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Ahead of the curve: next generation estimators of drug resistance in malaria infections

Nicole Mideo[1], David A. Kennedy[1,2], Jane M. Carlton[3], Jeffrey A. Bailey[4], Jonathan J. Juliano[5], and Andrew F. Read[1,2,6]

[1] Center for Infectious Disease Dynamics, Department of Biology, The Pennsylvania State University, University Park, PA 16802, USA
[2] Fogarty International Center, National Institutes of Health, Bethesda, MD 20892, USA
[3] Center for Genomics and Systems Biology, Department of Biology, New York University, New York, NY 10003, USA
[4] Division of Transfusion Medicine and Program in Bioinformatics and Integrative Biology, University of Massachusetts School of Medicine, Worcester, MA 01655, USA
[5] Division of Infectious Diseases, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA
[6] Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA

Drug resistance is a major obstacle to controlling infectious diseases. A key challenge is detecting the early signs of drug resistance when little is known about its genetic basis. Focusing on malaria parasites, we propose a way to do this. Newly developing or low level resistance at low frequency in patients can be detected through a phenotypic signature: individual parasite variants clearing more slowly following drug treatment. Harnessing the abundance and resolution of deep sequencing data, our 'selection differential' approach addresses some limitations of extant methods of resistance detection, should allow for the earliest detection of resistance in malaria or other multi-clone infections, and has the power to uncover the true scale of the drug resistance problem.

Drug resistance and clearance curves

The evolution of drug-resistant pathogens is a major challenge in the fight to control infectious diseases. Malaria parasites are a prime example of this: resistance has evolved to nearly every antimalarial drug in use [1] and appears to be emerging against the current front-line artemisinin derivatives [2–7]. Ensuring the continued efficacy of these important drugs requires good surveillance, early and rapid detection of resistance, and containment of its spread [3,8–13]. Ideally, resistance would be detected when it is at a low level (drug 'tolerance') and low frequency in a patient, well before it has become a clinical problem. Once a new drug resistance mutation has generated sufficient treatment failure to arouse suspicion, its spread is probably well advanced. Current methods used to detect resistance struggle to detect rare or low level resistance. Here we propose a new approach that in principle has substantially greater sensitivity.

Corresponding author: Mideo, N. (nlm12@psu.edu)
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Three main tools are used to detect drug-resistant malaria parasites. For simplicity, here we use the term 'resistance' to include any significant reduction in drug sensitivity below that found in wild type populations prior to drug use, including weak resistance or drug tolerance, although we note that there is disagreement over whether these phenotypes should be considered resistance [14,15]. These three tools are: (i) molecular markers of resistance, (ii) *in vitro* susceptibility tests, and (iii) therapeutic efficacy tests. Limitations of these approaches have been described in detail elsewhere (e.g., [12,16,17]). Briefly, searching for molecular markers requires knowledge of parasite candidate genes or pathways, yet little can be known in advance about the mechanisms underlying resistance when it is just starting to emerge. This is particularly so in malaria parasites where resistance mechanisms frequently involve loci other than those encoding drug targets [18]. Further, the list of known genetic markers of resistance will never be an exhaustive set of all pathways to resistance; new resistance mechanisms could regularly be evolving, and mechanisms need not be genetic. Testing the susceptibility of parasites to drugs *in vitro* is problematic because *in vivo* phenotypes do not necessarily correlate with *in vitro* performance (e.g., [4]), in addition to being technically challenging, laborious, and expensive. Although less technically demanding, therapeutic efficacy tests are logistically challenging and imprecise. The World Health Organization protocol [19] involves extensive follow up, monitoring patients for up to 1 month or more, during which time any estimate of resistance could be confounded by reinfections or relapses from liver stages. That protocol also sets out rigid criteria for parasitemia and other symptoms over the course of infections that indicate parasite resistance, thus failing to control for any possible sources of interindividual variation (e.g., host factors such as immunity, drug compliance, or coinfection) [8,11,16,20,21].

Recent studies of drug resistance in malaria parasites have addressed some of these problems by using a more

quantitative tool for assaying therapeutic efficacy, the parasite clearance curve [2,4,6,7,22,23]. The remarkable efficiency with which artemisinins act generates a pattern of log-linear decline in parasite densities on a very short timescale [24–26]. With sufficiently frequent sampling of parasite densities, the slope of their decline can be used to calculate a half-life (the time it takes for parasites to reach half of their initial density) [24,27,28]. Parasites that are cleared more slowly from a patient have longer half-lives and are deemed more 'resistant'. Comparisons of the average half-lives of parasites in different host populations have demonstrated artemisinin resistance emerging in Cambodia [4–6], Thailand [2], and Myanmar [7] and failing to emerge, for now, in Laos [23] and Mali [29].

