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 2; peer review: 3 approved]

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Abstract

**Background:** The human malaria parasite *Plasmodium falciparum* has evolved drug evasion mechanisms to allow all available antimalarials. 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 the ramp up approach to select amodiaquine resistance. We then employed the 4-Day Suppressive Test to measure the resistance level 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 the parasite to amodiaquine pressure yielded resistant line within thirty-six passages. The effective doses that reduced 90% of parasitaemia (ED90) of the sensitive and resistant lines were 4.29 mg/kg and 19.13 mg/kg respectively. The selected parasite retained resistance after ten passage cycles in the absence of the drug and freezing at -80ºC for one month with ED90 of 20.34 mg/kg and 18.22 mg/kg. The parasite lost susceptibility to chloroquine by (6-fold), artemether (10-fold), primaquine (5-fold), piperaquine (2-fold) and lumefantrine (3-fold).

Sequence analysis of *Plasmodium berghei* chloroquine-resistant transporter revealed His95Pro mutation. We found no variation in the nucleotide sequences of *Plasmodium berghei* multidrug resistance gene-1 (*Pbmdr1*), *Plasmodium berghei* deubiquitinating enzyme-1 or *Plasmodium*...
bergheri Kelch13 domain. However, high mRNA transcripts of essential transporters; Pbmdr1, V-type/H+ pumping pyrophosphatase-2 and sodium hydrogen ion exchanger-1 and Ca^{2+}/H+ antiporter accompanies amodiaquine resistance.

**Conclusions:** The selection of amodiaquine resistance yielded stable “multidrug-resistant” parasites and thus may be used to study shared resistance mechanisms associated with other antimalarial drugs. Genome-wide analysis of the parasite may elucidate other functionally relevant 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 highest 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). The majority of this burden is in sub-Saharan Africa, primarily in children under five years of age. With the newly introduced vaccine showing less than 50% reduction in the clinical cases and its efficacy waning with time (Olotu et al., 2013; RTS,S Clinical Trials Partnership, 2014), the use of drugs for prevention and treatment of malaria remains an essential alternative in malaria control. To date, the treatment of uncomplicated malaria relies on the artemisinin-based combination therapies (ACTs), comprising of the short-acting artemisinin derivative and a long-acting partner drug, a strategy intended to reduce the emergence of resistance (WHO, 2016). However, the genetically flexible malaria parasite has evolved drug evasion mechanisms to all available antimalarial drugs, including the artemisinins (Amaratunga et al., 2016; Amato et al., 2017; Miotto et al., 2015).

The ACTs are currently used widely in many African countries where malaria is endemic; however, the extensive use is against the backdrop of high malaria transmissions, exposing the long-acting partner drugs to intense selection pressures (White, 2002). For instance, the 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 Coarsucam™/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 metabolised to its active long-acting metabolite desethylamodiaquine (DEAQ), with a half-life of more than five days (Churchill et al., 1985). In some African countries, AQ-ASN is the first or a second line drug for treatment of uncomplicated malaria (Rwagacundo et al., 2004; Sondo et al., 2016; WHO, 2016). In areas of highly seasonal transmission, such as sub-Saharan region, the AQ and sulfadoxine/pyrimethamine (AQ-SP) is used as a prophylactic combination, in children below five years, of age (WHO, 2016). Thus, AQ remains a useful drug in the treatment and prevention of malaria infection.

Amodiaquine like chloroquine (CQ) belongs to 4-amino-quinolines class of the 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; Sa et al., 2009), suggesting that the mechanisms of resistance may be different. The resistance to 4-amino-quinoline drugs in *Plasmodium falciparum* strongly associate with polymorphisms in two essential 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 (Ferdig et al., 2004; Fidock et al., 2000; Holmgren et al., 2006; Wellens, 2002). Currently, the mechanisms of AQ resistance are poorly understood. To extensively study these mechanisms, one needs to obtain naturally occurring stable *P. falciparum* lines resistant to AQ, but such parasites are not available. This limitation is overcome by inducing resistance *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 and time-consuming process (Nzila & Mwai, 2010). On the other hand, stable-resistant parasites lines can be induced *in vivo*, with relative ease, 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), other mechanisms are similar. For instance, mefloquine (MQ) resistant *P. berghei* lines (Gervais et al., 1999) demonstrated overexpression on the *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 the *cytochrome b* gene associates with atovaquone resistance in *P. berghei, P. chabaudi* and
In this study, we report on the 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 the decreased susceptibility to AQ. Some markers of resistance to other quinoline drugs, such as lumefantrine (LM), piperaquine (PQ) and quinine (QN) modulate the susceptibility to CQ (Eastman et al., 2011; Mwai et al., 2012; Okombo et al., 2010; Witkowski et al., 2017). Since all these drugs are proffered to have common mechanisms of action, which is the inhibition of heme detoxification (Muller & Hyde, 2010; O’Neill et al., 2006; Robert et al., 2001). We hypothesised that selected resistance markers associated with the quinoline drugs mentioned above also modulate parasite susceptibility to AQ. These markers in addition to Pfcrt 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). The V-type H+ pumping pyrophosphatase 2 (vpg2) and Ca2+/H+ antiporter (vcx1) which modulate resistance to CQ, LM and PQ in P. falciparum and P. berghei (Gonzales et al., 2008; Kiboi et al., 2014). Also, the P. falciparum sodium-hydrogen ion exchanger 1 (Pfne1), which modifies pH gradient between the 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 assessed for the presence of synonymous SNP and measured the transcript levels of the markers mentioned above in AQ resistant P. berghei parasites. Finally, the role of the 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; Strainer et al., 2015) was also studied.

