Composition of the hypoxic gas mixture affects read-outs of the Ring-stage Survival Assay

2024 GMU CIDR

Poster Session

Standwell C. Nkhoma, Amel O.A. Ahmed, Sujatha Rashid, Rebecca Bradford and Robert E. Molestina

American Type Culture Collection, Manassas, VA. Email: snkhoma@atcc.org

Resistance to front-line antimalarials is a major impediment to malaria control and elimination efforts. The Ring-Stage Survival Assay (RSA) is an invaluable tool for assessing parasite resistance to artemisinin, the key component drug of Artemisinin-based Combination Therapies used for malaria treatment worldwide. Variation in the composition of the hypoxic gas mixture used for culturing parasites can profoundly impact their growth rate and Dihydroartemisinin (DHA) susceptibility as measured by RSA. This study was designed to evaluate the extent to which RSA read-outs differ between parasites cultured in a commercial hypoxic gas mixture (5% CO_2, 5% O_2, 90% N_2) versus candle jar gas (3% CO_2, 17-18% O_2, 79-80% N_2) commonly used in resource-constrained settings. Artemisinin-resistant and susceptible parasites used in this study are available from ATCC through BEI Resources (https://www.beiresources.org). These include (i) MRA-1315, an ART-resistant parasite bearing the C580Y K13 mutation; (ii) MRA-1254, an ART-sensitive line reverse-engineered to bear the wild type cysteine (C) residue at K13 codon 580; (iii) MRA-1317, an ART-resistant clone with the R539T K13 mutation; and (iv) MRA-1252, an ART-sensitive line featuring a reversion from the mutant tyrosine (T) to the wild type arginine (R) at K13 codon 539. Briefly, each parasite line was exposed to a pharmacologically relevant DHA dose (700nM for 6hrs) under the two different gas treatments. Following drug wash, parasites were allowed to proliferate in drug-free media. Parasite survival was determined by microscopic examination and counting of viable parasites in drug-treated wells versus control wells. A survival rate of ≥ 10% indicated DHA resistance. Parasites cultured in candle jars exhibited significantly reduced survival rates and impaired growth compared to those grown in commercial gas. These results have important implications for artemisinin resistance surveillance and comparing RSA readouts between studies. Parasite lines described herein have well-defined artemisinin susceptibility phenotypes and are available to registered malaria researchers through NIAID's BEI Resources Program, managed by ATCC.

INTRODUCTION

- The Ring-Stage Survival Assay (RSA) is a useful tool for assessing *in vitro* parasite resistance to artemisinin (ART), the key component of Artemisinin-based Combination Therapies (ACTs) used worldwide[1].
- Anecdotal evidence from our previous work and seminal work by Vicky Avery, Sandra Duffy and others has demonstrated that Kelch13 mutations modulate *in vitro* parasite replication and susceptibility to artemisinins in response to hyperoxia[2,3,4].
- We hypothesized that the degree of artemisinin drug tolerance as measured by RSA differs between parasites cultured in a commercial gas mixture (5% CO_2, 5% O_2, 90% N_2) versus candle jar gas (3% CO_2, 17-18% O_2, 79-80% N_2) commonly used in resource-constrained settings.
- The main goal of this project was to utilize a carefully selected set of parasites bearing specific K13 mutations to examine whether RSA readouts differ between normoxic and hyperoxic conditions.

METHODS

Parasite strains. The following strains were obtained from the BEI Resources, NIAID, NIH (www.beiresources.org): (i) MRA-1315, an ART-resistant parasite harboring the C580Y K13 mutation; (ii) MRA-1254, an ART-sensitive line reverse-engineered to bear the wild type cysteine (C) residue at K13 codon 580; (iii) MRA-1317, an ART-resistant parasite with the R539T K13 mutation; and (iv) MRA-1252, an ART-sensitive line featuring a reversion from the mutant tyrosine (T) to the wild type arginine (R) residue at K13 codon 539.

