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In vitro growth phenotypes of single parasite lineages cloned from multiclonal malaria isolates

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***ABSTRACT**: Measurement of malaria parasite proliferation in cultured erythrocytes is critical for elucidating key determinants of phenotypes, including drug susceptibility, virulence, and fitness. Multiple parasite lineages with different proliferation rates or fitness may coexist within a clinical isolate, resulting in complex growth interactions and variations in phenotype. We measured proliferation rates of three Plasmodium falciparum Cambodian isolates, including IPC_3445 (MRA-1236), IPC_5202 (MRA-1240), IPC_6403 (MRA-1285), and parasite lineages previously cloned from each of these isolates by limiting dilution. Following synchronization, in vitro cultures were maintained over four consecutive asexual parasite life cycles, with parasite sampling at the end of every cycle to estimate parasitemia and growth rate. In parallel with clinical isolates and component parasite lineages, growth rates and relative changes in parasitemia were measured for laboratory parasite lines 3D7 (MRA-102) and DD2 (MRA-150) as controls. We observed significant differences in fold-change in parasitemia (FC), and parasite growth rate (GR) between parasite isolates and clonal lineages that make up each isolate. For example, while isolate MRA-1240 exhibits similar a proliferation rate to one of its constituent lineages, MRA1240-hap1 (GR: 1.03 ± 0.02 vs. 1.02 ± 0.05; FC: 67 ± 6 vs. 62 ± 2; p > 0.05), the other two component lineages (MRA1240-hap2 and MRA1240-hap3) exhibit markedly different growth profiles. We observed that the most abundant parasite haplotype often dominates the growth phenotype, masking the effect of minority haplotypes akin to recent observations from drug susceptibility testing. Our results also show diminished proliferation of isolate MRA-1236 (GR: 0.90 ± 0.02; FC: 39 ± 4) relative to the component lineages MRA1236-hap1 (GR: 1.11 ± 0.02; FC: 101 ± 3) and MRA1236-hap2 (GR: 1.05 ± 0.04; FC: 88 ± 6) suggestive of competitive suppression. All parasite lines are available through BEI Resources and have well-defined in vitro growth phenotypes useful for both research and development of interventions against malaria.*

BACKGROUND

- Clinical malaria isolates often contain multiple parasite lineages exhibiting phenotypic variation
- Interaction amongst multiple lineages within a clinical isolate can drive evolution of important traits including growth rates, virulence and drug susceptibility
- We hypothesized that parasite lineages previously isolated from multiclonal isolates exhibit significantly variable proliferation rates under standard culture conditions
- To test this hypothesis, parasite growth rates and cell cycle times were measured for three *Plasmodium falciparum* Cambodian isolates, including IPC_3445 (MRA-1236), IPC_5202 (MRA-1240), IPC_6403 (MRA-1285), and parasite lineages previously cloned from each of these isolates by limiting dilution[1]

MATERIALS & METHODS

P. falciparum strains used and BEI Resources/MR4 catalog item numbers

- *P. falciparum* IPC_3445 (MRA-1236) and constituent haplotypes MRA1236-hap1 and MRA1236-hap2
- *P. falciparum* IPC_5202 (MRA-1240) and component haplotypes MRA1240-hap1, MRA1240-hap2, MRA1240-hap3
- *P. falciparum* IPC_6403 (MRA-1285) and its three component haplotypes MRA1285-hap1, MRA1285-hap2 and MRA1285-hap3
- *P. falciparum Dd2* (MRA-150)
- *P. falciparum* 3D7 (MRA-102)

Standard conditions of asexual *P. falciparum* culture

All parasites were grown in leukocyte-depleted human type O+ erythrocytes at 37˚C using a gas mixture containing 90% N_2, 5% O_2 and 5% CO_2