Materials and methods
Parasites, host and compounds
Male Swiss albino mice (6–7 weeks old) weighing 20±2g outbred at KEMRI Animal House (Nairobi, Kenya) were used to induce AQ resistance from sensitive parasite line of P. berghei ANKA (MRA-868, MRA, ATCC® Manassas, Virginia, 676m1cl1). 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 mouse experiments, at least three 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% and 90% effective doses that reduce parasitaemia by 50% (ED90) and 90% (ED90) 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 1×10⁶ parasites and then randomly allocated to the four test groups and the control group (five mice per group). Oral treatment with the drug started 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 ED50 and ED90 calculation was estimated microscopically (×100) on day 4 (96 hrs) post parasite inoculation using thin blood films made from tail blood snips. The parasite growth was 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). The ED50 and ED90 were then estimated using linear regression line.

Submission of the parasite to AQ pressure and testing the resistance levels
The AQ sensitive parasites were submitted to continuous AQ pressure. At least six mice (three for the control and three for the test group) were inoculated intraperitoneally each with 1×10⁶ parasitised red blood cells in a 0.2ml on day 0 (D0). The parasitaemia was then allowed to rise >5% when test mice were treated orally with AQ at a concentration equivalent to the ED90. The parasite growth was then monitored to between 2–7% when donor mice were selected for subsequent passage into the next naive group of three mice. The parasites were then exposed to an increasing concentration of AQ in the subsequent passages based on parasite growth. The level of acquired resistance was evaluated at an interval of four drug pressure passages by measuring the ED90 and ED90 in the standard 4DT. Two approaches were employed to confirm the stability of the acquired resistance; first by freezing the selected AQ resistant parasite at -80°C for at least one month, second the AQ resistant parasites were passaged for at least ten passages in the absence of the drug. The ED90 and ED90 values were determined after the freezing-thawing process and after the ten mechanical passages in the absence of the drug. The ED90 allowed us to calculate the 90% index of resistance (I90) from the ratio of the ED90 of the resistant line to that of sensitive parent line. Based on I90 value, resistance levels were classified into four categories: i) I90=1.0 (sensitive), ii) I90=1.01-10.0 (slightly resistant), iii) I90=10.01-100 (moderate resistance), iv) I90≥100 (high resistance) (Xiao et al., 2004).

Generation of the genetically homogeneous parasite by dilution cloning
During the selection of resistant lines using the ramp up approach, a high parasite density of approximately 1×10³ infected red blood cells is submitted to the increasing drug pressure. Consequently, the parasites accumulate mutations. To minimise the random variation occurring during the selection process, we generated a genetically homogeneous clone using the limiting dilution approach, as detailed by (Janse et al., 2004). Briefly, a mouse with parasitaemia between

