An analytical method for assessing stage-specific drug activity in *Plasmodium vivax* malaria: implications for *ex vivo* drug susceptibility testing

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Abstract

The emergence of highly chloroquine (CQ) resistant *P. vivax* in Southeast Asia has created an urgent need for an improved understanding of the mechanisms of drug resistance in these parasites, the development of robust tools for defining the spread of resistance, and the discovery of new antimalarial agents. The *ex vivo* Schizont Maturation Test (SMT), originally developed for the study of *P. falciparum*, has been modified for *P. vivax*. We retrospectively analysed the results from 760 parasite isolates assessed by the modified SMT to investigate the relationship between parasite growth dynamics and parasite susceptibility to antimalarial drugs. Previous observations of the stage-specific activity of CQ against *P. vivax* were confirmed, and shown to have profound consequences for interpretation of the assay. Using a nonlinear model we show increased duration of the assay and a higher proportion of ring stages in the initial blood sample were associated with decreased effective concentration (EC_{50}) values of CQ, and identify a threshold where these associations no longer hold. Thus, starting composition of parasites in the SMT and duration of the assay can have a profound effect on the calculated EC_{50} for CQ. Our findings indicate that EC_{50} values from assays with a duration less than 34 hours do not truly reflect the sensitivity of the parasite to CQ, nor an assay where the proportion of ring stage parasites at the start of the assay does not exceed 66%. Application of this threshold modelling approach suggests that similar issues may occur for susceptibility testing of amodiaquine and mefloquine. The statistical methodology which has been developed also provides a novel means of detecting stage-specific drug activity for new antimalarials.

Author Summary

The schizont maturation test (SMT) was developed to monitor drug resistance in malaria parasites. The SMT examines differences in the rate of parasite development when exposed to different drug concentrations, providing an estimate of drug efficacy. While the assay is effective when examining resistance in *Plasmodium falciparum*, there are concerns regarding its suitability for testing other malaria species, particularly if the drug only targets particular life-cycle stages of the parasite. Blood samples taken from *Plasmodium vivax* infected individuals exhibit significant heterogeneity in the parasite life-cycle stages present. If a drug targets the early stage parasites, but only late stage parasites are present in the sample, the test will show an erroneously high degree of resistance. In this study, we estimate thresholds which can be used to identify when test results can be considered accurate should the drug being tested only affect specific life stages of the parasites. Chloroquine is used as a case study but the method developed also allows the identification of stage-specific activity in other malarial drugs in *P. vivax*. For field researchers, this threshold modelling approach

will allow for increased confidence in the reliability of *P. vivax* resistance results and provides a novel means of detecting stage-specific drug activity for new antimalarials

Introduction

Malaria continues to pose a significant threat to human health globally. Currently, as many as 2.6 billion people are at risk of *P. vivax* infection, with an estimated 72-390 million cases per year [1,2]. Historically, malaria research has focussed on *P. falciparum,* due to its reputation as the most lethal human malaria parasite. Although *P. vivax* is less pathogenic than *P. falciparum*, it still poses a serious public health burden and is being increasingly recognised as a cause of severe and fatal disease, particularly in children and pregnant women [2,3].

A number of recent publications have highlighted the increasing recognition of the clinical importance of *P. vivax* and the renewed emphasis placed on research into this species [2,4,5,6]. Further, the emergence of highly chloroquine (CQ) resistant *P. vivax* in Southeast Asia (CQ remains a front-line treatment for vivax malaria as it is affordable, well tolerated and safe, and its long half-life ensures protection from early relapses [7,8]) has created an urgent need for an improved understanding of the mechanisms of drug resistance in these parasites, the development of robust tools for defining the spread of resistance, and the discovery of new antimalarial agents. To glean insights into the development of resistance in *P. vivax*, a modified form of the *ex vivo* Schizont Maturation Test (SMT) has been developed and applied to fresh isolates directly from patients [9,10,11,12].

The central tenant of the SMT is that drug activity on susceptible parasites will completely stop or slow the growth in a dose-dependent manner, with reduced susceptibility being manifest by an ability of parasites to mature to schizont stages in the presence of higher concentrations of drug. The standard assay is conducted for 30 hours, the time required for parasites to reach maturation without drug, and the proportion of schizonts at the conclusion of the assay is used as an indicator of parasite maturation.

