

Genetic Evaluation of the Performance of Malaria Parasite Clearance Rate Metrics

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Accurate measurement of malaria parasite clearance rates (CRs) following artemisinin (ART) treatment is critical for resistance surveillance and research, and various CR metrics are currently used. We measured 13 CR metrics in 1472 ART-treated hyperparasitemia infections for which 6-hour parasite counts and parasite genotypes (93 single nucleotide polymorphisms [SNPs]) were available. We used heritability to evaluate the performance of each metric. Heritability ranged from 0.06 ± 0.06 (SD) for 50% parasite clearance times to 0.67 ± 0.04 (SD) for clearance half-lives estimated from 6-hour parasite counts. These results identify the measures that should be avoided and show that reliable clearance measures can be obtained with abbreviated monitoring protocols.

Keywords. heritability; clearance rate; half-life; artemisinin; *Plasmodium falciparum*; single nucleotide polymorphism.

Artemisinin-based combination therapies (ACTs) are key to global malaria control efforts. However, the emergence of artemisinin-resistant malaria in western Cambodia [1–3] and along the Thai–Myanmar border [4] threatens these efforts. Artemisinin (ART) resistance is characterized by slow parasite

clearance in treated malaria patients [2] and has a strong genetic basis [5]. Although predictive molecular markers for ART resistance have not yet been identified, recent studies offer promise [6, 7]. There are currently no reliable in vitro tests for measuring ART resistance; standard growth inhibition assays fail to discriminate ART-sensitive from ART-resistant parasites [2]. Therefore, ART sensitivity must be measured in patients from the decline of parasitemia following ART treatment.

Several measures of parasite clearance have been used. Early studies used 24-hour sampling of parasites following treatment and examined time to the first negative blood slide or calculated a parasite reduction ratio (PRR), which is the proportional reduction in parasitemia at 24 or 48 hours [1, 2, 8–11]. Other studies used 50% and 90% parasite clearance times [12] (time taken for the initial parasite density to fall by 50% or 90%). More recently, 6-hour blood sampling following treatment until clearance has been advocated [13]; plots of log parasite clearance against time are used to measure parasite clearance rates or half-lives ($t_{1/2}P$) [3, 4]. Frequent quantification of parasitemia decline following treatment is clearly ideal. However, it is also intrusive, expensive, and labor intensive and is only feasible when patients remain hospitalized for several days following treatment [4]. This intensive sampling approach may not be possible in many clinical or field study situations. In addition, consensus on whether or not to account for the lag phase (the initial period following treatment when the relationship between log parasite density and time is not linear [13]) when estimating parasite clearance rates is lacking.

The central goal of this study was to evaluate the performance of different parasite clearance monitoring schemes and statistics in order to help others choose strategies that maximize accuracy while minimizing sampling effort and expense. Because there is no gold standard against which different measures of ART susceptibility can be compared, we used heritability (the proportion of variation in parasite clearance attributable to parasite genetic factors) as a benchmark for evaluating the utility of each metric [10]. The rationale for this approach is that accurate measures of clearance will show strong heritability, while inaccurate measures will show poor heritability. Parasite clearance rates following ART treatment are highly heritable [5]. On the Thai–Myanmar border, 66% of the variation in $t_{1/2}P$ is attributable to parasite genetic factors [4]. Therefore, we can assess the usefulness of each clearance metric by estimating the proportion of the maximal genetic signal that it captures.

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MATERIALS AND METHODS

Ethics Statement

The ethics review boards of the faculty of Tropical Medicine at Mahidol University, Bangkok, Thailand, approved these studies.

Parasite Clearance Data

Parasite clearance data were collected from 1472 hyperparasitemic *Plasmodium falciparum* malaria patients presenting to 4 clinics on the Thai–Myanmar border (2007–2010) and treated with ART-combination therapy [4]. The Thai–Myanmar border is a region of emerging artemisinin resistance [4] and declining malaria transmission (VI Carrara, et al, unpublished data) [14]. We derived 13 parasite clearance metrics for each infection and used a subset of infections ($n = 1057$), genotyped at 96 genome-wide single nucleotide polymorphisms (SNPs) [4], to measure the heritability of each clearance metric.

