that the extended haplotype—the DNA surrounding the K13 gene—was very different from that in the Cambodian isolates, demonstrating clearly that the Myanmar strains evolved the same K13 SNPs independently and had not simply spread from Cambodia. Preventing the spread of parasites that carry a few key alleles of K13 is not the only threat: new artemisinin-resistant foci can also evolve.

The identification of K13 in the Mekong region immediately spurred researchers in other regions to begin to search for isolates that carry suspect SNPs in the K13 paddle region. Even in the Mekong region, >30 different alleles with single changes in the K13 paddle region have now been identified [22]. The previous strategy of identifying a few markers associated with SP or CQ resistance will not be sufficient. Taylor et al have proposed a novel strategy for scanning simultaneously the sequence of the whole K13 paddle region in many P. falciparum isolates. This group had previously designed an approach in which DNA isolated from multiple different parasite isolates was combined and sequenced as a pool, using newer in-depth sequencing methods. They showed that the method was sufficiently sensitive to detect a SNP different from the published wild-type DNA sequence with a frequency of only 1% in the pooled DNA [23].

In the current study, they assembled a large group of collaborators who contributed dried blood samples collected between 2010 and 2012 from P. falciparum–infected patients from 14 different sites in Africa. They devised a detailed set of control experiments to filter the primary sequence output and ensured that they counted genuine novel SNPs but excluded sequencing errors. They then used the strategy to examine the prevalence of SNPs different from the wild type in the pooled DNA from each site.

Among the 1100 isolates, they found 24 different alleles carrying suspect SNPs in the K13 paddle region; 15 were not among those that had been reported in the initial K13 publication. Four of these had been deposited previously from whole-genome sequences of African parasites as part of the MalariaGEN project. In 13 of 14 sites, one particular SNP was observed at low levels of around 1%–2%, indicating a sort of pan-African allele, but all sites had at least 1 other private allele seen only in that single location. Two areas had elevated frequencies of single suspect SNPs in the K13 paddle region. Two sites in Mali each had 5 different alleles, some at about 5% frequency, but only the pan-African allele was shared between the 2 Malian sites. Isolates from the Democratic Republic of the Congo also stood out. In the province of Bas-Congo, 5 alleles were also observed, and 1 allele was at 36% and another at 7%; only the pan-African allele was shared with any other site. The allele present at 37% frequency was confirmed by standard bidirectional sequencing and was identified in 1 of 13 isolates randomly chosen from a pool of 50. Kinshasa, an adjacent Democratic Republic of the Congo province, also had 4 alleles, one at almost 10% frequency and another that was shared with the 2 Mali sites. However, isolates taken from Kinshasa province a year before carried only a low frequency of the pan-African allele.

If these K13 alleles resulted from artemisinin selection, population genetics analysis should reveal signatures of strong directional selection of the kind characteristic of populations of P. falciparum associated with resistance to CQ and SP. Moreover, one would expect that resistance-associated alleles might be geographically clustered. No such patterns were uncovered in this study. The simplest hypothesis is that most of these alleles have arisen de novo at the various sites. What have we learned from these 2 studies? First, there is apparently a deep reservoir of diverse alleles among P. falciparum populations in both Southeast Asia and Africa that carry SNPs that may confer reduced susceptibility to artemisinins.
Their association with slow clearance of parasites is solid in the Mekong region but unknown in Africa. However, 3 alleles in this African study are shared with the list recently published by Ashley et al [22], and one of these is shared with the current article by Takala-Harrison et al. These 3 alleles clearly are linked with slow clearance, at least in the Mekong parasites, but it is still possible that the Southeast Asian parasites share common genetic determinants required for the proteins encoded by the K13 alleles to convey the slow-clearance phenotype. If these K13 alleles are the molecular marker of artemisinin resistance, the genotype-phenotype association will need to be validated in other regions. Second, we must design efficient approaches to determine the phenotype of a rare isolate that carries a suspect allele of K13 by using clinical studies [22], a recently developed laboratory assay [24], or some novel strategy. Finally, a strategy that depends on tracking the spread of a few key SNPs will not be adequate to contain artemisinin resistance. Any strategy must also include the reality that new foci can arise. Mutations in the K13 paddle region are clearly not rare, so it will be crucial to identify the conditions that favor their selection and target those sites for elimination.

Retrospective analyses of molecular markers have provided a detailed understanding of *P. falciparum* resistance to CQ and SP. We now are challenged to do more than chronicle retrospectively the rise of resistance to another antimalarial. In areas outside the Mekong region, artemisinin resistance still seems to be rare, so new approaches will be needed [25]. Rapid and concerted action is needed to sort out the complexities revealed in these 2 articles. The science is fascinating, but surveillance for artemisinin resistance is going to be far more complicated than we expected. It will take modern methods and cooperative action to identify the location of and, if possible, eliminate resistant parasites. The stakes are high, and a practical strategy needs to be deployed urgently.

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**References**