The SMT was initially developed for testing drug susceptibility in *P. falciparum* [13,14], where almost all parasites in the peripheral circulation are at the immature ring stage. However, in infections due to non-falciparum species, trophozoite and schizont stages are commonly present in the peripheral circulation. To accommodate this, a modified SMT has been developed in which the control wells

are monitored until the number of schizonts exceeds 40% of parasites prior to harvest (i.e., assays are conducted for variable lengths of time)[15].

For the results of the SMT to be valid, a sample, irrespective of the drug resistance phenotype of the parasites, must have had sufficient exposure to the drug to affect a response. The diversity in parasite life cycle stages in *P. vivax* infections creates a significant confounding factor, particularly since there is an apparent marked variation of drug susceptibility in different erythrocytic life cycle stages. In previous work, it has been demonstrated that the trophozoite stages of *P. vivax* are almost completely resistant to CQ, and continue to mature no matter how high the concentration of drug [15,16]. It follows that, if a drug only acts on ring stage parasites, but the sample contains a majority of trophozoites and schizonts, the parasite is likely to be erroneously categorised as resistant simply because there were no susceptible life cycle stages present in the assay.

In this study we develop a statistical methodology to identify stage specific drug activity in the SMT. We use CQ against *P. vivax* as a case study and demonstrate that stage specific drug activity has profound consequences for the interpretation of SMT results. We also examine stage-specific drug effects for other commonly used drugs and simulate the growth dynamics of *P. vivax* parasites within the SMT to provide recommendations on how to improve the reliability of SMT results.

Methods

Ethics Statement

Ethical approval for the collection of blood samples for drug susceptibility testing was obtained from the ethics committees of the National Institute of Health Research and Development, Ministry of Health (Jakarta, Indonesia), and the Human Research Ethics Committee of NT Department of Health & Families and Menzies School of Health Research (Darwin, Australia). Written informed consent was obtained from adult patients and parents and/or guardians of enrolled children.

Data collection and ex vivo drug susceptibility testing

Blood samples were collected from patients attending outpatient clinics in Timika, Papua Province, Indonesia, as previously described [15]. Only patients infected with a single species of Plasmodia were included in the study; the majority of samples contained *P. vivax* or *P. falciparum*, although a limited number of *P. malariae* and *P. ovale* isolates were also available [17].

SMTs were conducted on these samples following World Health Organisation guidelines for drug susceptibility testing [18], with modifications developed to aid the application of the test to *P. vivax*

127 [15]. Venous blood (5mL) was collected by venipuncture, and after removal of host white blood cells
128 using a CF11 column, 800µL of packed infected red blood cells (IRBC) were used for the SMT. Assays
129 conducted for up to 7 drugs: CQ, artesunate, amodiaquine, lumefantrine, mefloquine, piperaquine
130 and pyronaridine [15].
131
132 The proportion of parasite life cycle stages (i.e., rings, trophozoites and schizonts, as defined by
133 Russell et al [12,15]) in each isolate was assessed at 0 hours, 24 hours, and then at non-uniform
134 times until 40% of the parasites in the control well reached mature schizonts. At this point, the
135 assay was terminated, and wells under serial drug concentrations were harvested. The proportion
136 of parasites in each stage of the erythrocytic cycle at time of harvest was reported for each drug
137 concentration, as was the duration of the assay.
138
139 *Dose Response Modelling*
140 The estimated drug response (*R)* of each isolate was derived from the ratio between the proportion
141 of schizonts at harvest in the treatment well compared to that in the control well. Only data
142 satisfying the following criteria were included in the dose response modelling:
143 1. $R < 1.5$ for all drug concentrations,
144 2. $R > 0$ for the lowest drug concentrations,
145 3. $R < 0.5$ for at least one drug concentration.
146
147 The sigmoid E_{max} dose-response curve (equation 1) was fitted to all available data simultaneously,
148 with mixed-effects modelling used for CQ data.