Parasite Clearance Measures

For each infection, we calculated $t_{1/2}P$ from parasite counts at 6-hour intervals following ART treatment until clearance. $t_{1/2}P$ is the first-order parasite clearance half-life and is estimated from the plot of natural log parasite density vs time as $t_{1/2}P = \ln(2)/-k$, where k is the slope. We also calculated $t_{1/2}P$ from parasite counts taken at 12- or 24-hour intervals following treatment until clearance; $t_{1/2}P$ from 6-hour parasite counts only during the first 24 or 48 hours of treatment; and 24- and 48-hour parasite reduction ratios (PRR₂₄ and PRR₄₈), calculated as follows: $(1 + \text{parasite density at 24 or 48 hours}) / (1 + \text{parasite density before treatment})$. In addition, 50% and 90% parasite clearance times (PC50 and PC90), defined as the time taken for the initial parasite density to fall by 50% or 90%, and parasite clearance time (time until the first negative slide) were calculated. We also estimated $t_{1/2}P$ by incorporating or excluding the lag phase. We used the parasite clearance estimator [13], developed by the WorldWide Antimalarial Resistance Network (WWARN), to account for the lag phase when estimating $t_{1/2}P$. Furthermore, we estimated $t_{1/2}P$ by modeling parasite clearance data using both standard regression, which excludes zero parasite counts, and tobit regression, which accounts for uncertainty in the measurement of parasitemia below the microscopy detection threshold [13]. Data from patients showing poor fit to a linear model ($r^2 < 0.75$) or with < 3 data points were excluded. Hence, sample sizes varied for the different clearance measures.

Parasite Genotyping

We genotyped parasite infections at 93 polymorphic SNPs across the *P. falciparum* genome using Illumina GoldenGate [4]. Of 1057 infections genotyped, 267 contained multiple parasite genotypes and were excluded from heritability analyses. SNP data from the remaining 790 single-genotype infections were used to identify groups of patients infected with identical multilocus parasite genotypes (MLGs). MLGs recovered from

multiple patients are considered to be identical-by-descent and can be used for heritability estimation [4, 5, 10].

Estimation of Heritability

We measured the heritability of parasite clearance metrics by comparing the variance of each metric within and among MLGs found in ≥ 2 patients. We then estimated heritability (H^2) from the mean squares terms in 1-way analysis of variance (ANOVA) [4, 5]. To account for other covariates that could influence clearance measures, we performed multiple regression analysis with each metric as the dependent variable and sampling date, location, treatment type, admission parasite density, patient age, and sex as independent variables. We then used residuals from the regression analysis in 1-way ANOVA to estimate H^2 .

To assess whether lags are heritable, we treated the lag phase as a binary character and compared the incidence of lags in the population with the incidence among identical parasite MLGs with lags [15]. We estimated H^2 using Edward's approximation [16] as $H^2 = 2 \times [0.57 \ln(\varphi_r/\varphi_p)] / [-\ln(\varphi_p) - 0.44 \ln(\varphi_r/\varphi_p) - 0.18]$ [15]. φ_p is the incidence of lags in the population calculated as $\varphi_p = \sum n_i / Nn$, where n_i is the number of infections bearing lags in the i th MLG, and n is the total number of infections scored for lags in N MLGs, each found in ≥ 2 infections. φ_r is the incidence of lags among infections bearing identical MLGs, calculated as $\varphi_r = \sum (n_i - 1) / [N_a(n - 1)]$, where N_a is the number of infections with lags.

RESULTS AND DISCUSSION

Data Summary

The parasite clearance data analyzed will be made available through WWARN (<http://www.wwarn.org/>). There were 790/1057 (75%) infections that contained a single predominant genotype; 516 MLGs were found in these infections. Of these, 129 MLGs were observed in 2–14 patients ($n = 403$); these are used in our heritability analysis. Table 1 shows the results from ANOVAs comparing clearance metrics within and between MLGs, and Figure 1 shows heritability estimates for each metric. These results allow direct comparison of the robustness of parasite clearance measures derived using different sampling schemes and slope-fitting procedures.

