RESEARCH ARTICLE

Amodiaquine resistance in *Plasmodium berghei* is associated with *PbCRT* His95Pro mutation, loss of chloroquine, artemisinin and primaquine sensitivity, and high transcript levels of key transporters [version 1; peer review: 3 approved with reservations]

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Abstract

Background: The human malaria parasite *Plasmodium falciparum* has evolved complex drug evasion mechanisms to all available antimalarials. To date, the combination of amodiaquine-artesunate is among the drug of choice for treatment of uncomplicated malaria. In this combination, a short acting, artesunate is partnered with long acting, amodiaquine for which resistance may emerge rapidly especially in high transmission settings. Here, we used a rodent malaria parasite *Plasmodium berghei* ANKA as a surrogate of *P. falciparum* to investigate the mechanisms of amodiaquine resistance. **Methods:** We used serial technique to select amodiaquine resistance by submitting the parasites to continuous amodiaquine pressure. We then employed the 4-Day Suppressive Test to monitor emergence of resistance and determine the cross-resistance profiles. Finally, we genotyped the resistant parasite by PCR amplification, sequencing and relative quantitation of mRNA transcript of targeted genes. **Results:** Submission of *P. berghei* ANKA to amodiaquine pressure yielded resistant parasite within thirty-six passages. The effective dosage that reduced 90% of parasitaemia (ED₉₀) of sensitive line and resistant line were 4.29mg/kg and 19.13mg/kg, respectively. After freezing at -80°C for one month, the resistant parasite remained

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stable with an ED₉₀ of 18.22mg/kg. Amodiaquine resistant parasites are also resistant to chloroquine (6fold), artemether (10fold), primaquine (5fold), piperazine (2fold) and lumefantrine (3fold). Sequence analysis of *Plasmodium berghei chloroquine resistant transporter* revealed His95Pro mutation. No variation was identified in *Plasmodium berghei multidrug resistance gene-1 (Pbmdr1)*, *Plasmodium berghei deubiquitinating enzyme-1* or *Plasmodium berghei Kelch13 domain* nucleotide sequences. Amodiaquine resistance is also accompanied by high mRNA transcripts of key transporters; *Pbmdr1*, *V-type/H⁺ pumping pyrophosphatase-2* and *sodium hydrogen ion exchanger-1* and Ca²⁺/H⁺ antiporter.

Conclusions: Selection of amodiaquine resistance yielded stable “multidrug-resistant” parasites and thus may be used to study common resistance mechanisms associated with other antimalarial drugs. Genome wide studies may elucidate other functionally important genes controlling AQ resistance in *P. berghei*.

Keywords

Malaria, Resistance, Plasmodium berghei, Amodiaquine, Cross-resistance

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Introduction

The malaria parasite *Plasmodium falciparum* causes the greatest disease burden and death in developing countries. In 2015, the World Health Organization reported 200 million clinical malaria cases with 400,000 cases resulting in death (WHO, 2016). Majority of this burden is in sub-Saharan Africa, primarily in children under 5 years of age. With the newly introduced vaccine showing less than 50% reduction in clinical cases and its efficacy waning with time (Olotu *et al.*, 2013), the use of drugs for prophylaxis and treatment of malaria remains a key alternative in malaria control. To date, strategies for treatment of uncomplicated malaria is the use of artemisinin based combination therapies (ACTs), comprising of short acting artemisinin derivative and a long acting partner drug, a strategy intended to reduce the emergence of resistance (WHO, 2015). However, the genetically flexible malaria parasite has evolved drug evasion mechanisms to all available antimalarial drugs, including the artemisinins (Miotto *et al.*, 2015).

Currently the ACTs are used widely in all malaria endemic countries; however, this is against the backdrop of high malaria transmissions, exposing the long acting partner drugs to strong selection pressures (White, 2002). For instance, combination of amodiaquine and artesunate (AQ-ASN) is among the five recommended ACTs for treatment of uncomplicated malaria (WHO, 2016). This combination is available as a fixed combination CoarsucamTM/Winthrop®, Sanofi-Aventis (Gil, 2008). The ASN is a short acting drug with a half-life of <2hours (Robert *et al.*, 2001; Tilley *et al.*, 2016). On the other hand, AQ is a prodrug that is rapidly metabolized to its active long acting metabolite desethylamodiaquine (DEAQ), with a half-life of more than 5 days (Churchill *et al.*, 1985). In a number of African countries, AQ-ASN is the first or a second line drug for treatment of uncomplicated malaria (Rwagacondo *et al.*, 2004; Sondo *et al.*, 2016; WHO, 2016). In addition, AQ and sulfadoxine/pyrimethamine (AQ-SP) is used as a prophylactic combination, in children below 5 years of age in areas of highly seasonal transmission, such as sub-Sahel region (WHO, 2015). Thus, AQ remains a useful drug in the treatment and prophylaxis of malaria.

AQ like chloroquine (CQ) belongs to 4-amino-quinolines class of antimalarial drugs and their mechanisms of resistance are predicted to be similar; however, AQ is active against some CQ resistant parasite strains (Basco & Ringwald, 2003; Gorka *et al.*, 2013; Sá *et al.*, 2009), thus the mechanisms of resistance may be different. The 4-amino-quinoline resistance in *Plasmodium falciparum* resistance is strongly associated with polymorphisms in two key genes. First, *Plasmodium falciparum* chloroquine resistance transporter (*Pfcr*) Lys76Thr change is associated with CQ resistance and decreased sensitivity to AQ (Ecker *et al.*, 2012; Fidock *et al.*, 2000; Ochong *et al.*, 2003). Second, in the presence of *Pfcr* Lys76Thr mutation, *Plasmodium falciparum* multidrug resistance gene 1 (*Pfmdr1*), Asn86Tyr mutation enhances CQ resistance and decreases AQ sensitivity (Cooper *et al.*, 2005; Holmgren *et al.*, 2006; Wellem, 2002). Currently, the mechanisms of AQ resistance are poorly understood. To fully investigate these mechanisms, one needs to obtain naturally occurring stable *P. falciparum* lines resistant to AQ, but such parasites are not available. To circumvent this limitation, resistance can be induced *in vitro* using *P. falciparum* or *in vivo*

using murine malaria parasites. However, exposing drug-sensitive *P. falciparum* parasite to drug concentrations to select stable drug resistant lines is a cumbersome, time-consuming process, and generally the selected resistant phenotypes are not stable, thus not amenable for further studies (Nzila & Mwai, 2010). On the other hand, stable resistant parasites lines can be induced *in vivo*, with relative success, using a rodent model in mice, and these rodent parasites can be used as a surrogate of *P. falciparum* to study the mechanisms of drug resistance (Carlton *et al.*, 2001). Although some drug resistance mechanisms between *P. falciparum* and murine malaria do not correlate (Afonso *et al.*, 2006; Carlton *et al.*, 2001; Hunt *et al.*, 2007), others mechanisms are similar. For instance, mefloquine (MQ) resistant *P. berghei* lines, (Gervais *et al.*, 1999) demonstrated over-expression on *mdr1* gene, the gene associated with MQ resistance in *P. falciparum*, *P. berghei* and *P. chabaudi* (Cravo *et al.*, 2003; Price *et al.*, 2004). Similarly, non-synonymous mutations in *cytochrome b* gene are associated with atovaquone resistance in *P. berghei*, *P. chabaudi* and *P. falciparum* (Afonso *et al.*, 2010; Srivastava *et al.*, 1999b; Syafruddin *et al.*, 1999). Mutations in dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes are associated with sulphadoxine and pyrimethamine resistance in *P. chabaudi* and *P. falciparum* (Culleton *et al.*, 2005; Martinelli *et al.*, 2011). These studies support the utility of murine malaria as surrogate models for identifying drug resistance genes in *P. falciparum*.