P. falciparum (Afonso et al., 2006; Srivastava et al., 1999; Syafruddin et al., 1999). Mutations in the dihydrofolate reductase (dhfr) and dihydropterotate 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.
0.5 and 1% was selected as a donor mouse. Five microlitres of infected blood were collected from the tail snip of the mouse in 1µl of heparin and diluted in 1ml of 1× PBS. The number of infected erythrocytes per 1µl was estimated from 20µl of the diluted blood. The cell suspension was then diluted further with 1×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 considered successful when 3 to 6 mice had a parasitaemia of between 0.3–0.5% at day eight post-infection. The fastest growing clone was selected for the subsequent 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 measuring the ED\textsubscript{50} and ED\textsubscript{90} in the 4DT assay (Fidock et al., 2004). The ED\textsubscript{50} and ED\textsubscript{90} of the resistant parasite were compared to the ED\textsubscript{50} and ED\textsubscript{90} of sensitive parental line. To this purpose, four different drug concentrations were selected for each of the test drugs 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 \textit{Pbmdr1}, \textit{Pbcrt}, \textit{Pbubp1} and \textit{PbKelch13}
Evaluation for the presence of SNPs in \textit{Pbmdr1}, \textit{Pbcrt}, \textit{Pbubp1} and \textit{PbKelch13} genes was carried out by sequencing, after PCR amplification from genomic DNA (gDNA) or cDNA generated from the mRNA. As illustrated in Figure 1a–b, target fragments corresponding to specific regions of interest from the \textit{Pbubp1} (PBANKA_0208800) and \textit{PbKelch13} (PBANKA_1356700) were PCR amplified from gDNA and sequenced using primers commercially synthesised from Inqaba Biotechnical Industries (Pty) Ltd, South Africa. The whole coding regions of the \textit{Pbcrt} (PBANKA_1219500) and \textit{Pbmdr1} (PBANKA_1237800) genes were amplified from the cDNA or gDNA template using primers listed in Table 1a. In extracting parasite genomic DNA (gDNA), 500µl of infected mouse blood with 5–10% parasitaemia was diluted with 500µl of 1×PBS, and the solution spun for 1 min at 500xg. After aspiration of the supernatant, the pellet was resuspended in a 30ml volume of cold 4°C 1×erythrocytes lysis buffer for 30 minutes, followed by spinning at 500xg for 10 min. The parasite pellet was washed twice with 30ml 1×PBS with centrifugation at 500xg 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. For the PCR amplification, 1µl of gDNA was used as the template in 25µl PCR reactions using the DreamTaq Master Mix or Phusion Flash High Fidelity Master Mix (ThermoScientific™). Table 1b shows the optimised cycling conditions. The PCR products were first analysed in 1.5% agarose gel, purified using the GeneJet™ PCR purification kit (Thermo Scientific™) and then sequenced 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).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Genome view of drug resistance genes and the target regions. (a) \textit{Plasmodium berghei} ubiquitin carboxyl-terminal hydrolase 1, and (b) \textit{Plasmodium berghei kelch} 13 protein, putative showing targeted positions (*), annealing positions for PCR and sequencing primers and the sizes of amplified PCR products.}
\end{figure}
Table 1. PCR methods. (A) Primer sequences for the PCR amplification 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 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.

<table>
<thead>
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<th>Primer Name</th>
<th>PCR primers sequence (5' to 3')</th>
<th>Primer annealing position</th>
</tr>
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<tbody>
<tr>
<td>Pbcrt - Forward</td>
<td>GGA CAG CCT AAT AAC CAA TGG</td>
<td>69-89</td>
</tr>
<tr>
<td>Pbcrt - Reverse</td>
<td>GTT AAT TCT GCT TCG GAG TCA TTG</td>
<td>1230-1253</td>
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**Sequencing primers (5' to 3')**

<table>
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<tr>
<td>Pbcrt - Forward</td>
<td>GGA CAG CCT AAT AAC CAA TGG</td>
<td>69-89</td>
</tr>
<tr>
<td>Pbcrt - Reverse</td>
<td>CGA CCA TAG CAT TCA ATC TTA GG</td>
<td>751-729</td>
</tr>
<tr>
<td>Pbcrt - Reverse</td>
<td>TCA GGA AGA AGT TGT GTC A</td>
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<td>Pbcrt - Reverse</td>
<td>GAT AAG GAA AAA CTG CCA TC</td>
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<td>Pbcrt - Forward</td>
<td>CCT AAG ATT GAA TGC TAT GGT CGT</td>
<td>729-751</td>
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<tr>
<td>Pbcrt - Reverse</td>
<td>GGA CAG CCT AAT AAC CAA TGG</td>
<td>69-89</td>
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**PCR primers (5' to 3')**

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<td>Pbkelch13 - Reverse</td>
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**Sequencing primers (5' to 3')**

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<td>1272-1291</td>
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<td>Pbkelch13 - Reverse</td>
<td>AGC TTC TAA TAA TGC ATA TGG</td>
<td>1899-1879</td>
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**PCR primers (5' to 3')**

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<td>Pbmdr1 - Forward</td>
<td>GTCTAAATGTTGTAATTTGTTGTCCT</td>
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<td>Pbmdr1 - Reverse</td>
<td>GACATTATCAATTTGCTCATCGCATGTCAGGCT</td>
<td>180bp downstream</td>
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**Sequencing primers (5' to 3')**