Composition of media used

RPMI 1640 media (Gibco; Cat # 21870-084) supplemented with 4µg/mL Gentamicin solution (Gibco; Cat # 15750-060), 0.21% Sodium Bicarbonate (Gibco; Cat # 21870-084), 22mM HEPES buffer (Gibco; Cat # 15630-080), 0.18mM Hypoxanthine (Sigma; Cat # H9636), 0.18% Glucose (Sigma; Cat # G7021), 1.77mM L-Glutamine (Gibco; Cat # 25030-149) and 10% pooled human serum[2]

Establishing highly synchronous cultures for growth rate studies

- Culture-adapted parasites (>3% parasitemia, >70% ring-stage) were synchronized by sorbitol treatment & incubated at 37˚C for 42 hrs.
- Following incubation, cultured samples were passed through percoll gradients to enrich for schizonts
- Schizonts were grown for three hours & the resulting post-invasion rings (0-3 hr. old) were synchronized by sorbitol treatment, followed by multiple washes

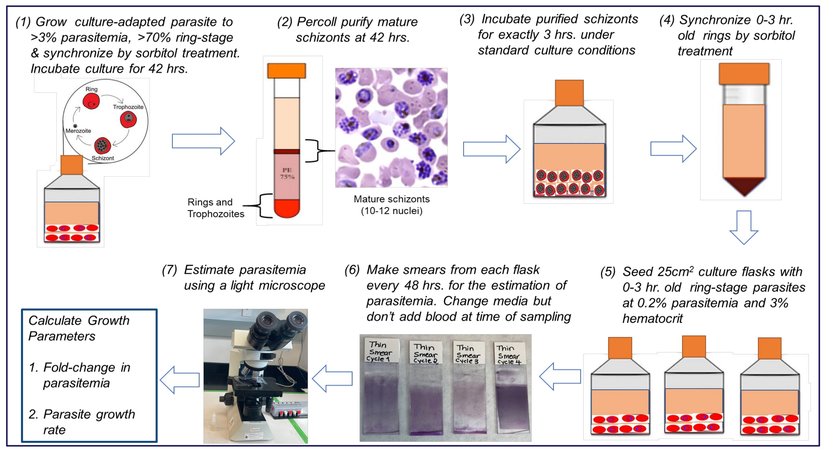
Setting up growth rate experiments

- 0-3 hr. old rings were seeded at 0.20% parasitemia and 3% hematocrit in 25cm^2 culture flasks & incubated at 37˚C for 48 hrs.
- Thin smears were made from each culture every 48 hrs. with fresh media changes made to each culture but without any blood added
- Smears were stained with 10% geimsa and read by examination of at least 500 cells with a light microscope

Estimation of growth phenotypes

- For each parasite line we estimated both the fold-change in parasitemia & growth rate
- Fold-change describes change in parasitemia over time and is calculated as final parasitemia at the end of growth monitoring (in our case, after the 4th growth cycle) divided by the initial parasitemia used to seed the cultures
- Growth rate per asexual cycle is estimated as the slope of a plot of log-transformed parasite counts against number of cycles

TECHNICAL APPROACH SUMMARY



RESULTS

Different parasite lines exhibit variability in growth under standard culture conditions

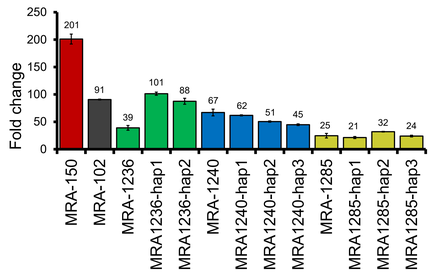


***Figure 1.** Fold-change (FC) in parasitemia for each parasite line. FC is calculated and expressed as final parasitemia at the end of monitoring parasite growth (4th asexual cycle, 192 hrs.) divided by the initial parasitemia at the time of seeding parasites in culture. Significant variation in FC is observed between parasite isolates and component parasite lineages of each multiclonal isolate studied. Error bars represent the standard error of the mean for at least three independent experiments.*

Robust modelling of parasite growth captures variation in parasite proliferation rates