In this study, we report on *in vivo* selection of stable AQ resistant murine malaria *Plasmodium berghei* ANKA parasite lines, and their use in investigating the mechanisms of AQ resistance. As discussed earlier, AQ and CQ are quinoline based drugs and resistance to CQ is associated with decreased activity to AQ. Some markers of resistance to other quinoline drugs, such as lumefantrine (LM), piperazine (PQ) and quinine (QN), modulate the activity of CQ (Eastman *et al.*, 2011; Mwai *et al.*, 2009; Okombo *et al.*, 2010; Witkowski *et al.*, 2017). We hypothesised that some resistance markers associated with the aforementioned quinoline drugs could also modulate AQ activity, since all these drugs are proffered to have a common mechanism of action, which is the inhibition of heme detoxification (Müller & Hyde, 2010; O'Neill *et al.*, 2011; Robert *et al.*, 2001). These markers, in addition to *Pfcr* and *Pfmdr1*, are the deubiquitinating enzyme 1 (*ubp1*), which is linked with resistance to CQ and artesunate in *Plasmodium chabaudi* (Hunt *et al.*, 2007; Hunt *et al.*, 2010) and artemisinin tolerance in *P. falciparum* (Henriques *et al.*, 2014); V-type H⁺ pumping pyrophosphatase 2 (*vp2*) and Ca²⁺/H⁺ antiporter (*vcx1*), which modulate resistance to CQ, LM and PQ in *P. falciparum* and *P. berghei* (Gonzales *et al.*, 2008; Jiang *et al.*, 2008; Kiboi *et al.*, 2014); *P. falciparum* sodium hydrogen ion exchanger 1 (*Pfnhe1*), which modifies pH gradient between digestive vacuole and cytosol milieu and regulates quinine resistance in *P. falciparum* (Bennett *et al.*, 2007). Thus, using the selected stable AQ resistant parasite line, we investigated the impact of SNP and/or transcript levels of *P. berghei* ortholog genes of the aforementioned markers on AQ resistance. Finally, the role of *Kelch13* propeller, a protein domain involved in detecting intracellular oxidative stress resulting from artemisinin and other endoperoxides action and a marker for artemisinin resistance in *P. falciparum* (Leroy, 2017; Miotto *et al.*, 2015; Straimer *et al.*, 2015) was also studied.

Materials and methods

Parasites, host and compounds

Male Swiss albino mice (6–7 weeks old) weighing 20 ± 2 g outbred at KEMRI Animal House (Nairobi, Kenya) were used to induce AQ resistance from sensitive parasite line of *P. berghei* ANKA (MRA-868, MR4, ATCC® Manassas, Virginia, 676m1c11). The animals were kept in the animal house in standard polypropylene cages and fed on commercial rodent feed and water *ad libitum*. AQ, CQ, primaquine (PMQ), LM, artemether (ATM) and PQ were prepared freshly by dissolving in a solvent containing 3% ethanol and 7% Tween-80. In all the experiments involving use of laboratory mice, at least 3 mice were used per experimental group to allow the calculation of averages, standard deviation and statistical analysis.

Determination of 50% and 90% effective doses

The 50% (ED_{50}) 90% (ED_{90}) and 99% (ED_{99}) effective doses, the doses that reduce parasitaemia by 50%, 90% and 99% respectively, after four consecutive drug dosages were determined following quantitative standard 4-Day Suppressive Test (4DT; [Fidock et al., 2004](#)). Briefly, twenty-five mice were randomly infected intraperitoneally each with 1×10^6 parasites and then randomly allocated to the four test groups and the control group (five mice per group). Oral treatment with drug was initiated on day 0, (2–4 hrs post-infection) and continued for four days, days 0–3 (24, 48 and 72 hrs post-infection). Parasite density for ED_{50} and ED_{90} calculation was estimated microscopically ($\times 100$) on day 4 (96 hrs) post parasite inoculation using thin blood films made from tail blood snips. Parasite growth was also monitored on D2, D3, D4, D7, D9, D11 and D15 days post infection. Percentage chemo-suppression of each dose was calculated following the formula ([Fidock et al., 2004](#)): $A/B \times 100$. Where A = the mean parasitaemia in the negative control group and B the parasitaemia in the test group. The ED_{50} and ED_{90} were then estimated using linear regression line.

Submission of parasite to AQ pressure and Resistance Level Test

AQ sensitive parasite was submitted to continuous AQ pressure. Six mice (three for the control and three for the test group) were intraperitoneally inoculated each with 1×10^6 parasitized red blood cells in a 0.2ml on day 0 (D0). Parasitaemia was then allowed to rise $>5\%$ when test mice were treated orally with AQ at a concentration equivalent to ED_{99} . Parasite growth was then monitored to 2–7% when donor mice were selected for subsequent passage into the next naïve group of three mice. The parasites were then exposed to an increasing concentration of AQ in the subsequent passages based on parasite growth. Resistance level was evaluated at interval of four drug pressure passages by measurement of ED_{50} and ED_{90} in the standard 4DT. To determine the stability of the resistance, AQ selected parasite line was frozen at -80°C for at least one month followed by determination of ED_{50} and ED_{90} . This allowed us to calculate the 90% index of resistance (I_{90}) from the ratio of the ED_{90} of the resistant line to that of sensitive parent line. Based on I_{90} value, resistance levels were classified into four categories: i) $I_{90} = 1.0$ (sensitive), ii) $I_{90} = 1.01-10.0$ (slightly resistance), iii) $I_{90} = 10.01-100$, (moderate resistance), iv) $I_{90} \geq 100$ (high resistance) ([Xiao et al., 2004](#)).

Generation of genetically homogenous parasite by dilution cloning

During the selection of resistant lines, a high parasite density (1×10^6) is submitted to drug pressure, even though this parasite population may share common resistant markers, they however accumulate random mutations that may or not associate with resistance. We thus sought to generate a genetically homogeneous clone using a limiting dilution approach, as detailed by [Janse et al., 2004](#). Briefly, a mouse with parasitaemia between 0.5 and 1% was selected as a donor mouse. 5 μl of infected blood was collected from the tail snip of the mouse in 1 μl of heparin and diluted in 1ml of $1 \times \text{PBS}$. The number of infected erythrocytes per 1 μl was estimated from 20 μl of diluted blood. The cell suspension was then diluted further with $1 \times \text{PBS}$ to an estimated final concentration of 0.5 parasites/0.2ml PBS. 12 mice were then intravenously injected with the infected blood. Cloning was deemed successful when 3 to 6 mice become positive with a parasitaemia of between 0.3–0.5% at day 8 post infection. The fastest growing clone was selected for cross resistance and molecular studies.

Evaluation of cross resistance profiles

The sensitivity of the selected AQ-resistant parasites line against other antimalarial drugs, DEAQ, CQ, PMQ, PQ, ATM and LM, was also investigated by assessing the ED_{50} and ED_{90} in a the 4DT assay, as detailed previously, and the results compared to the ED_{50} and ED_{90} of sensitive parental line. To this purpose, four different drug concentrations were selected for each of the test drug and administered orally, except for DEAQ which was administered intraperitoneally. The 50% and 90% indices of resistance were calculated as previously discussed.