Parasite clearance curves are important tools for defining host population level distributions of parasite half-lives, and they have the advantage of being able to account for some of the variation that occurs between hosts. For example, some infections show a lag phase before parasite densities start to drop after treatment is initiated, or a tail phase where parasite densities remain above the detectable threshold after a period of log-linear decline [24,27]. The Parasite Clearance Estimator [27] controls for these nonlinearities when estimating a half-life. Although these patterns could be the result of host effects, they could also be measurement errors or, importantly, could indicate parasite subpopulations within a host that respond differently to treatment [24]. Parasite clearance curves are not able to give finer resolution on this sort of within-host variation because they are not designed for making inferences at this level. A parasite clearance curve offers a single estimate of the clearance rate of parasites: a weighted average of the clearance trajectories of all parasites within a patient [24]. Malaria infections in humans seldom consist of one parasite clone; many parasite genotypes share their hosts with other genotypes (e.g., [30–38]). A consequence of this is that resistant parasites will often share their host with sensitive parasites, particularly early in the evolutionary process as resistance is spreading. Attempts to interpret clearance curves for individual patients are affected by this within-host diversity.

Consider a patient that harbors two parasite clones: a fast-clearing, drug-sensitive clone and a slow-clearing, drug-resistant clone. When the clearance of these individual clones cannot be directly tracked, two opposing conclusions can be reached, depending on the relative frequency of the clones. If these two clones are at equal frequencies in a patient when drug treatment is initiated, then the clearance curve observed follows the trajectory of the resistant parasite, because it quickly becomes numerically dominant in a drug-treated infection (Figure 1A). If, however, the resistant clone is initially at low frequency in the patient, then our ability to 'see' those resistant parasites in the infection is vastly diminished. The clearance curve now follows the trajectory of the sensitive clone (Figure 1B). From clearance curves alone, it is impossible to tell that the patient in Figure 1B is harboring resistant parasites and potentially transmitting those parasites. Current approaches may therefore underestimate the scale of the drug resistance problem.

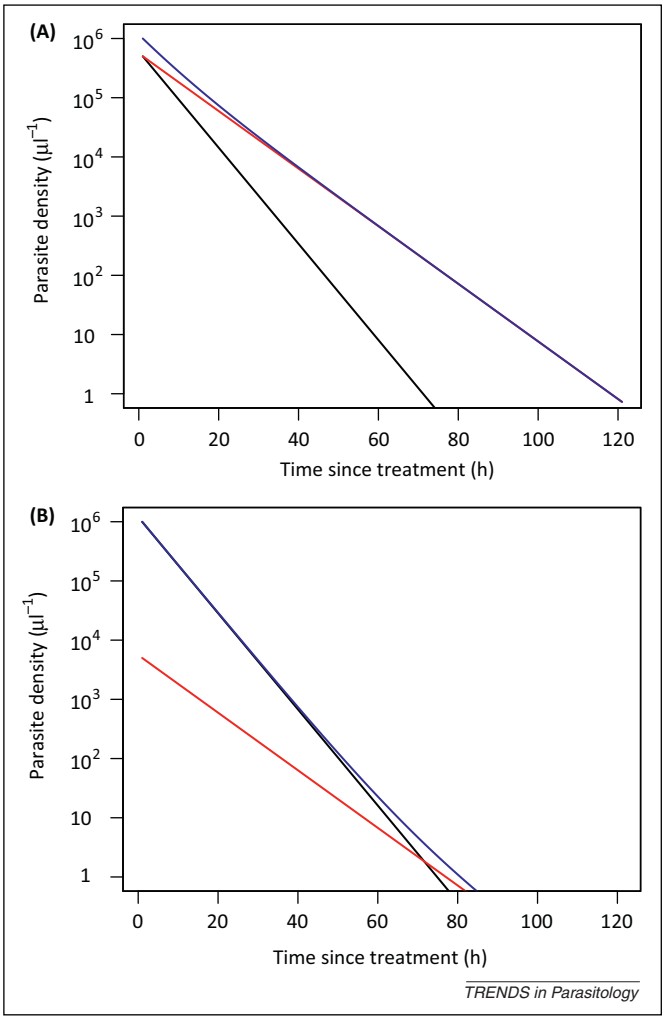

Figure 1. Clearance curves of diverse infections. Predicted clearance curves of sensitive (black) and resistant (red) parasite clones in hypothetical infections after drug treatment is initiated (ignoring any possible lag or tail phases [24,27]). The blue line shows the clearance curve that would actually be measured for an individual patient using standard approaches (i.e., it shows the change in the total parasite density over time). Initial frequencies of the resistant clone are **(A)** 50% and **(B)** 0.5%. Half-lives for sensitive and resistant clones are taken from [2] (3.7 h and 6.2 h, respectively). Despite harboring resistant parasites in both cases, the infection in (A) appears resistant whereas in (B) it appears sensitive.

Capitalizing on within-host diversity with next generation approaches

We propose that, rather than being an obstacle to identifying drug resistance in individual patients, the within-host diversity of infections can instead be exploited to detect it. When drug treatment is applied to mixed infections, clones with any degree of resistance will rise in frequency in the parasite population within that host. Even weakly drug tolerant clones will become more frequent because fully susceptible parasites will be cleared at faster rates. Thus, an increase in the frequency of any clone-specific genetic marker is indicative of up-selection by drugs of a tolerant or resistant clone in a sea of susceptible parasites. The critical requirement for identifying such a change in frequency is a detection technique that can quantify the relative abundance of different clones and be sufficiently sensitive to detect rare variants in a mixed population. Next generation sequencing (NGS) technologies, when they are used to deeply sequence a single highly