149 $$R_{ij} = E_{\max_i} - (E_{\max_i} - E_0) \times \frac{c_{ij}^{\gamma_i}}{(c_{ij}^{\gamma_i} + EC_{50_i}^{\gamma_i})} \qquad \text{(Equation 1)}$$

150 R_{ij} represents the drug mediated growth inhibition response (the ratio between the proportion of
151 schizonts at harvest in the treatment well compared to the control well) for the ith isolate at the jth
152 concentration, c_{ij} represents the drug concentration. E_{max} and E_0 represent the maxima and minima
153 of the dose response curve and γ the slope of this curve. The EC_{50} value represents the effective
154 concentration at which 50% of the parasite population exhibit a response to the drug. Inter-isolate
155 variability was included for E_{max}, EC_{50} and γ. The drug plate batch was also incorporated as a random
156 effect, to control for batch-to-batch variability. Predicted values for EC_{50} for each isolate were
157 calculated from Empirical Bayes estimates.
158

159 EC_{50} values for parasite samples collected from April 2004 and May 2007 have previously been
160 reported (Russell 2008). However the methodology used to calculate EC_{50} for CQ differs between the
161 previous report and the current study due to the use of mixed-effects modelling.
162
163 *Statistical analysis*
164
165 For each of the seven drugs tested, linear regression models were used to characterise the
166 relationship between the EC_{50} values derived for each isolate and the following independent
167 variables: assay duration, the proportion of rings at 0 hours, and delay between venepuncture and
168 assay. The EC_{50} values were not normally distributed and therefore, $\ln(EC_{50})$ was used as the
169 dependent variable.
170
171 Where a relationship between one of the independent variables and $\ln(EC_{50})$ was found a non-linear
172 threshold model was constructed to determine the threshold at which the association ceased to
173 exist (Equation 2).
174 $$EC_{50} = b + (\text{Duration} < c) * a * (\text{Duration} - c), \qquad \text{(Equation 2)}$$
175 *a* represents the rate of decline in EC_{50} when the duration of the assay is less than the threshold, *b*
176 the mean EC_{50} values for samples where the duration exceeds the threshold, and *c* the threshold. A
177 threshold model was preferred over other non-linear models due to the greater interpretability of
178 parameters.
179
180 A threshold model of the same form was also fitted to define the relationship between the
181 proportion of rings at time 0 and EC_{50}. The threshold models were fitted to the data using nonlinear
182 regression.
183
184 *Characterising the duration of the erythrocytic cycle*
185 A simulation approach was used to estimate the duration of each stage of the erythrocytic life cycle
186 in both *P. vivax* and *P. falciparum.* Estimates were also made of stage durations using the limited
187 data available for *P. malariae* and *P. ovale* to validate the methodology. Poisson distributions were
188 selected to represent the duration of each stage, as random deviates produced by sampling the
189 distribution are always positive, and the parameters of the distributions allow for biologically
190 meaningful interpretation. We assumed the duration of each stage could be drawn from a
191 probability distribution, and estimate the mean of each Poisson distribution (λ).
192

One hundred simulations of the growth dynamics within a culture well, each containing 200 parasites, were conducted. It was assumed each parasite began life as a ring, transitioned to a trophozoite, then to a schizont. The length of time spent in each stage was determined by sampling from the relevant probability distribution. At selected time points, the proportion of parasites in each stage was calculated. As parasites completed the schizont stage, they were assumed to die, and subsequently, proportions were calculated based only on the remaining surviving parasites.

A systematic search of the parameter space for the mean duration in each life cycle stage (λ) was conducted to determine the optimal values for each Poisson distribution. The mean model fit from three simulation experiments (totalling 300 simulations) for each combination of parameters was used and the search examined potential parameters in increments of 0.5 hours.

The optimal fit was determined by minimising the sums-of-squares between the simulation results and data on proportion of parasite at each life cycle stage from the control wells, for a subset of the original field samples. The subset of field samples used had 1) 100% rings at 0 hours, 2) data for at least 3 time points, and 3) an assay duration >42 hours. This subset was used to ensure only samples with young ring stage parasites were included in the fitting process. A penalty equivalent to a difference of 20 between data and simulation results was applied for any later time points where the death of the entire simulated parasite population meant that no direct comparison could be made to the data.