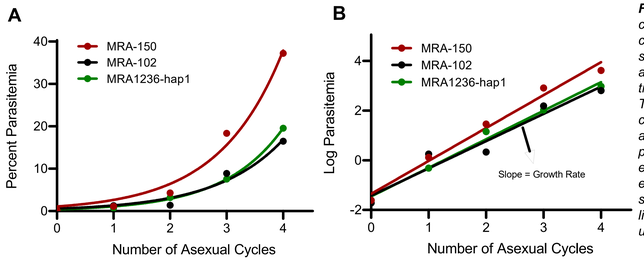


***Figure 2.** Panel A shows classical exponential growth curves for some parasite lines studied. Panel B is a plot of asexual cycles against log-transformed parasite counts. This yields a linear growth curve whose slope provides an empirical estimate of parasite growth rate. To ensure the robustness of our estimates, only datasets showing excellent fit to the linear model ($r^2 \geq 0.90$) were used.*

RESULTS…

Table 1: *In vitro* growth phenotypes of parasite lines studied

PARASITE ISOLATE	FOLD CHANGE (MEAN ± SEM)	GROWTH RATE (MEAN ± SEM)
MRA-102 3D7	91 ± 1	1.08 ± 0.01
MRA-150 Dd2	201 ± 9	1.27 ± 0.04
MRA-1236 IPC_3445	39 ± 4	0.90 ± 0.02
MRA1236-hap1	101 ± 3	1.11 ± 0.02
MRA1236-hap2	88 ± 6	1.01 ± 0.04
MRA-1240 IPC_5202	67 ± 6	1.03 ± 0.02
MRA1240-hap1	62 ± 2	1.02 ± 0.05
MRA1240-hap2	51 ± 1	0.94 ± 0.01
MRA1240-hap3	45 ± 1	0.93 ± 0.01
MRA-1285 IPC_6403	25 ± 4	0.77 ± 0.06
MRA1285-hap1	21 ± 2	0.80 ± 0.02
MRA1285-hap2	32 ± 1	0.84 ± 0.02
MRA1285-hap3	24 ± 3	0.82 ± 0.04

Data show the mean and standard error of the mean (SEM) for at least three independent experiments. An unpaired t-test was used to examine whether empirical values for the fold-change or growth rates of any two parasite lines are significantly different ($p < 0.05$). Comparisons that are significantly different for the mean fold-change are: MRA-1236 vs MRA1236-hap1, MRA-1236 vs MRA1236-hap2, MRA-1240 vs MRA1240-hap3, MRA1240-hap1 vs MRA1240-hap2, MRA1240-hap1 vs MRA1240-hap3, MRA1240-hap2 vs MRA1240-hap3, MRA1285-hap1 vs MRA1285-hap2 and MRA1285-hap2 vs MRA1285-hap3. Comparisons that are significantly different ($p < 0.05$) for the growth rate are: MRA-1236 vs MRA1236-hap1, MRA-1236 vs MRA1236-hap2, MRA-1240 vs MRA1240-hap2 and MRA-1240 vs MRA1240-hap3.

SUMMARY

- This study reveals significant variation in proliferation between co-infecting parasite lineages
- In some cases, the most predominant lineage dominates the growth profile, masking the contribution of minor frequency lineages
- In others, individual parasite lineages exhibit greater proliferation than the isolate from which they are cloned, suggestive of competitive suppression
- Parasite lines studied here are available to the malaria research and control community through the MR4 collection of NIAID's BEI Resources Program (https://www.beiresources.org)

REFERENCES

1. Nkhoma SC, Ahmed AO, Zaman S, Porier D, Baker Z and Stedman TT. Dissection of haplotype-specific drug response phenotypes in multiclonal malaria isolates. *Int. J. Parasitol. Drugs Drug Resist.* 2021:152-161.
2. MR4/ATCC and EVIMalaR. Methods in Malaria Research. (2013) 6th ed., Manassas, VA.

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