DNA extraction, PCR and sequencing of *Pbmdr1*, *Pbcrt*, *Pbubp1* and *PbKelch13*

The identification of SNP in *Pbmdr1*, *Pbcrt*, *Pbubp1* and *PbKelch13* was carried out by sequencing, after DNA extraction and PCR amplification. Briefly, to extract parasite DNA, 500 μl of blood from a mouse with 5–10% parasitaemia was diluted with 500 μl of $1 \times \text{PBS}$, the solution was spun for 1 min at $500 \times g$. The supernatant was discarded and the pellet resuspended in 30ml volume of cold 4°C $1 \times$ erythrocytes lysis buffer for 30 minutes, followed by spinning at $500 \times g$ for 10 min. The parasite pellet was washed twice with 30ml $1 \times \text{PBS}$ with centrifugation at $500 \times g$ for 5 min at 4°C . Genomic DNA (gDNA) was extracted using a commercial QIAamp® Blood DNA extraction kit (Qiagen) following the manufacturer's instructions. As illustrated in [Figure 1a–d](#), target fragments corresponding to specific regions of interest from *P. falciparum* ortholog genes, *Pbcrt* (PBANKA_1219500), *Pbmdr1* (PBANKA_1237800), *Pbubp1* (PBANKA_0208800) and *PbKelch13* (PBANKA_1356700) were PCR amplified and sequenced using primers commercially synthesised at Inqaba Biotechnical Industries (Pty) Ltd, South Africa ([Table 1a](#)). Briefly, 1 μl of gDNA was used as the template in 25 μl PCR reactions using DreamTaq (Thermo-Scientific™). Other reagents, MgCl_2 , dNTPs, primers and the cycling conditions, were optimized accordingly ([Table 1b](#)). Products from PCR were first analysed in 1.5% agarose gel, purified using the GeneJet™ PCR purification kit (Thermo Scientific™) and then sequenced

Figure 1. Genome view of drug resistance genes and the target regions. (a) *Plasmodium berghei* chloroquine resistance transporter, (b) *Plasmodium berghei* multidrug resistance gene 1, (c) *Plasmodium berghei* ubiquitin carboxyl-terminal hydrolase 1, putative, and (d) *Plasmodium berghei* kelch 13 protein, putative, showing targeted positions (*), annealing positions for PCR and sequencing primers and the sizes of amplified PCR products.

Table 1. PCR methods. (A) Primer sequences for PCR amplifying and sequencing of *Plasmodium berghei* chloroquine resistance transporter (*Pbcrt*), *Plasmodium berghei* multidrug resistance gene 1 (*Pbmdr1*), *Plasmodium berghei* ubiquitin carboxyl-terminal hydrolase 1 (*Pbubp1*) and *Plasmodium berghei* kelch 13 protein, putative (*Pbkelch13*) genes. (B) Optimized condition for PCR amplification of *Pbcrt*, *Pbmdr1*, *Pbubp1* and *Pbkelch13* genes.

TABLE 1A				
Primer name	PCR primers sequence (5' to 3')		Primer annealing position	
<i>Pbcrt</i> - Forward	GGA CAG CCT AAT AAC CAA TGG		69-89	
<i>Pbcrt</i> - Reverse	CGA CCA TAG CAT TCA ATC TTA GG		751-729	
<i>Pbcrt</i> - Forward	CCT AAG ATT GAA TGC TAT GGT CGT		729-751	
<i>Pbcrt</i> - Reverse	GTT AAT TCT GCT TCG GAG TCA TTG		1230-1253	
Sequencing primers (5' to 3'):				
<i>Pbcrt</i> - Forward	TCA GGA AGA AGT TGT GTC A		109-127	
<i>Pbcrt</i> - Reverse	GAT AAG GAA AAA CTG CCA TC		383-402	
<i>Pbcrt</i> - Forward	GTG TTG GCA TGG TCA AAA TG		908-927	
<i>Pbcrt</i> - Reverse	CTT GGT TTT CTT ACA GCA TCG		1124-1104	
PCR primers (5' to 3')				
<i>Pbkelch13</i> - Forward	AGT CAA ACA GTA TCT CTA ACT		20-40	
<i>Pbkelch13</i> - Reverse	ACG GAA TGT CCA AAT CTT G		2198-2180	
Sequencing primers (5' to 3')				
<i>Pbkelch13</i> - Forward	TCC ACT AAC CAT ACC TAT AC		1272-1291	
<i>Pbkelch13</i> - Reverse	AGC TTC TAA TAA TGC ATA TGG		1899-1879	
PCR and sequencing primers (5' to 3')				
<i>Pbmdr1</i> - Forward	GTG CAA CTA TAT CAG GAG CTT CG		176-198	
<i>Pbmdr1</i> - Reverse	CAC TTT CTC CAC AAT AAC TTG CTA CA		742-717	
<i>Pbmdr1</i> - Forward	GGA TTT TTA TCG TCG CAT ATT AAC AG		2647-2672	
<i>Pbmdr1</i> - Reverse	TAG CTT TAT CTG CAT CTC CTT TGA AG		3259-3234	
<i>Pbmdr1</i> - Forward	CTT CAA AGG AGA TGC AGA TAA AGC TA		3234-3259	
<i>Pbmdr1</i> - Reverse	GAT TCA ATA AAT TCG TCA ATA GCA GC		3887-3862	
PCR and sequencing primers (5' to 3')				
<i>Pbubp1</i> - Forward	AGT TCC AAT GAA TAT ATT CAT GTG AA		1990-2015	
<i>Pbubp1</i> - Reverse	CTA AGT TGC ATA GCT TTA TCA TTT TC		2621-2596	
TABLE 1B				
PCR amplifying profiles	Temperature (°C)/Time (min)			
	<i>Pbcrt</i> 1 st and 2 nd Fragment	<i>Pbmdr1</i> 1 st , 2 nd and 3 rd fragments	<i>Pbubp1</i>	<i>Pbkelch13</i>
Initial denaturation	95°C, 5 min	95°C, 5 min	95°C, 5 min	95°C, 5 min
Denaturation	95°C, 1 min	95°C, 30 secs	95°C, 30 secs	95°C, 1 min
Annealing Temperature	50°C, 30 secs	52°C, 30 secs	50°C, 30 secs	51°C, 30 secs
Elongation	72°C, 3 min	72°C, 1 min	72°C, 1.5 min	72°C, 1.5 min
Primer (Forward & reverse)	2.5µM each	2.5µM each	2.5µM each	2.5µM each
MgCl2 (mM)	2.0	1.5	1.5	2.0
dNTPs (mM)	2.0	2.0	2.0	2.0
Cycles	35	30	30	30
Final elongation	62°C, 10 min	62°C, 10 min	62°C, 10 min	62°C, 10 min

using a 3730xlsequencer based on BigDye v3.1. DNA sequences were analysed using Lasergene 11 Core Suite and CLUSTAL Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) and PlasmoDB (<http://plasmodb.org/plasmo/>) (PlasmoDB, 2017).

RNA extraction, cDNA synthesis and qRT-PCR assays

The quantification of the mRNA transcripts of *Pbmdr1*, *Pbvp2*, *Pbvcx1*, and *Pbnhe1* genes was carried out after cDNA synthesis from mRNA. Before extraction of RNA, all buffers and solutions for parasite preparation were first treated with 0.1% (v/v) of diethyl pyrocarbonate (DEPC). Total RNA was prepared from approximately 1×10^6 fresh parasites pellet. In preparation of parasite pellet, parasitized red blood cells were first washed in 1×PBS and then lysed in 5 volumes of ammonium chloride solution. The parasite pellet was washed twice in 10ml of 1×PBS and then resuspended in 200µl of 1×PBS. Total RNA was isolated using Quick-RNA™ MiniPrep (Zymo Research™) following the manufacturer's instructions. The first strand cDNA synthesis was performed in a final volume of 20µl using RevertAid First Strand cDNA synthesis kit and oligo-DT as primers. 5µg of total RNA, 1µl of oligo-DT and water were mixed with 4µl Reaction buffer (5×), 1µl RiboLock RNase Inhibitor (U/µl), 2µl of dNTPs (10mM) and 1µl of RevertAid M-MuLV RT (200U/µl). The reaction mix was incubated at 42°C for 60min, then at 70°C for 5min and finally chilled on ice. The cDNA was then used as template for qRT-PCR assays.