Routine parasite culture and maintenance. All parasites were grown in leukocyte-depleted human type O+ erythrocytes at 37˚C using a hypoxic gas mixture containing 90% N_2, 5% O_2 and 5% CO_2. Growth media used is 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]

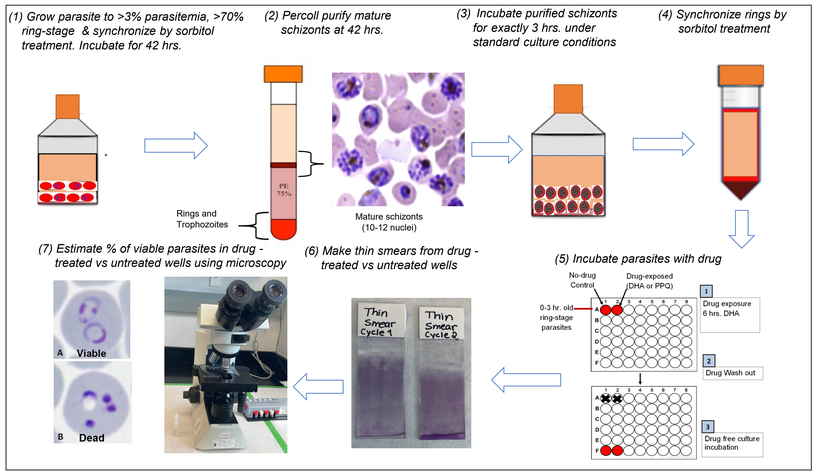
Establishing highly synchronous cultures for the Ring-Stage Survival Assay

- Culture-adapted parasites (>3% parasitemia, >70% ring-stage) were synchronized by sorbitol treatment & incubated at 37˚C for 42 hrs.
- Samples were then passaged through percoll gradients to enrich for schizonts
- Schizonts were grown for three hours & the resulting 0-3 hr. old post-invasion rings were synchronized by sorbitol treatment, washed multiple times and used in RSAs

Experimental set up of the RSA at two different treatments of the hypoxic gas

- Two identical innocula of each parasite line were seeded in 48-well plates and exposed to a pharmacologically relevant dose of Dihydroartemisininin (700nM DHA for 6 hrs) under two gas treatments: commercial gas (5% CO_2, 5% O_2, 90% N_2) versus candle jar gas (3% CO_2, 17-18% O_2, 79-80% N_2).
- Following incubation, drug was washed off, and parasites were allowed to recover and proliferate in drug-free growth media for another 66 hrs.
- Thin smears were made from resuspended cells, stained with 10% geimsa and at least 10,000 cells were examined for both morphology and parasite positivity using a light microscope
- Percent parasite survival was determined by microscopic examination and counting of viable parasites in the drug-treated wells versus the untreated to which no drug was added.
- Parasites were considered resistant to DHA if they showed a survival rate of ≥ 10%.