Effect of Sampling Frequency

Clearance half-lives obtained by 6-hourly sampling until clearance show high heritability ($H^2 = 0.66 \pm 0.04$ (SD), $P = 5.12 \times 10^{-41}$), as previously shown [4]. This decreases slightly to 0.62 ± 0.04 (SD) after removing the effects of significant covariates (Figure 1). To assess whether less frequent sampling can generate equally robust estimates of $t_{1/2}P$, we subsampled the 6-hour dataset and recalculated $t_{1/2}P$ for 12- and 24-hour intervals and at 6-hour intervals for the first 24 and 48 hours

Table 1. Effect of Parasite Genotype on Different Measures of Parasite Clearance

Clearance Metric	N	Total Variance			Residual Variance		
		F Value	DF(x,y)	PValue	F Value	DF(x,y)	PValue
T1/2 tobit regression + lag	1429	7.307	123, 258	5.12×10^{-41}	5.838	123, 258	6.41×10^{-33}
T1/2 tobit regression no lag	1419	6.664	123, 258	1.33×10^{-37}	5.331	123, 258	7.27×10^{-30}
T1/2 standard regression + lag	1429	6.878	123, 258	9.25×10^{-39}	5.486	123, 258	8.17×10^{-31}
T1/2 standard regression no lag	1419	6.773	123, 258	3.40×10^{-38}	5.400	123, 258	2.74×10^{-30}
T1/2 6-hour sampling for 24 hours	1295	3.613	103, 221	7.14×10^{-16}	3.203	103, 221	2.49×10^{-13}
T1/2 6-hour sampling for 48 hours	1431	7.069	122, 257	1.43×10^{-39}	5.654	122, 257	1.16×10^{-31}
T1/2 12-hour sampling to clearance	1114	4.899	99, 190	3.49×10^{-21}	4.374	99, 190	1.45×10^{-18}
T1/2 24-hour sampling to clearance	605	5.317	57, 109	4.21×10^{-14}	4.217	57, 109	5.74×10^{-11}
24-hour PRR	1170	1.769	96, 193	.0004	1.547	96, 193	.0056
48-hour PRR	1158	3.084	96, 193	1.63×10^{-11}	2.506	96, 193	3.36×10^{-8}
50% parasite clearance time	1229	1.145	101, 205	.2085	1.174	101, 205	.1688
90% parasite clearance time	1442	2.509	121, 252	4.73×10^{-10}	2.318	120, 251	1.31×10^{-8}
Time to the first negative slide	1415	3.438	119, 247	1.51×10^{-16}	3.125	119, 247	2.45×10^{-14}

We estimated the amount of variation in each clearance metric explained by parasite genetic factors using 1-way analysis of variance on log-transformed parasite clearance data. We also examined the effect of parasite genotype on residual variance in each metric after removing the effects of significant covariates.

Abbreviations: DF(x,y), degrees of freedom in the x-variable (parasite genotype) and the y-variable (clearance metric or residuals after removing effect of significant covariates); N, number of patients with good clearance rate estimates ($r^2 \geq 0.75$); PRR, parasite reduction ratio; T1/2, parasite clearance half-life.

after treatment. Both 12-hour and 24-hour sampling generated $t_{1/2}P$ with marginally reduced heritability ($H^2 = 0.56 \pm 0.06$, $P = 3.49 \times 10^{-21}$ and $H^2 = 0.62 \pm 0.07$, $P = 4.21 \times 10^{-14}$, respectively). These abbreviated sampling approaches capture 85% and 94% of the genetic component of $t_{1/2}P$ variation identified from 6-hour parasite sampling until clearance (Figure 1). However, sampling every 24 hours yields sparse data and cannot be recommended because many samples fit poorly to the linear model. Hence, in this dataset, 59% of samples ($n = 867$) had $r^2 < 0.75$ or < 3 data points and were rejected (Table 1).

Six-hour monitoring of parasite density over the first 48 hours was very successful. This approach captured 100% of the maximal genetic signal in $t_{1/2}P$ variation ($H^2 = 0.66 \pm 0.04$) and performed as well as 6-hour parasite monitoring until clearance (Figure 1). This approach will be cost effective in malaria-endemic areas where ART-resistant infections are found and could result in considerable reductions in time and expense. In this dataset, 18 116 slides were read for all patients from treatment to clearance. Reading slides from only the first 48 hours reduces this number by 33%. In addition, this sampling approach yields parasitemia data with a good fit to the linear model. Of the 1472 samples analyzed, 97.2% ($n = 1431$) had $r^2 \geq 0.75$ compared with 97.1% ($n = 1429$) for the full dataset (Table 1). This abbreviated strategy will be beneficial only in locations where resistance is present, because in most endemic countries parasites are cleared within 48 hours. In contrast, 6-hour monitoring of parasite density over the first 24 hours of treatment does not provide such robust results. This approach yields parasite clearance half-lives with moderate heritability

($H^2 = 0.45 \pm 0.06$) and results in considerable (12%) reduction of sample size ($n = 1295$) (Table 1).