The mRNA transcript levels were evaluated using qRT-PCR in a final volume of 20µl using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific™). Oligonucleotide for *Pbmdr1*, *Pbvp2*, *Pbvcx1* and *Pbnhe1* were designed to run using similar cycling conditions relative to *Pbβ-actin 1*, as the housekeeping gene (Table 2). Briefly, 12µl of Maxima SYBR mix, 2.0µl (0.25µM) of forward and reverse primers each, 1µl cDNA and 3µl water were mixed. The reaction mix was run for pre-treatment at 50°C, for 2 min; initial

denaturation at 95°C for 10 min; denaturation at 95°C for 15 secs; and annealing at 60°C for 60 secs for 45 cycles.

Statistical analysis

The means of expression levels of each gene from three independent experiments and from triplicate assays obtained from AQ resistant were compared to AQ sensitive using Student's t-test; p value was set at 0.05. The relative expression level results were normalized using *Pbβ-actin 1* as the housekeeping using the formula $2^{-\Delta\Delta CT}$ based on Livak & Schmittgen, 2001. The means for cross resistance profiles for each drug from at least four different drug concentrations was analysed using Student's t-test, with p value set at 0.05.

Ethical approval

This study was conducted at KEMRI. All animal work was carried out as per relevant national and international standards, as approved by KEMRI-Animal Use and Care Committee. Permission to carry out this study and ethical clearance were approved by KEMRI's Scientific Ethics Review Unit (No 3378).

Results and discussion

Amodiaquine drug pressure induces stable resistant phenotypes

The current introduction of AQ as a fundamental component of ACT therapy (Gil, 2008) has spurred studies on understanding the mechanisms of AQ resistance. Using 2% Relapse Technique, AQ resistance has previously been selected in *P. berghei* and *P. yoelii* by submitting the parasite to 60 and 100mg/kg, respectively (Peters & Robinson, 1992); however, the stability, resistance indices and molecular mechanisms were not determined. Here we demonstrate that stable AQ resistant *P. berghei* ANKA can be achieved by submitting sensitive parasites to thirty-six continuous drug pressure passages (Dataset 1). To initiate selection of resistance, we first

Table 2. Oligonucleotides for quantitative Real-Time PCR. These were used to measure the transcriptional level profiles of *Plasmodium berghei* multidrug resistance gene 1 (*Pbmdr1*), *Plasmodium berghei* V-type H⁺ pumping pyrophosphatase (*Pbvp2*), *Plasmodium berghei* Ca²⁺/H⁺ antiporter (*Pbvcx1*), *Plasmodium berghei* sodium hydrogen exchanger (*Pbnhe1*) genes with *Plasmodium berghei* β-actin 1 gene (*Pbβ-actin 1*) as housekeeping using Maxima SYBR Green chemistry in quantitative Real-Time PCR.

Name	Primer sequence (5' – 3')	Position	Tm
<i>Pbmdr1</i> - Forward	ACGGTAGTGGCTTCAATGGA	917-936	54.2
<i>Pbmdr1</i> - Reverse	CTGTCGACAGCTGGTTTTCTG	1082-1062	54.7
<i>Pbnhe1</i> - Forward	TGGAGAGTTTGATTAGGCTTACC	2022-2045	54.0
<i>Pbnhe1</i> - Reverse	GCTAGGCGATGTTTTGTTAGGAG	2202-2180	55.3
<i>Pbvp2</i> - Forward	TGCAGCAGGAAATACAACAGC	1449-1469	55.2
<i>Pbvp2</i> - Reverse	GTCGTACTTTTGCCTACTTGCGT	1558-1535	56.5
<i>Pbvcx1</i> - Forward	TCAAATTGCTCTTTTGTGTACCAA	1101-1126	57.9
<i>Pbvcx1</i> - Reverse	ACACCTTCTAGCCAATTACTTTCACC	1265-1240	57.1
<i>Pbβ-actin 1</i> - Forward	CAGCAATGTATGTAGCAATCAAGC	392-416	56.8
<i>Pbβ-actin 1</i> - Reverse	CATGGGGTAATGCATATCCTTCATAA	523-498	58.9

determined the ED_{50} , ED_{90} and ED_{99} of AQ against the sensitive *P. berghei* ANKA. The ED_{50} , ED_{90} , ED_{99} were 0.95, 4.29 and 5.05mg/kg/day, respectively. We adopted the serial technique, in which drug pressure is increased sequentially. 5.05mg/kg was selected as the starting drug pressure dose that was administered once percentage parasitaemia rose to 2–7%. At the onset, average parasitaemia reached 2–7% on day 3–4 post infection, after which dosage equivalent to ED_{99} was administered. Figure 2 shows parasite responses to AQ at different passages during resistance selection process. The drug pressure dose was increased by a factor of ED_{99} or based on parasite growth at different passage levels. Within the first 12 passages, administration of single 5mg/kg of AQ, after attaining >2% parasitaemia, cleared the parasite to below detectable levels by microscopy. Recurrence of >2% parasitaemia was only attained after 7–10 days on average; therefore, the drug pressure dose was not increased for the first 12 passages. From the 13th passage, the parasite recrudescence after drug treatment reduced from 7 days to 3–4 days. We henceforward increased the drug pressure dose by a factor of 1.5 of ED_{99} (equivalent to 2.5mg/kg) every two passages up to the 20th passage when the dosage was further increased by a factor of 2 of ED_{99} (equivalent to 5mg/kg) every two passages. By the 36th passage, the drug pressure dosage had risen to 50mg/kg, which was 50 and 10 times higher than the ED_{50} and ED_{99} respectively, we thus expected a higher ED_{50} and ED_{90} . Surprisingly the I_{50} and I_{90} was only 12 and 4 folds respectively (Table 3a). The resistant line remained stable after freezing at -80°C for at least one month, with ED_{50} and ED_{90} of 5.86 mg/kg and 18.22mg/kg respectively. The effective doses were equivalent to I_{50} and I_{90} of 6- and 4-fold respectively, meaning that stable AQ resistant *P. berghei* line was successfully selected and the resistance mechanisms were probably encoded in the cell genome.

