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Meeting report

Malaria genomics meets drug-resistance phenotyping in the field

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Abstract

A report of the 2nd Wellcome Trust Conference on Genomic Epidemiology of Malaria, Hinxton, UK, 14-17 June 2009.

New genomic technologies and analyses present opportunities for understanding the evolution of drug resistance in malaria parasites and for identifying associated genetic markers. In addition, such techniques may be of use in tracking and containing the evolution of resistance. Given the appearance of field reports of reduced sensitivity to new artemisinin-based drugs, the second of the Wellcome Trust conferences on the application of genomics to malaria epidemiology provided a timely opportunity to review scientific and public-health developments and to discuss future research, surveillance and intervention priorities. At this meeting the focus was on genomics and drug resistance. Here we report a few highlights.

Is artemisinin resistance already a reality?

The control of malarial disease by drug treatment is at a critical stage. The old therapies such as chloroquine and antifolates have largely failed and we are increasingly dependent upon artemisinin combination therapies (ACTs). Some scientists have questioned whether resistance to artemisinin would ever arise. Recently, however, reports of reduced susceptibility to artemisinin derivatives, such as artesunate, artemether and dihydroartemisinin, are accumulating from Thailand and Cambodia. The continued emergence of artemisinin resistance would represent a grave risk to public health.

Arjen Dondorp (Mahidol University, Bangkok, Thailand) described recent data comparing two different artesunate therapies in Pailin (in western Cambodia) and Wang Pha (on the northwestern Thailand-Myanmar border). He reported significantly longer parasite clearance times in Pailin for both treatments relative to Wang Pha. There were no significant differences between measured drug levels *in vivo* in the two areas, and no relationship between these measures and parasite clearance in individuals. Conventional *in vitro* tests appeared to be insufficiently sensitive to fully identify the artesunate-resistance

phenotype. No molecular markers for resistance were identified. Dondorp interpreted these data as clearly establishing the presence of artemisinin resistance in Western Cambodia. Chansuda Wongsrichanalai (USAID, Bangkok, Thailand) outlined the work of National Malaria Control Programs in six countries of the Greater Mekong Subregion, where multi-drug resistance foci exist. She explained how endemic foci along national borders and migrant populations might obstruct elimination policies and how amplification of the *mdr1* gene for multi-drug resistance is believed to play a major role in a loss of artesunate-mefloquine efficacy in that region.

Steffen Boormann (University of Heidelberg, Germany) described surveillance for ACT resistance in East Africa, comparing data from 2005 to 2006 and 2007 to 2008 after treatment with artemether-lumefantrine and dihydroartemisinin-piperaquine. Parasite clearance times, 24-hour parasite reduction ratios, and rates of recrudescence by day 84 all suggested that the 2007 to 2008 parasites were being controlled less well by the artemisinin component of the ACT relative to the 2005 to 2006 parasites.

These studies present valuable and hard-won data suggesting that the evolution of artemisinin resistance may already be under way, although the question arises of whether these changes represent selection of pre-existing response variability or the occurrence of novel mutations. Recurrent themes of the meeting included an emphasis on the crucial importance of measuring both *in vivo* and *in vitro* resistance traits - which was captured succinctly by Xin-Zhuan Su (National Institutes of Health, Bethesda, USA) in his phrase 'phenotype, phenotype, phenotype'. Another theme emphasized by several speakers was the importance of building a panel of molecular markers of resistance and their use in surveillance and resistance management.

Genetic markers for artemisinin resistance

Rachel Hallett (London School of Hygiene and Tropical Medicine, UK) and Shannon Takala (University of Maryland Medical School, Baltimore, USA) explained how candidate markers will be integrated into two collaborative

projects for surveying artemisinin resistance in the field. Hallett described the structure of the MALACTRES consortium, a European Union-funded initiative that aims to investigate resistance to artemisinin combination therapy in Nigeria, Burkina Faso and Tanzania. One aim is to sequence candidate genetic markers such as *mdr1*, *atp6* and *ubp1* in *Plasmodium falciparum* parasites not cleared by ACTs, and to evaluate how they contribute to gametocyte carriage and mosquito infectivity in the presence of the drug.