PC50, PC90, PRR₂₄, PRR₄₈, and parasite clearance time have been widely used due to logistical simplicity [1, 2, 8–12]. However, these approaches capture significantly less heritability and cannot be recommended. For example, heritability for PC50 ($H^2 = 0.06 \pm 0.06$) is not significant (Table 1), while heritabilities for PC90, PRR₂₄, PRR₄₈, and clearance time range from 0.21 ± 0.07 to 0.45 ± 0.05 (Figure 1). We caution against using these metrics for assessing ART susceptibility because they have high error and poorly capture the genetic signal in the clearance data.

Effect of Lag Phase

Previous approaches for measuring parasite clearance rates did not account for the lag phase [5]. However, there is concern that exclusion of the lag phase may misrepresent the clearance profile and that the lag phase may contain important pharmacodynamic information. Of profiles passing our quality thresholds, 367 of 1429 had lags, ranging from 4 to 41 hours. While the presence of the lag phase slightly overestimated $t_{1/2}P$, it had a major influence on PC50, PC90, and 48-hour PRRs. This is because the latter are associated with initial parasite densities (Supplementary Table 1). In contrast, $t_{1/2}P$ values from 6-hour parasite monitoring were not associated with admission parasitemia (Supplementary Table 1). Heritability of $t_{1/2}P$ did not differ significantly whether or not the lag phase was incorporated in estimating $t_{1/2}P$ (Figure 1). Although lags were significantly more common ($\chi^2 = 24.981$; $df = 1$; $P = 5.79 \times 10^{-7}$) among fast-clearing infections with $t_{1/2}P < 6.15$ hours (28%;

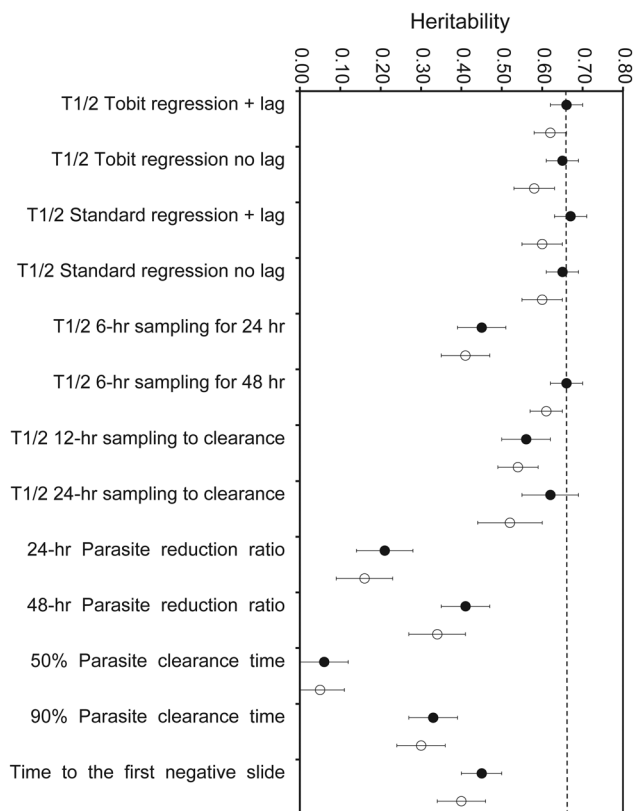


Figure 1. Heritability of 13 measures of parasite clearance on the Thai–Myanmar border. The proportion of variation in each parasite clearance metric attributable to parasite genetic factors (heritability) is shown for infections sampled from 2007 to 2010. Filled circles show total heritability; open circles show heritability of residuals obtained after removing effects of significant covariates. The dotted line shows heritability estimated from 6-hour parasite counts until clearance. The error bars are 1 SD.