Amodiaquine resistance associated with cross resistance to CQ, LM, PMQ, PQ and ATM

Selection of stable AQ resistant line allowed us to study whether AQ resistance is associated with reducing efficacy of other anti-malarial drugs (Dataset 2). Using dilution cloned parasite, we determined the ED_{90} of PQ, LM, PMQ and ATM against both AQ sensitive and AQ resistant parasite. To our surprise, AQ resistance was associated with moderate and slight resistance to ATM (I_{90} = 10.2) and PMQ (I_{90} = 5.8), respectively. Interestingly, AQ resistant line was less resistant to PQ (I_{90} = 2.2-fold) than LM (I_{90} = 3.5-fold), despite PQ and AQ belonging to the same chemical class of 4-aminoquinoline and LM belonging to different chemical class of aryl-alcohols (Table 3b). This means that AQ resistant phenotype is accompanied by mechanisms that confer resistance to ATM, LM, PQ, PMQ and CQ. The cross-resistance profile is not surprising for drugs such as CQ and PQ, since they are quinoline based compounds molecules, and chemically related to AQ, thus may share some resistance mechanisms. Indeed, selection of CQ resistance in *P. berghei* has previously been shown to confer cross-resistance to AQ, mefloquine and PMQ, two quinoline based drugs (Platel *et al.*, 1998). Similarly, we expect PMQ (8-amino quinoline) and LM (an aryl-alcohol) to share certain mechanisms with 4-amino quinoline based on similarity of modes of action. However, the high cross resistance levels for ATM (I_{90} = 10fold) is entirely surprisingly. ATM is mechanistically and chemically unrelated to AQ (Robert *et al.*, 2001; Tilley *et al.*, 2016), while PMQ is an 8-aminoquinoline but predicted to have similar mode of action with AQ and other aminoquinoline drugs (O'Neill *et al.*, 2006). AQ inhibits heme polymerization within the digestive vacuole, thus killing the parasite by the accumulation of toxic heme (O'Neill *et al.*, 2006). On

Figure 2. Log10 average parasitaemia of *Plasmodium berghei* ANKA during selection of resistance. This was measured on day four in the 4-Day Suppressive Test at different passage levels under 2.5mg/kg and 5mg/kg of amodiaquine relative to the untreated control, in at least five male Swiss albino mice per dosage.

Table 3. Amodiaquine resistance and cross resistance levels. (A)

The 50% and 90% Effective Dose (ED_{50} and ED_{90}) in mg/kg/day of amodiaquine resistant *Plasmodium berghei* ANKA line at different passage levels. Index of resistance at 50% (I_{50}) and 90% (I_{90}) from the ratio of ED_{50} or ED_{90} of the resistant line with ED_{50} or ED_{90} of sensitive line respectively. (B) Cross resistance profiles of antimalarial drugs; primaquine, piperazine, lumefantrine, artemether, chloroquine and desethylamodiaquine (DEAQ) against the amodiaquine resistant line *Plasmodium berghei* ANKA line and the sensitive parent line as measured in the 4-Day suppressive Test using at least four different dosages and at least four Swiss mice per dose. The Index of resistance (I_{90}) calculated from the ratio of ED_{90} of resistant line to that of sensitive parent line.

TABLE 3A				
Passages No.	50% and 90% effective dose		Index of resistance	
	ED_{50}	ED_{90}	I_{50}	I_{90}
1 st	0.95	4.29	1.00	1.00
4 th	1.07	3.59	1.13	0.84
8 th	1.90	4.06	2.00	0.95
12 th	2.26	4.13	2.38	0.96
20 th	2.63	4.55	2.76	1.06
28 th	5.00	11.44	5.26	2.67
36 th	12.01	19.13	12.64	4.46
Stability after freezing for 1 month	5.86	18.22	6.17	4.24

TABLE 3B			
Antimalarial drug	Sensitive parental line	Amodiaquine resistant line	Index of resistance
	ED_{90}	ED_{90}	I_{90}
Primaquine	1.33 (Langat et al., 2012)	7.76 [†]	5.83
Piperazine	3.52	7.90 [*]	2.24
Lumefantrine	3.93 (Kiboi et al., 2009)	13.8 [*]	3.51
Artemether	3.28	33.4 [‡]	10.2
Chloroquine	4.47	27.0 [‡]	6.04
DEAQ	3.44	18.40 [‡]	5.33

Using Student's t-test the differences between the sensitive parental line and amodiaquine resistant line were significant: ^{*} $p < 0.01$; [†] $p < 0.001$; [‡] $p < 0.0001$.

the other hand, early studies on mechanism of artemisinins action proposed the heme digestion pathway (Klonis et al., 2011), inhibition of the translationally controlled tumour protein (TCTP) and the *PfATP6*, a sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) (Eckstein-Ludwig et al., 2003; Krishna et al., 2008). Recently, phosphatidylinositol-3-kinase was validated as a key artemisinin target with high levels of its product phosphatidylinositol-3-phosphate associating with artemisinin resistance in *P. falciparum* (Mbengue et al., 2015). Since the mechanisms of action and resistance of ATM are different from that of AQ, the cross resistance between these two drugs may be due to the

alteration of the mechanisms of drug transport, drug metabolism and/or drug accumulation within the cells. To date, combination of ATM/LM (Coartem®), dihydroartemisinin/PQ (Artekin®) and ASN/AQ are in use in malaria endemic countries (WHO, 2006). Assuming the mechanism of resistance between *P. falciparum* and *P. berghei* are similar, then our results would suggest that selection of AQ resistance, a component of Coarsucum™ would compromise the efficacy of Artekin® and Coartem®. However, so far studies in *P. falciparum* do not indicate a correlation between decrease in AQ and artemisinin activity (Borrmann et al., 2013; Nsoby et al., 2010).

Evaluation of point mutation in *Pbcrt*, *Pbmdr1*, *Pbubp1* and *PbKelch13* (Dataset 3)

To investigate the possible mechanisms of AQ resistance, we evaluated polymorphisms in two *P. berghei* orthologs genes, *Pbcrt* and *Pbmdr1*, known to be associated with quinoline resistance in *P. falciparum*, and our study focused on regions of the genes that contained the reported point mutations in *P. falciparum* (Figure 1a and b). To date, several studies have demonstrated the association between resistance or decrease susceptibility to 4-amino-quinoline resistance and mutation in *crt* gene, changes in expression profiles and/or copy number variation in *mdr1* gene (Borges *et al.*, 2011; Duraisingh & Cowman, 2005; Holmgren *et al.*, 2006). Single nucleotide polymorphism (SNP) in *Pfcr* (codon 76) is associated with CQ and AQ resistance in *P. falciparum* (Ecker *et al.*, 2012; Fidock *et al.*, 2000; Ochong *et al.*, 2003). Recent studies also identified potential *crt* background mutations; Ile356Thr and Asn326Ser that associate with artemisinin resistance (Miotto *et al.*, 2015). In the present study, nucleotide codons corresponding to amino acid position 76, 326 and 356 of the *PbCRT* protein were found not to harbour any mutation in AQ resistant line (compared to sensitive line). However, a substitution of A → C 284 was observed, resulting in His95Pro mutation in *PbCRT* protein. His95Pro mutation is located within the second transmembrane domain in close proximity to the food vacuole compartment (Valderramos & Fidock, 2006). Thus, the functional role and biological consequence of His95Pro mutation in AQ resistance requires further investigation.

Mutation at positions 86, 184, 1034, 1042, and 1246 in *Pfmdr1* mediate and/or modulate CQ, LM and mefloquine resistance (Ecker *et al.*, 2012; Price *et al.*, 1999; Price *et al.*, 2004; Sisowath *et al.*, 2005). Studies in the rodent malaria *Plasmodium chabaudi* found no association between *crt* and CQ resistance (Afonso *et al.*, 2006; Hunt *et al.*, 2004). Similarly, our recent investigation using LM and PQ resistant *P. berghei* parasite found no polymorphisms in *crt* and *mdr1* genes (Kiboi *et al.*, 2014). Sequencing of *PbMDR1* regions from *P. berghei* AQ resistant and sensitive line did not reveal any sequence variation. The absence of hitherto known mutations within *crt* and *mdr1* genes indicate either the existence of mutations in regions outside our targets positions or that these two genes are not involved in this resistance phenotype. Thus, full sequence analysis of these genes would provide additional insights on mechanisms of resistance in this cell line.