The ARC3 project is a Gates Foundation-funded study of potential artesunate resistance in western Cambodia, northwestern Thailand and Bangladesh. Takala explained how it will track possible pathways of migration of resistant parasites from Cambodia. The molecular-marker/genomic module of ARC3 will use candidate-gene and genome-wide approaches, exploiting whole-genome resequencing and microarray analysis of single nucleotide polymorphisms (SNPs), to conduct population genetic studies on *P. falciparum* parasites in order to detect signatures of drug selection, migration patterns and genome-wide associations.

New approaches for identifying molecular markers of drug resistance were described by a number of speakers. Su described a comprehensive experimental system for analyzing responses of parasites to new drugs and for identifying the genetic determinants of variation. High-throughput genotyping arrays use a novel molecular inversion probe technology that allows the identification of genetic elements contributing to differential responses to chemicals or drugs in a wide variety of parasite strains. This system can be used to perform rapid analysis of quantitative trait loci on the progeny of genetic crosses or parasite isolates collected from the field.

One of us (PH) described how specific mutations underlying chloroquine resistance and artemisinin resistance were identified in a congenic lineage of multi-drug resistant mutants of the rodent malaria *Plasmodium chabaudi*. Loci associated with drug resistance were mapped using genome-wide scans of genetic crosses. Within these loci, mutations in an amino acid transporter (*aat1*) and a deubiquitinating enzyme (*ubp1*) were identified by Solexa genome resequencing of mutant and wild-type parasites. Importantly, this approach is rapid. It could, therefore, be used for proactive nomination of candidate resistance genes before resistance to future drugs arises in *Plasmodium* species that cause disease in humans.

Genomic studies of drug resistance

Central to the meeting was the impressive progress made in the application of single molecule deep sequencing and high-density genotyping arrays for the investigation of field samples, and their relevance to the discovery and control of drug resistance. Sarah Auburn and Dominic

Kwiatkowski (Sanger Institute, Hinxton, UK) described how whole-genome resequencing of clinical parasite isolates is being used to identify patterns of genome variation in natural *Plasmodium* populations. They also detailed how challenges associated with sequencing high-quality samples directly from the field and resolving mixed infections are being tackled to improve the application of this technology to parasites collected directly from infected people.

Philip Awadalla (University of Montreal, Quebec, Canada) and Sarah Volkman (Harvard School of Public Health, Boston, USA) extended the theme of using genome-wide variation data to understand global patterns of *P. falciparum* parasite diversity. Awadalla has found that high-coverage parasite sequence data suggest a greater extent of diversity than previously anticipated, and described how rare variants could provide insights into malaria evolutionary history, especially for the most recent processes. For instance, regarding the core haplotype around the chloroquine-resistance marker gene *crt*, one can ask whether the rare alleles underlying this variation are the remnant of previous balancing selection or whether they represent the appearance of new resistance variants?

Volkman demonstrated the versatility of high-density, genome-wide genotyping arrays in determining the geographic population structure of the *P. falciparum* parasite, relationships between linkage disequilibrium and transmission intensity, and the detection of selective sweeps. She described preliminary results from genome-wide association studies combining genotyping data with robust drug-resistance phenotypes using cultured parasites. This approach detected known loci of resistance to chloroquine and pyrimethamine (*crt* and *dhfr*, respectively) and two putative genes underlying resistance to chloroquine or halofantrine.

New web tools facilitating display and analysis of deep sequencing and genotyping data were introduced. Magnus Manske and Susana Campino (Sanger Institute, Hinxton, UK) presented LookSeq: a web-based application for visualizing and comparing sequence read alignments. LookSeq [<http://www.sanger.ac.uk/Software/analysis/lookseq>] features an intuitive browsing environment with easy detection of SNPs, indels and other structural variants between samples. Olivo Miotto (Mahidol University, Bangkok, Thailand) introduced MapSeq, a tool to integrate genotype data browsing with geographical distributions, statistical and comparative analysis and exploration of associations.

The genomic studies challenge us to ask how these data and insights regarding genome-wide selection, population and evolutionary genetics can serve the public-health agenda. Since 2002, it has been understood that chloroquine resistance (conferred by *crt* mutations) arose and

spread a limited number of times, producing a selective sweep. Now, extended haplotype analysis suggests that the same is true of multiple mutations in *dhfr* and *dhps*, which underlie resistance to the antifolates. One of us (CR) described how microsatellite analysis has defined extended haplotypes around *dhfr* and *dhps* in a large number of African field samples. She and her colleagues observe one dominant *dhfr* triple-mutant haplotype of Asian origin throughout Africa. A small number of *dhps* haplotypes of African origin have strong geographic associations. These data underline the importance of dispersal in the evolution of resistance, and suggest that surveillance for artemisinin resistance in South-East Asia and coordinated multi-disciplinary containment measures might reduce the local and global spread of resistant parasites. Indeed, this possibility is specified in the ARC3 project.