All statistical analyses and simulations were conducted using the R statistical computing software package [19].

Results

Description of initial samples (at 0 hours)

SMT results for parasites sourced from 784 patients with single-species infections of either *P. vivax* (n = 345) or *P. falciparum* (n = 439) were analysed; 289 (84%) *P. vivax* and 331 (75%) *P. falciparum* isolates met the inclusion criteria for statistical analysis. Among these, 141 *P. vivax* (49%) and 216 *P. falciparum* (65%) isolates reached the 40% schizont threshold at harvest. The time between venepuncture and start of the assay (the 'delay') was significantly correlated with the duration of the assay for isolates of *P. falciparum* (correlation co-efficient (r) = -0.211, $p < 0.001$), but not *P. vivax* (r = -0.065, p = 0.226).

227 There was a significantly higher mean proportion of ring stage parasites in samples at the start of the
228 assay (0 hours) for *P. falciparum* (0.922) compared to *P. vivax* (0.588) (*Wilcoxon rank sum test*, p
229 <0.001). 87.5% (189/216) of *P. falciparum* isolates contained 100% ring stage parasites at 0 hours,
230 compared to only 2.8% (4/141) of *P. vivax* isolates.
231
232 *Predictors of CQ susceptibility*
233 There was a significant negative association between the CQ assay duration (hours) and the ln (EC_{50})
234 values for *P. vivax* (r^2 = 0.219, p < 0.001; Figure 1a). A similar, but weaker relationship was observed
235 for *P. falciparum* (r^2 = 0.097, p < 0.001; Figure 1b). Figure 1 suggests some bimodality in the
236 distribution of results, but this is predominantly due to the lack of sampling between ~32 and 40
237 hours. The proportion of rings at the start of the assay was also significantly negatively associated
238 with the ln(EC_{50}) values for both *P. vivax* (r^2 = 0.245, p < 0.001) and *P. falciparum* isolates (r^2 = 0.206,
239 p < 0.001; Figure 2). In both species there was a significant negative correlation between the
240 proportion of rings at the onset of assay and assay duration (r^2 = 0.621, p < 0.001; r^2 = 0.185, p <
241 0.001, for *P. vivax* and *P. falciparum*, respectively).
242
243 The threshold model for assay duration was successfully fit to the *P. vivax* data (Figure 3, Table 1).
244 The threshold value, c, represents the point at which the assay duration was no longer significantly
245 associated with estimates of EC_{50}. We interpret this threshold as the point at which assay duration
246 was sufficiently long to guarantee that the target parasite stage/s were present and exposed to the
247 drug. Although there was a relationship between assay duration and EC_{50} for *P. falciparum* we were
248 unable to fit a threshold model as fitting procedures did not identify an appropriate non-linear
249 model and associated threshold point.
250
251 A threshold model to determine the relationship between the initial proportion of rings and the EC_{50}
252 was also only possible for *P. vivax*; we were unable to fit a threshold model to the *P. falciparum* data.
253 For *P. vivax* the proportion of rings at the onset of assay was found to have a significant non-linear
254 relationship with EC_{50} (Figure 4; Table 2). Samples with less than 65% ring stage parasites at time 0
255 had higher and more variable EC_{50} values than those isolates with a greater proportion of ring stage
256 parasites.
257
258 *Comparisons with other drugs*