The AQ resistant line had significantly reduced sensitivity to ATM with an ED_{50} of 33.4mg/kg compared with an ED_{50} of 3.28 mg/kg for AQ sensitive, translating to 10-fold difference. Recent reports have validated *Kelch13* propeller domain, Met476Ile, Tyr493His, Arg539Thr, Ile543Thr and Cys580Tyr mutations as markers for artemisinin resistance (Miotto *et al.*, 2015; Straimer *et al.*, 2015). We hypothesised that *PbKelch13* may possess SNPs, and thus mediate this cross resistance. Our data showed no mutation in *PbKelch13* domain of AQ resistant line, thus AQ and ATM resistance observed *in vivo* is not associated with SNPs in *Kelch13* domain. We focused our study on *Kelch13*, however other genes such as *TCTP*, *SERCA* and *PI3P* reported to associate with mechanisms of artemisinins action or resistance in *P. falciparum* (Eckstein-Ludwig *et al.*, 2003) may also be linked with our selected AQ resistant line. As the index of resistance to ATM (I_{90} = 10.2) was double that of AQ (I_{90} = 4.2)

indicate that AQ and ATM could share some resistance mechanisms in *P. berghei*. Thus, these AQ resistant lines could be used to define these common mechanisms and some of them may be *TCTP*, *SERCA* and *PI3P* or other unknown genes.

The V739F and V770F mutations in *ubp1* C-terminal conserved region are associated with artesunate resistance in *P. chabaudi* (Hunt *et al.*, 2010). Similarly, Tyr835Ly and Ser836Gln mutations were mapped in both LM and PQ resistant *P. berghei* (unpublished data; Kiboi, Irungu, Orwa, Kamau, Ochola-Oyier, Ng'ang'a and Nzila). In our current study, the analysis of the sequence fragments flanking 739, 770, 834 and 835 position of the *PbUBP1* protein revealed no amino acid changes in the selected AQ resistant line. Studies in *P. falciparum* *in vitro* also found no association between artemisinin resistance and mutation in *ubp1* (Chavchich *et al.*, 2010); however analysis of field *P. falciparum* isolates from Western Kenya associated *Pfubp1* Glu1528Asp mutation with tolerance to artemisinin (Henriques *et al.*, 2014). We thus envisage complex mechanisms controlling loss of ATM efficacy in the AQ resistant phenotype. Examining the whole genome and transcriptome profile may expose these complex networks.

High mRNA transcripts of *Pbmdr1*, *Pbnhe1*, *Pbvp2* and *Pbcvx1* associated with AQ resistance

To further probe other possible mechanisms of AQ resistance, we hypothesised that major transporters or ion exchangers, *Pbmdr1*, *Pbnhe1*, *Pbvp2* and *Pbcvx1* could mediate AQ resistance via altered mRNA transcript levels (Dataset 3). The results show that the mRNA transcript of *Pbmdr1* and *Pbvp2* were elevated 3.0fold ($p < 0.0001$) and 2.3fold ($p < 0.0001$), respectively (Figure 3). In relation to *Pbnhe1* and *Pbcvx1*, AQ resistant parasites have a significantly high amount of *Pbnhe1* mRNA transcripts of 2.6fold compared to sensitive line ($p < 0.0001$), and similar results were observed on *Pbcvx1*, 1.7fold ($p < 0.001$) (Figure 3). Therefore, elevated *mdr1*, *vp2*, *cvx1* and *nhe1* transcript level is associated with AQ resistance. First, overexpression of *mdr1* is a common marker for *P. falciparum* resistant to MQ, AQ, CQ and ATM (Borges *et al.*, 2011; Gonzales *et al.*, 2008). In addition, the *mdr1* regulates transcription of other drug resistance genes (Gonzales *et al.*, 2008; Jiang *et al.*, 2008). For instance, augmenting CQ resistance in parasites harbouring *Pfcr* K76T mutation (Fidock *et al.*, 2000). We thus argue that *mdr1* overexpression may play a direct role in mediating AQ resistance.

Two genes, *vp2* and *cvx1*, are H^+ channel molecules that play two roles in CQ resistance: regulation of *pH* balance in the parasite's food vacuole and a compensatory role (adaptive changes in response to mutation in drug resistance genes) in a mutated *Pfcr* protein (Jiang *et al.*, 2008). In a recent report, PQ resistance was associated with high *vp2* and *cvx1* expression in *P. berghei*, though there was no mutation in *Pbcrt* gene (Kiboi *et al.*, 2014). The AQ resistant line carried His95Pro mutation in *PbCRT* protein, thus elevation of *vp2* and *cvx1* may compensate for this mutation, as it has been reported with the Lys76Thr *crt* mutation in *P. falciparum*. To date, the proffered mode of action for CQ, AQ and PQ is the inhibition of heme polymerization within the food vacuole (O'Neill *et al.*, 2011). Based on this mode of action, some resistance mechanisms associated with AQ may involve proteins within the food vacuole. We thus argue that high *vp2* and *cvx1* expression may play a role in

Figure 3. Expression levels of target drug resistance genes. The multidrug resistance gene 1 (*mdr1*), sodium hydrogen exchanger (*nhe1*), V-type H⁺ pumping pyrophosphatase (*vp2*) and Ca²⁺/H⁺ antiporter (*cvx1*), as measured from cDNA amount derived from 5 µg of total RNA isolated from amodiaquine resistance (AQR), relative to the wild type amodiaquine sensitive (AQS) clones. The differential expression from a mean of three independent experiments were significantly different for *mdr1* ($p < 0.0001$), *nhe1* ($p < 0.0001$), *vp2* ($p < 0.0001$) and *cvx1* ($p < 0.001$), after Student's *t*-test analysis with p-value set at 0.05.

regulating pH balance in AQ resistance. Lastly, we report a 2.6-fold increase in *nhe1* mRNA transcript in AQ resistance in *P. berghei* ANKA. A report in *P. falciparum* has shown that quinine resistance can be associated with increased expression of *nhe1* in the presence of mutations in *Pfprt* and *Pfmdr1* (Nkrumah *et al.*, 2009). Since the role of the *nhe1* is to regulate Na⁺/H⁺ exchange, this ion exchange may also contribute in AQ resistance phenotype.

In conclusion, we provide important evidence in relation to AQ resistance in *P. berghei* ANKA. First, emergence of AQ resistance is associated with cross resistance to ATM, PMQ, LM, PQ and CQ, thus this is a “multi-drug” resistant line. Second, novel His95Pro mutation in *PbCRT* is associated with AQ resistance and may well mediate the cross-resistance profiles. Third, one route for acquiring AQ resistance is via increased transcription of *mdr1*, *nhe1*, *vp2* and *cvx1* genes. These genes are associated with increasing resistance levels and conferring physiological advantage to drug resistance genes that may possess biologically deleterious mutations (Gonzales *et al.*, 2008). The elevated expression of these genes is consistent with *P. falciparum* resistance to CQ, LM and ATM (Gonzales *et al.*, 2008; Jiang *et al.*, 2008; Mwai *et al.*, 2012), suggesting that some mechanisms between *P. falciparum* and *P. berghei* are similar. Finally, AQ resistance and its associated cross resistance profiles are independent of SNPs in *ubp1* and *Kelch13* genes. Studies are underway to explore whole genome to reveal other possible SNPs and copy number variants associated with AQ resistance.

Data availability

The raw data for this study are deposited in OSF as follows:

Dataset 1: Parasite densities in the 4DT used for determination

of 50% and 90% effective dose, <https://doi.org/10.17605/OSF.IO/NWPXK> (Kiboi, 2017a).