SUMMARY OF THE TECHNICAL APPROACH



RESULTS

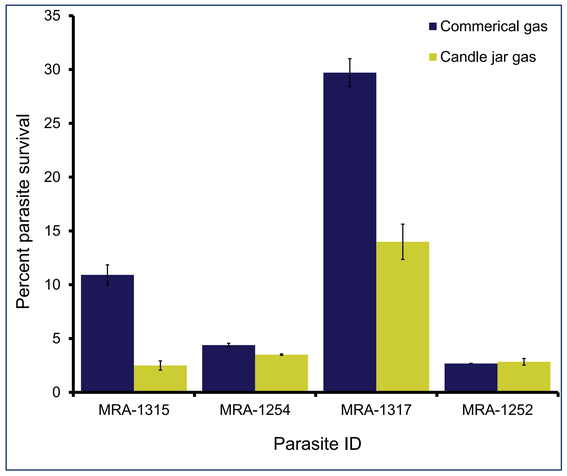


Figure 1. RSA readouts for parasites cultured in commercial gas (normoxic physiological state) versus candle jar gas (hyperoxic state). *Identical 48-well plates pre-dosed with 700nM DHA were seeded with parasites at 0.5% parasitemia and 2% hematocrit. Plates were placed in parasite culture chambers and incubated with commercial gas or candle jar gas. Significant variation in parasite survival is observed between ART-resistant parasites incubated at normoxic vs hyperoxic conditions (Unpaired t-test; $p<0.05$). ART-sensitive parasites MRA-1252 and MRA-1254 do not exhibit such variation in drug tolerance at the two gas states (Unpaired t-test; $p>0.05$). Error bars represent the standard error of the mean for at least three independent RSA experiments.*

RESULTS

Table 1: Details about parasites used in this study and their K13 genotype profile

Parasite Strain	BEI Catalog Number	Parasite type	Sampling Location*	Sampling Year*	K13 mutation profile
MRA1236-hap1	MRA-1315	Dilution cloned from culture-adapted isolate IPC_3445	Pailin Province, Western Cambodia	2010	C580Y
Cam2rev	MRA-1254	Genetically modified at K13 codon 580	Pailin Province, Western Cambodia	2010	Wild type
MRA1240-hap1	MRA-1317	Dilution cloned from culture-adapted clinical isolate IPC_5202	Battambang Province, Western Cambodia	2011	R539T
Cam3.1rev	MRA-1252	Genetically modified at K13 codon 539	Battambang Province, Western Cambodia	2011	Wild type

Sampling year denotes the year when the original isolate was sampled from the malaria-infected individual. Similarly, sampling location denotes the place where the original clinical isolate was sampled.

SUMMARY

- The level of O_2 under which the RSA is performed has a significant impact on assay readouts.
- The artemisinin resistance status of the parasite line is associated with the degree to which these readouts are affected with artemisinin-sensitive parasites showing no differential survival under different hypoxic states
- These results have important implications for artemisinin resistance surveillance and comparing RSA readouts between studies.

References

1. Witkowski, B., Amaratunga, C., Khim, N., Sreng, S., Chim, P., Kim, S., Lim, P., Mao, S., Sopha, C., Sam, B., Anderson, J.M., Duong, S., Chuor, C.M., Taylor, W.R., Suon, S., Mercereau-Puijalon, O., Fairhurst, R.M., Menard, D., 2013. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: *in-vitro* and *ex-vivo* drug-response studies. Lancet Infect Dis 13, 1043-1049.
2. Nkhoma SC, Ahmed AOA, Porier D, Rashid S, Bradford R, Molestina RE, Stedman TT. Dynamics of parasite growth in genetically diverse Plasmodium falciparum isolates. Mol Biochem Parasitol. 2023 Jun;254:111552.
3. Duffy S, Avery VM. Naturally Acquired Kelch13 Mutations in Plasmodium falciparum Strains Modulate *In Vitro* Ring-Stage Artemisinin-Based Drug Tolerance and Parasite Survival in Response to Hyperoxia. Microbiol Spectr. 2022 Oct 26;10(5):e0128221.
4. Hott A, Casandra D, Sparks KN, Morton LC, Castanares GG, Rutter A, Kyle DE. Artemisinin-resistant Plasmodium falciparum parasites exhibit altered patterns of development in infected erythrocytes. Antimicrob Agents Chemother. 2015;59(6):3156-67.

ACKNOLEDGEMENTS

This work was funded by ATCC Internal Research and Development Program. The following strains were obtained from BEI Resources, NIAID, NIH (www.beiresources.org): MRA1236-hap1 (MRA-1315) and MRA1240-hap1 (MRA-1317) cloned from IPC_3445 and IPC_5202 respectively and contributed to BEI by Dr Didier Menard. Cam3.1rev (MRA-1252) and Cam2rev (MRA-1254) were both contributed by Dr David Fidock. We acknowledge the depositors of these materials.