259 Of the six other drugs tested, threshold models were successfully fitted to data for amodiaquine and
260 mefloquine in *P. vivax*. Confidence intervals around the estimated threshold points showed distinct
261 overlap, suggesting no significant differences between the two drugs, or CQ (Table 3).
262
263 *Characterising the duration of the erythrocytic cycle*
264 In the simulations of the growth dynamics under *ex vivo* assay conditions, the λ parameter for the
265 Poisson distributions represented the duration of each parasite life cycle stage in *P. vivax*. The mean
266 λ was 19.1 hours (95% CI 11 , 29) for rings, 23.1 hours (95% CI 11, 36) for trophozoites, and 2.2 hours
267 (95% CI 0, 7) for the schizonts. The total duration of the *P. vivax* erythrocytic cycle was estimated as
268 44.5 hours (95% CI 29, 62). Similar simulations of *P. falciparum* growth gave mean estimates of the λ
269 parameter of 23.3 hours (95% CI 14, 33) for ring stage parasites, 21.4 hours (95% CI 11, 33) for
270 trophozoites, and 3.7 hours (95% CI 0, 10) for schizonts; estimates produce a total duration of 48.4
271 hours (95% CI 32, 66). These estimates of an *ex vivo* life cycle do not include the age of the rings
272 before the start of the assay and the growth time of schizonts after the end of the assay, thus the
273 true life cycle is expected to be longer than reported.
274
275 To validate the methodology, the growth dynamics of a small number of SMT results were also
276 simulated for *P. malariae* (n=39) and *P. ovale* (n=13) isolates. The total duration of the erythrocytic
277 cycle was estimated to be 75.6 hours (95% CI 59, 93) in *P. malariae* and 53.9 hours (95% CI 34, 79) in
278 *P. ovale*.
279
280 **Discussion**
281 Despite being developed initially for *P. falciparum* parasites, the Schizont Maturation Test (SMT) has
282 seen considerable use in testing drug susceptibility of *P. vivax*. However, little consideration has
283 previously been given to whether the application of the SMT to *P. vivax* is appropriate. In this study,
284 we demonstrate that the stage-specific dynamics of drug activity may impact on the validity of SMT
285 results. If a particular drug targets ring stage parasites, but is tested in an isolate predominantly
286 containing mature stage parasites, the drug will appear to be ineffective, resulting in an
287 overestimate of resistance. Assay duration and the proportion of rings in the initial sample both
288 provide proxy indicators of the likelihood that early stage parasites will be exposed to drugs.
289 Previous experimental work has identified that trophozoite stage *P. vivax* parasites are insensitive to
290 CQ [15,16]; our results support this finding. We apply statistical analysis techniques to show that the
291 heterogeneity of erythrocytic life cycle stages present in peripheral blood samples taken from

292 patients infected with *P. vivax* necessitates additional criteria be applied to the SMT to ensure the
293 validity of the results.
294
295 The significant negative relationship between the time of venepuncture and start of the SMT and
296 duration of the SMT for *P. falciparum* was not unexpected. The onset of fever is usually associated
297 with the start of a new erythrocytic cycle, meaning the sum of the time elapsed between
298 establishment of the SMT and SMT duration will be more indicative of total duration of the
299 erythrocytic cycle than the SMT duration alone. Hence, parasites which have a delay between
300 venepuncture and start of SMT will enter the SMT in a more advanced state, requiring less time to
301 develop to 40% schizonts. This effect is most likely more dominant in *P. falciparum* samples due to
302 the high synchronicity in parasites when obtained from the patient, compared to *P. vivax*.
303
304 For both *P. vivax* and *P. falciparum* isolates, there were significant negative relationships between
305 the duration of the assay and estimated EC_{50} values (i.e., short duration assays were associated with
306 reduced susceptibility to drugs). There are a number of possible explanations for these results, none
307 of which is mutually exclusive and all of which are specific to the drug/parasite combination:
308 1. Later stage parasites are more tolerant to the drug then ring stage parasites; the higher the
309 proportion of ring stage parasites the lower the EC_{50},
310 2. The drug effect is cumulative over time which results in longer exposure times having a
311 greater relative effect for the same drug concentration,
312 3. Resistant parasites grow faster.
313
314 When stage-specific drug activity is a consideration, as with CQ against *P. vivax* [15,16], we
315 hypothesised that samples more advanced in their development, as characterised by short assay
316 duration or a low proportion of ring stage parasites in the initial blood sample, would appear to be
317 less susceptible to a drug because a significant number of parasites present in the assay had
318 developed beyond the target stage. Support for this hypothesis is provided by the threshold
319 modelling. While the linear models we have described are appropriate for the *P. falciparum* SMTs,
320 examination of the trends in *P. vivax* suggest a distinctly non-linear pattern for some drugs, including
321 CQ. The threshold modelling indicates that the EC_{50} for CQ in *P. vivax* parasites stabilises once the
322 sample has been exposed to the drug for at least 33.7 hours (CI 28.2, 39.3). The simulation
323 modelling of parasite development time suggests that *P. vivax* parasites spend an average of 25.3
324 hours as trophozoites and schizonts before termination of the assay. If the SMT is terminated when
325 the control well reaches 40% schizonts, and this occurs after 33.7 hours (mean duration threshold),