Dataset 2: Parasite densities for cross resistance profiles, <https://doi.org/10.17605/OSF.IO/KTSYB> (Kiboi, 2017b).

Dataset 3: Expression level profiles and sequence data of resistance genes, <https://doi.org/10.17605/OSF.IO/VH9RY> (Kiboi, 2017c).

Competing interests

No competing interests were disclosed.

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Version 1

Reviewer Report 07 September 2017

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Richard T. Eastman

National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, MD, USA

The emergence and spread of drug resistance parasites remains a constant concern and threatens to reverse the reduction of malaria related morbidity and mortality. Amodiaquine is widely used to treat malaria episodes, combined with artesunate, and as a prophylactic, combined with sulfadoxine-pyrimethamine. As such the elucidation of genetic determinates underlying decreased drug susceptibility would permit genetic surveillance for the emergence and spread of amodiaquine-resistant parasites, facilitating adequate public health measures to assure proper utilization of antimalarial therapy. Ndung'u *et al.* report the *in vivo* drug selection and characterization of amodiaquine-resistant *Plasmodium berghei* parasites. This represents an essential first-step in the elucidation of genetic determinants underlying the resistance phenotype. As the authors note, further characterization of the lines (whole genome sequencing/RNA-seq/genetic backcross), along with validation studies will be required to further support this association based initial characterization.

1. From the Methods section, it is unclear if the *P. berghei* ANKA line was cloned prior to selection experiments. This would limit the impact that initial sub-populations contribute to the identified pre-selection/post-selection genetic variances. If the line wasn't cloned prior to the selection this may partially explain the multiple genetic differences identified (Pbcrt SNP along with expression variance in four distinct transporters).
2. It is unclear from the Methods section if independent PCR reactions/sequencing of both strands were performed on the target loci indicated in Figure 1, to address polymerase/sequencing errors.
3. The depiction of the drug selection procedure in Figure 2 is confusing as the parasites were subjected to an increasing drug concentration selection (initial oral treatment of the AQ ED99 concentration and "increasing concentrations...based on parasite growth"), not two groups (either 2.5 or 5mg/kg/day) as indicated. The figure should be revised to indicate the

selection concentration used for each passage and day 4 parasitemia. Also unclear from the methods/figure is the robustness of the selection for each passage. As three mice were inoculated per passage, was there any variance in the positivity/parasitemia of the mice upon selection pressure? If these lines/sub-passages are preserved they may represent an exciting tool to dissect the evolution of AQ resistance (in a similar manner that Hunt *et al.* discerned drug resistance in *P. chabaudi*).

4. The authors utilize a single parasite freeze/thaw to assess stability of the drug resistance phenotype. It is suggested that serial passage in naïve, non-drug treated mice is a more stringent evaluation of the resistance stability. Another method would be the passage of the line through the mosquito stage (which could also be run in parallel with genetic back-crossing of the line). The lower IC50 and IC90 values after the freeze/thaw suggest some instability in the drug-resistance phenotype.
5. As the drug dose response is sigmodal a non-linear regression analysis is usually preferred. Although a linear regression analysis would typically have good estimation of the IC50 value, due to the linear nature of the slope, there is disparity in calculation of the IC90 value using a linear regression analysis.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 May 2018

Daniel Kiboi, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Author's Response

1. The parent line used for AQ resistance was a clonal parasite (676m1cl1). We have added this statement in the Method section (Parasite, Host and Compounds subsection). After the selection of the resistant line, parasite lines were cloned by limiting dilution before PCR amplification and sequencing of the *Pbmdr1*, *Pbcrt*, *Pbubp1* and *PbKelch13* genes.

2. We amplified independent amplicons and sequenced in both forward and reverse direction. To have repeated the experiment by PCR amplifying and sequencing the whole coding region for both *Pbcrt* and *Pbmdr1* genes. To minimise the possible polymerase errors, we used a proof-reading polymerase (Phusion Flash High Fidelity PCR Master Mix, Thermo Fisher Scientific). We have added these statements in the methodology and results section

3. We have used percentage parasitaemia recorded during the drug selection process to revise Figure 2. We have revised the Figure 2 to show the percentage parasitaemia during selection process relative to the increasing drug pressure dosage. In response to the second question on the parasitaemia variance during the selection process. We recorded variances in the parasitaemia between the three mice used for selecting AQ resistance at each of the drug pressure passage. Parasite lines for each mouse and at each drug pressure passage were cryopreserved. We agree with the reviewer that one robust way of dissecting the mechanisms of AQ resistance is to use the Linkage Group Selection (Culleton et al. 2005; Hunt et al. 2007; 2010) followed by whole genome and transcriptome sequencing. We hope to use these approaches in our current studies.

4. We concur with the reviewers that performing drug-free passage is a more stringent approach for ascertaining the stability of the AQ resistant. We therefore evaluated and confirmed the stability of the AQ resistant parasite by culturing the resistant parasite for a total of ten passages in the absence of the AQ. We then determined the ED₅₀ and ED₉₀ using the standard 4DT test. Figure 3 illustrates this data on the stability of the mutant parasites. Table 3a also contains the new computed ED₉₀ and indices of resistance.

5. We consistently used the linear regression analysis in estimation of the ED₅₀ and ED₉₀. Since, the linear regression would provide a good estimate, we presume our results on the ED₅₀ and ED₉₀ would correlate well across the different assays we conducted.

Competing Interests: No competing interests were disclosed.

Reviewer Report 17 July 2017

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David A. Fidock

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This is an interesting report on an important topic. The authors have made an important contribution by selecting a rodent malaria parasite line (in *Plasmodium berghei*) that is resistant to amodiaquine (ADQ), an antimalarial combination therapy partner drug. The results implicate a novel mutation in the *Plasmodium berghei* *PbCRT* ortholog of the *Plasmodium falciparum* chloroquine resistance transporter *Pfcr*. This P95H is associated with reported changes in parasite sensitivity to multiple antimalarials including ADQ and its metabolite monodesethyl-ADQ, as well as piperazine, lumefantrine, artemether and chloroquine.

This is a preliminary assessment of this drug-resistant parasite line. One important caveat is that the authors only targeted certain regions of *PbCRT* or the other likely resistance determinant *Pbmdr1* (*P. berghei* multidrug resistance gene-1). Other mutations might therefore have appeared that were not detected. The authors have not performed gene editing to confirm whether or not the *PbCRT* H95P mutation can account for the full extent of altered antimalarial susceptibilities that were observed in their mutant line.

To provide a comprehensive assessment of the genetic basis of resistance, the authors should perform whole-genome sequence analysis of the mutant compared to the parental line. If that is not feasible, the authors should at the very least complete their sequencing of the entire coding sequence for both *PbCRT* and *Pbmdr1*. For *PbCRT*, full-length sequences can be obtained from reverse-transcribed cDNA (they already report making RNA for some of their qRT-PCR studies).

Other points:

1. Introduction: It is not entirely correct that selected resistant parasites in *P. falciparum* are generally not stable. This argument should be removed.
2. Figure 2 should show what regions were adequately sequenced.
3. Concluding that the lines have a stable resistant phenotype after being stored at -80°C for one month is an overstretch. Stability usually means that the phenotype persists for one month or more of continuous propagation without drug pressure. Especially as the ED₅₀ and I₅₀ values post-thawing are ~1/2 that of the pre-freezing line on passage #36 (see Table 3A). The authors should either test for true stability in the absence of drug, or remove this as a central finding of their study.
4. Table 3B – the authors cannot base any changes in mutant parasite susceptibility to primaquine or lumefantrine based on a comparison with earlier published data for those drugs with the sensitive parental line. If the parent was not tested here in parallel with the mutant for these drugs, then those data should be removed, or at the very least they should attenuate their statements and list the caveat that data for the parental line were from separate studies and thus shifts in susceptibility have not been directly demonstrated. Also for Table 3, the authors need to list how the number of independent experiments and mouse group sizes.