$n = 1297$) compared with slow-clearing infections with $t_{1/2}P \geq 6.15$ hours (8%; $n = 132$), we found no evidence that lags are heritable ($H^2 = -0.06 \pm 0.10$). The lag phase is most likely due to the age distribution of the parasite population, as suggested previously [13, 17].

Effect of Using Different Slope-Fitting Procedures

Both tobit and standard regression models captured the same amount of variation in $t_{1/2}P$ due to parasite genetic factors (Figure 1). However, this dataset is unusually complete. Tobit regression will improve fit to datasets with fewer data points or with long gaps between observations, and we recommend use of this slope-fitting procedure in preference to simple regression [13].

Conclusions

Our results demonstrate that 6-hourly sampling of parasitemia for the first 48 hours following treatment provides robust clearance rate measures that are equal to 6-hourly monitoring until

clearance. This abbreviated approach has considerable advantages in Southeast Asian countries that harbor ART-resistant parasites because parasites may be present for up to a week following treatment. While the current dataset involves hyper-parasitemic patients, we expect that 6-hourly sampling of parasitemia for the first 48 hours following treatment will be equally effective for uncomplicated parasitemia cases [3]. Methods based on time to clearance or PRRs performed poorly and should be avoided. Because we observed no indication that parasite genetics determines the lag phase observed in some clearance curves, we suggest that both surveillance and genetic studies of ART resistance focus on the linear part of clearance curves. More generally, these results demonstrate the utility of heritability for assessing the robustness of phenotype measures [10].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

- Noedl H, Se Y, Schaefer K, et al. Evidence of artemisinin-resistant malaria in Western Cambodia. *N Engl J Med* **2008**; 359:2619–20.
- Dondorp AM, Nosten F, Yi P, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* **2009**; 361:455–67.
- Amaratunga C, Sreng S, Suon S, et al. Artemisinin-resistant *Plasmodium falciparum* in Pursat province, Western Cambodia: a parasite clearance rate study. *Lancet Infect Dis* **2012**; 12:851–8.
- Phyo AP, Nkhoma S, Stepniewska K, et al. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *Lancet* **2012**; 379:1960–6.
- Anderson TJ, Nair S, Nkhoma S, et al. High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in western Cambodia. *J Infect Dis* **2010**; 201:1326–30.
- Cheeseman IH, Miller BA, Nair S, et al. A major genome region underlying artemisinin resistance in malaria. *Science* **2012**; 336:79–82.

7. Takala-Harrison S, Clark TG, Jacob CG, et al. Genetic loci associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment in Southeast Asia. *Proc Natl Acad Sci U S A* **2013**; 110:240–5.
8. Carrara VI, Zwang J, Ashley EA, et al. Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. *PLoS One* **2009**; 4: e4551.
9. Stepniewska K, Ashley E, Lee SJ, et al. *In vivo* parasitological measures of artemisinin susceptibility. *J Infect Dis* **2010**; 201:570–9.
10. Anderson TJ, Williams JT, Nair S, et al. Inferred relatedness and heritability in malaria parasites. *Proc Biol Sci* **2010**; 277:2531–40.
11. Bethell D, Se Y, Lon C, et al. Artesunate dose escalation for the treatment of uncomplicated malaria in a region of reported artemisinin resistance: a randomized clinical trial. *PLoS One* **2011**; 6:e19283.
12. Beshir KB, Hallett RL, Eziefula AC, et al. Measuring the efficacy of anti-malarial drugs *in vivo*: quantitative PCR measurement of parasite clearance. *Malar J* **2010**; 9:312.
13. Flegg JA, Guerin PJ, White NJ, Stepniewska K. Standardizing the measurement of parasite clearance in falciparum malaria: the parasite clearance estimator. *Malar J* **2011**; 10:339.
14. Nkhoma SC, Nair S, Al-Saai S, et al. Population genetic correlates of declining transmission in a human pathogen. *Mol Ecol* **2013**; 22: 273–85.
15. Lynch M, Walsh B. Threshold characters. In: *Genetics and Analysis of Quantitative Traits*. Sunderland, MA, USA: Sinauer Associates, **1998**: 727–744.
16. Edwards JH. Familial disposition in man. *Brit Med Bull* **1969**; 25:58–63.
17. White NJ. The parasite clearance curve. *Malar J* **2011**; 10:278.