326 it follows that the SMT must have exposed sufficient ring stage parasites to the drug, resulting in
327 EC_{50} reaching its minimum. Combining the results from the threshold and life cycle modelling, an
328 assay lasting 33.7 hours will have exposed 86.5% of the parasites to CQ for at least one hour at ring
329 stage. Using the upper 95% confidence limit for mean assay duration, a more conservative threshold
330 estimate of 39 hours, we can predict that 95% of the parasites were exposed to CQ for at least 2
331 hours at ring stage. We propose that this threshold represents the duration of the assay which is
332 sufficient to guarantee that a significant proportion of the ring stage *P. vivax* parasites have been
333 exposed to the drug (i.e. there is no longer any association between duration and EC_{50}). Assay
334 durations shorter than this threshold expose a greater proportion of tolerant mature stage parasites
335 to the drug, and thus the EC_{50} values derived from these samples will be artificially elevated.
336
337 Similar dynamics are apparent when examining the composition of the initial blood samples. The
338 disparity in the degree of developmental stage heterogeneity in the initial samples between *P. vivax*
339 and *P. falciparum* is striking. *P. falciparum* isolates are markedly more synchronous, presumably
340 because the mature stages (trophozoites and schizonts) are sequestered in deep tissues and organs,
341 rather than in the peripheral circulation. Nearly all *P. falciparum* samples contain only rings, and are,
342 therefore, ideal for SMT. In contrast, *P. vivax* isolates tend to show significantly greater
343 heterogeneity in the life cycle stages, with trophozoites and schizonts regularly occurring. The
344 presence of these advanced stages at the onset of SMT makes the interpretation of drug
345 susceptibility results more difficult. Our modelling suggests that the initial sample should contain a
346 minimum of 66% ring stage parasites, preferably >90% ring stage parasites (upper 95% confidence
347 interval of the threshold parameter), to ensure the target life cycle stages are sufficiently present in
348 a sample. However, this significantly reduces the number of samples from which drug sensitivity
349 data can be obtained potentially introducing a sampling bias. It should be noted that, by selecting
350 only those samples that achieve 40% schizonts, we also introduce bias against those parasites that
351 do not grow well in culture.
352
353 Processes for synchronising parasitemia have also been proposed as a means of decreasing stage
354 heterogeneity of *Plasmodium* isolates for *ex vivo* characterisation [20,21]. Although such methods
355 may have utility and permit testing of some field isolates that would otherwise be excluded from
356 testing, the removal of mature trophozoite parasites inevitably results in a reduction in parasite
357 count, which is itself another important parameter for reliable quantification of parasite growth.
358