5. On page 10 the authors state that *Pfmdr1* overexpression is a common marker of resistance to chloroquine. Results presented in Sidhu *et al* 2007 J Infect Dis showed no change in chloroquine IC₅₀ in isogenic lines with different *Pfmdr1* copy numbers and that work should be cited.
6. Figure 2 is hard to understand as it seems to indicate that selection was only performed at two fixed concentrations, whereas resistance was obtained using a ramping procedure. The authors should clarify what is being shown.
7. Figure 3 is also non-intuitive. Are the data shown relative to a reference gene? This should be listed in the legend. Were these three independently prepared and harvested cultures? Or are these technical triplicates from the same set of cultures?

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 May 2018

Daniel Kiboi, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

We concur that performing whole genome sequencing of the resistant line may comprehensively dissect AQ resistance markers. This objective is in our current study plan; however, we have sequenced full-length of the *Pbcrt* and *Pbmdr1* genes from cDNA. We have edited the methods section to highlight the sequencing approach and included this data in the results section. We have also revised the Figure 1 to portray the sequenced region of the other two genes; *Pbubp1* and *Pbkelch13*. We have included the new sequence

data in dataset 3.

Response to other comments

1. We have removed the statement on "that selected resistant parasites in *P. falciparum* are generally not stable."
2. We have edited the Figure 1 to portray the regions of the genes that were adequately sequenced
3. We tested the stability of the AQ resistant parasite by culturing the resistant parasite for a total of ten passages in the absence of the AQ. We then determined the 50% and 90% effective dosages (ED₅₀ and ED₉₀) using the standard 4DT test. In the MATERIALS AND METHODS section, we have included a statement on the stability assays, under the subsection "Submission of the parasite to AQ pressure and Resistance Level Test. In the RESULTS AND DISCUSSION section, we have included the ED₅₀ and ED₉₀ values in Table 3a to illustrate the stability of the AQ-resistant parasites. We have included Figure 3 to show the drug response profile of the AQR, drug-free parasite and drug sensitive parent line. This data is under subsection on "Amodiaquine drug pressure induces stable-resistant phenotypes" We have included a new figure (Figure 3) in our revised version.
4. We have retested the ED₉₀ for primaquine and lumefantrine against the sensitive parent parasite. We have included the new data on ED₉₀ in Table 3b and the raw data in dataset 2. We used at least four different drug concentrations and at least four Swiss mice per drug concentration. We have clarified this statement in Table 3a.
5. We have included the statement on the lack of association between CQ resistance and amplification of the *Pfmdr1* gene. We have cited Sidhu et al. 2007 study.
6. Using data from drug pressure and at different passage stages, we have revised the Figure 2 to show the percentage parasitaemia during selection process relative to the increasing drug pressure dosage.
7. We have included the reference gene used to normalise the expression level data. We used technical triplicates from three independently prepared cultures. We have clarified this statement in the figure legend. Since we added a new figure on the stability of the amodiaquine resistant parasite, Figure 3 in our first version changes to Figure 4.

Competing Interests: No competing interests were disclosed.

Reviewer Report 12 July 2017

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Axel Martinelli

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² Biological and Environmental Sciences and Engineering (BESE) Division, King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia

The article presents the selection of a line of the rodent malaria parasite *Plasmodium berghei* for amodiaquine (AQ) resistance. This is potentially interesting work, due to the primary role played by AQ as a partner drug in artemisinin combination therapy (ACT).

There are however two main issues that the authors must address before the paper can be accepted for publication.

The first is unfortunately experimental. Persistence of drug resistant phenotypes can be unstable in malaria parasites and testing it after recovering parasites from deep freeze is not enough to guarantee that the phenotype is due to mutations rather than transient epigenetic effects. The fact that the ED₅₀ doses are half or less than those measured before deep freezing further emphasises this concern.

A far better way to ensure stability of the phenotype is either through passaging the resistant line for several rounds in mice in the absence of any drug pressure and/or passaging the resistant line through mosquitoes (e.g. Hayton *et al*, 2002; Afonso *et al* 2006; Kiboi *et al*, 2009). After the passaging protocol has been satisfied, the line can be tested for drug resistance.

I urge the authors to perform this test. I do realise that this step will take a couple of months, but I am afraid it is necessary in order to ensure the stability of the phenotype.

The second main issue is the quality of the written English. The manuscript is peppered with grammatical and style errors. This results in sometimes confusing and awkward sentences that affect a proper review of the content. The authors should consider rewriting the manuscript with the help of a native English speaker to ensure it meets the standards required for a scientific publication.

A minor issue is that references should be ordered chronologically in the main manuscript when used together (e.g. Duraisingh and Cowman, 2005; Holmgren *et al*, 2006; Borges *et al*, 2011). At the moment the order appears to be rather random.

I believe that selecting lines of malaria parasites for drug resistance to understand its genetic basis is essential to provide effective therapies for the treatment of this disease. Thus the work presented here is of interest to the scientific community, but only if the authors address the aforementioned issues.

I also understand that the authors are in the process of sequencing the whole genome (and I presume transcriptome) of their AQ resistant line and it will be interesting to see what mutations

may have arisen. If the line is indeed phenotypically stable, the authors should consider crossing it with a genetically distinct susceptible strain and then apply Linkage Group Selection (Culleton *et al*, 2005) to identify mutations underlying the phenotype. I could provide more details about how to proceed, should the authors decide to do so.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Jul 2017

Daniel Kiboi, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

We thank the referee for insightful comments.

To address the experimental concern raised, we are currently carrying out further experimentation on stability test. We choose to pass the parasite line through mice for at least five- ten drug free passages. We will then determine the resistance level after the drug free passages.

We recognize that passing the resistant line through mosquitoes as one of the ways of verifying stability of the phenotype. Since this is an ongoing project, we hope to use this approach as well before sequencing the genome and the transcriptome of the resistant line.

To address the second major concern, we have requested a native English speaker to assist in rewriting and improving the manuscript to the required publication standards.

Competing Interests: No competing interests were disclosed.

Author Response 23 May 2018

Daniel Kiboi, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

1. We tested the stability of the AQ resistant parasite by culturing the resistant parasite for a total of ten passages in the absence of the AQ. We then determined the 50% and 90% effective dosages (ED₅₀ and ED₉₀) using the standard 4DT test. In the materials and methods section, we have included a statement on the stability assays, under the subsection "Submission of the parasite to AQ pressure and Resistance Level Test. In the results and discussion section, we have included the ED₅₀ and ED₉₀ values in Table 3a to illustrate the stability of the AQ-resistant parasites. We have included a new figure (Figure 3) to show the drug response profile of the AQR, drug-free parasite and drug sensitive parent line. This data is under subsection on "Amodiaquine drug pressure induces stable-resistant phenotypes. We have uploaded the new data on stability in dataset 1.

2. We have improved the quality of the written English

3. We have corrected the order of the references

4. We plan to get in touch in with the reviewer as we embark to dissect further the molecular signatures associated with AQ resistance through sequencing the whole genome and transcriptome of the resistant parasites.

Competing Interests: No competing interests were disclosed.