The genetic architecture of phenotype-genotype relationships

Michael Ferdig (University of Notre Dame, Notre Dame, USA), Su, and Chris Plowe (University of Maryland School of Medicine, Baltimore, USA) all discussed the different possible quantitative relationships between phenotype and genotype. For example, Ferdig addressed the limitations of our (historically necessary) simple 'one gene-one phenotype' paradigms head-on by pointing out that whereas atovaquone resistance is dramatically bimodal (contingent on one mutation) and chloroquine less so (Figure 1), we should not assume that the same may be true of responses to other drugs, such as artemisinin. Su showed data exemplifying phenotype distributions for a number of drugs. For instance, both chloroquine and sulfadoxine/pyrimethamine showed discontinuities in the phenotype distribution, presumably reflecting the effect of one dominant mutation, whereas other drugs such as quinine and dihydroartemisinin showed continuous distributions, perhaps reflecting the small effects of more than one mutation.

Plowe focused our attention onto the consequences of resistance for the disease itself, using the apparently well characterized examples of *dhfr* and *dhps* mutations and resistance to antifolate drugs. Although the impact of these mutations on *in vitro* IC_{50} (the concentration of drug showing 50% (of the maximum) inhibition of parasite growth) accumulates gradually, it appears that, *in vivo*, parasites with all mutations are selected during drug treatment. A different pattern occurs with chloroquine. *Crt* accounts for only a small part of the variance in chloroquine resistance, yet it appears to be an excellent predictor of clinical resistance. Such data can be used to make predictions regarding drug failure rates and, hence, guide drug use policy.

Immediate questions are: what 'distributions' of artemisinin resistance will be observed, and how will they

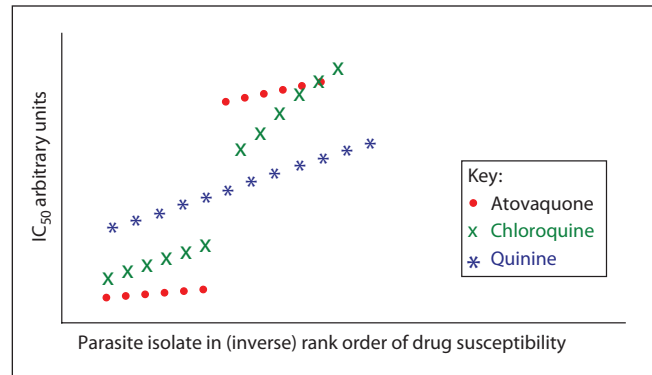


Figure 1

Possible modes of distribution of drug-resistance phenotypes. Responses to quinine are presumed to be continuous and unimodal, while atovaquone (and chloroquine) may show bimodal character with parasite isolates falling into two distinct groups (characterized by low IC_{50} or high IC_{50}), each with their own distribution and variance. Courtesy of Michael Ferdig and Xin-Zhuan Su.

relate to the current range of variation in Cambodia. And what implications will this have for treatment failure and evolution of resistance in the future?

Abdoulaye Djimde (University of Bamako, Mali) reminded us that to turn research into practical application, we need to go beyond 'the parasite' and 'the genes'. *In vivo* phenotypes such as quinine sensitivity are a consequence not only of parasite genotype but of other factors, including the age of the patient, their nutritional status, their immune status and their pharmacogenetics. We should expect our understanding of variation to go beyond the genotype of the parasite: optimal strategies will then require holistic, and necessarily complex, approaches.

This resonates with one of the enduring themes of the meeting; the value of multidisciplinary research between genome scientists and malarial biologists in laboratory and field studies. For artemisinin, there is both anxiety and hope. There is growing evidence that parasites with reduced susceptibility are arising in specific foci in South-East Asia. On the other hand, multidisciplinary research in the laboratory and the field will optimize treatments, clarify relevant phenotypes, identify and evaluate genetic markers, monitor resistance evolution in time and space and stimulate resistance-containment practices.