359 An alternate approach to specifying a definitive threshold is to apply the same types of threshold
360 models which we have developed here using all available data. Such an approach would have three
361 advantages. First, it would allow all the field samples to be used, thus reducing the potential for bias
362 in the SMT samples. Second, it would allow the development of resistance to be monitored over
363 time by looking for changes in the threshold duration and minimum EC_{50}. Third, it can be used to
364 look for stage-specific drug action in current and new antimalarial drugs. Similar patterns and
365 threshold values were found for CQ, amodiaquine and mefloquine, suggesting all of these drugs
366 have their main effect on ring stage *P. vivax* parasites. Such a relationship was not observed for the
367 other antimalarials investigated (i.e., artesunate, lumefantrine, piperaquine and pyronaridine).
368 Differences between the stage specific activity of each drug and its variation between parasite
369 species may prove highly informative in elucidating the mechanisms of arug action as well as innate
370 and acquired drug resistance. While it is always possible to investigate stage-specific drug activity
371 using carefully planned laboratory experiments, as reported by Russell et al. [15], the methodology
372 presented here can identify stage specificity through far less laborious means, and can use
373 previously collated results.
374
375 Our simulation of parasite development in the SMT and subsequent estimate for the duration of
376 each life cycle stage is the first attempt to model parasite development times for *P. vivax*. It is
377 important to note that the estimates are relative to the parasite development in the restricted
378 conditions of the SMT control well and may not represent the length of the life stages *in vivo*, or
379 indeed in potential *in vitro* culture. It should also be expected that the estimated duration of the
380 ring stage underestimates the true duration due to the delay in obtaining the blood sample after
381 parasite rupture and establishing the SMT. More expansive sampling over the first 24 hours of the
382 assay would likely reduce the confidence intervals of the estimated development times.
383
384 In summary, a threshold modelling approach was applied to data from a modified SMT to investigate
385 resistance to CQ in *P .vivax*. We identified patterns which suggest a non-linear relationship between
386 drug susceptibility in the parasite and both the duration of an assay and the proportion of ring stage
387 parasites in the initial sample, which signifies tolerance of late stage parasites to CQ. Consequently,
388 we recommend that *P. vivax* isolates should contain a minimum of 66% ring stage life cycle stages,
389 and that assay duration should exceed 34 hours to ensure this stage-specific effect does not
390 artificially inflate the reported EC_{50}. More conservative thresholds would require a minimum of 90%
391 ring stage parasites and a minimum assay duration of 40 hours. An alternative approach would be
392 to use the statistical methodology which has been developed. For field researchers, this threshold

modelling approach will allow for increased confidence in the reliability of resistance results. This approach also provides a novel means of detecting stage-specific drug activity for new antimalarials, as demonstrated by our analysis of the susceptibility to amodiaquine and mefloquine.

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467 **Figure legends**

468 Figure 1. Relationship between assay duration and ln(EC_{50}) for (A) *P. vivax* (n = 141) and (B) *P.*
469 *falciparum* (n = 216)

470

471 Figure 2. Relationship between the proportion of ring stage parasites at start of assay (0 hours) and
472 ln(EC_{50}) for (A) *P. vivax* and (B) *P. falciparum*

473

474 Figure 3. Threshold modelling results for the relationship between assay duration and estimated CQ
475 EC_{50} values in *P. vivax*

476

477 Figure 4. Threshold modelling results for the relationship between the proportion of rings in the
478 initial sample and estimated CQ EC_{50} values in *P. vivax*

479

480 **Table 1. Parameter estimates for threshold modelling of the relationship between assay duration**

481 **and CQ EC_{50} in *P. vivax***

Parameter	Estimate	95% Confidence Intervals		P-value
		Lower	Upper	
Threshold (*c*, in hours)	33.73	28.15	39.31	< 0.001
Change in EC_{50} per increase of one hour assay duration when assay duration < *c* (*a*)	-82.01	-131.69	-32.33	0.002
Mean EC_{50} when duration > *c* (*b,* in nmols/l)	141.91	-19.05	302.87	0.086

482

483

484 Table 2. Parameter estimates for threshold modelling of the relationship between the proportion of
485 rings samples at hour 0 and CQ EC_{50} in *P. vivax*

Parameter	**Estimate**	**95% Confidence Intervals**		**P-value**
		Lower	Upper	
Threshold (*c*)	0.65	0.42	0.89	< 0.001
Change in EC_{50} when proportion of rings at hour 0 < *c* (*a*)	-1693.2	-2470.7	-9 15.7	< 0.001
Mean EC_{50} when proportion of rings at hour 0 > *c* (*b,* in nmols/l)	139.69	-7.49	286.87	0.065

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487

488 Table 3. Comparison of the threshold points for amodiaquine, chloroquine and mefloquine, when
489 analysing the relationship between assay duration and EC_{50} in *P. vivax*

Drug	**Threshold Estimate (*c*, hrs)**	**95% Confidence Intervals**		**P-value**
		Lower	Upper	
Chloroquine	33.73	28.15	39.31	< 0.001
Amodiaquine	31.13	27.84	35.7	0.01
Mefloquine	39.14	32.94	45.34	< 0.001

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