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<th>PCR primers sequence (5' to 3')</th>
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<td>Pbmdr1 - Forward</td>
<td>CAGTATGCTCAATTTTCTCC</td>
<td>250-271</td>
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<td>Pbmdr1 - Forward</td>
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<td>176-198</td>
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<td>Pbmdr1 - Forward</td>
<td>CACCTTCTCCAAACAATTACTCTACTCA</td>
<td>717-742</td>
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<tr>
<td>Pbmdr1 - Reverse</td>
<td>GCAGCCTCTACTATGTAATAAAGGGTCC</td>
<td>611-636</td>
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<tr>
<td>Pbmdr1 - Reverse</td>
<td>GTCGAACGCTGGATTATTCTCTCTCTG</td>
<td>1062-1080</td>
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<td>Pbmdr1 - Forward</td>
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<tr>
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<td>Pbmdr1 - Forward</td>
<td>TGGAGTATGTTGTAATGCAAAGATCT</td>
<td>1362-1383</td>
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<tr>
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<td>Pbmdr1 - Forward</td>
<td>TCAAAGTAGAGATCAAGAGTCAACAGG</td>
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<td>2247-2269</td>
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<td>Pbmdr1 - Reverse</td>
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<td>2803-2823</td>
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<td>2647-2672</td>
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<td>Pbmdr1 - Reverse</td>
<td>TAGCCTTTTCTCGATCCTCTTCAGG</td>
<td>3234-3259</td>
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<td>TGCAATAGATTATGCACTTCAAAGGGG</td>
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<td>Pbmdr1 - Reverse</td>
<td>ATCCCTTCAATCGTAGATCATCGCAT</td>
<td>3513-3538</td>
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<tr>
<td>Pbmdr1 - Forward</td>
<td>CCTCAAGAGGAGTGCAGATAAAAGCTA</td>
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<td>Pbmdr1 - Reverse</td>
<td>GAATCTATCAACGTGACAAGACTCAGG</td>
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<td>TGCAGTTAATACCAAAGAACATGCT</td>
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<tr>
<td>Pbmdr1 - Reverse</td>
<td>TAGATGCAATTCTGTGAGAATCGCAT</td>
<td>4100-4123</td>
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**PCR and Sequencing primers (5' to 3')**

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<td>Pbubp1 - Forward</td>
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<td>1990-2015</td>
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<tr>
<td>Pbubp1 - Reverse</td>
<td>CTA AGT TGC ATA GTC TTA TCA TTT TC</td>
<td>2621-2596</td>
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RNA extraction, cDNA synthesis and qRT-PCR assays
The quantity of the mRNA transcripts of Pbdmrl, Pbvp2, Pbvcx1, and Pbneh1 genes was carried out after cDNA synthesis from mRNA. Before the extraction of RNA, all the buffers and solutions for parasite preparation were treated with 0.1% (v/v) of diethyl pyrocarbonate (DEPC). The total RNA was isolated from approximately 1x10⁸ fresh parasites pellet. In preparation of parasite pellet, parasitised red blood cells were first washed in 1xPBS and then lysed in 5 volumes of ammonium chloride solution. The parasite pellet was washed twice in 10ml of 1xPBS and then resuspended in 200µl of 1xPBS. 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. Five micrograms of the total RNA, 1µl of oligo-DT and water were mixed with 4µl Reaction buffer (5x), 1µl RiboLock RNase Inhibitor (U/µl), 2µl of dNTPs (10mM) and 1µl of RevertAid M-MuLV RT (200U/µl). The reaction mix was first incubated at 42°C for 60min, then at 70°C for 5min and finally chilled on ice. The cDNA was used as the 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 Pbdmrl, Pbvp2, Pbvcx1 and Pbneh1 were designed to run using similar cycling conditions relative to the Pbβ-actin I, as the housekeeping gene (Table 2). Briefly, 12µl of Maxima SYBR mix, 2.0µl (0.25mM) 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 I as the housekeeping using the formula 2^{-ΔΔCT} based on Livak & Schmittgen, 2001. The means for cross-resistance profiles for each drug from at least four different drug concentrations were 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 was 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 component of the ACT therapy (Gil, 2008) has spurred studies on understanding the mechanisms of AQ resistance. Using the 2% Relapse approach; the AQ resistant P. berghei and P. yoelii were generated by submitting the parasites to 60mg/kg and 100mg/kg respectively (Peters & Robinson, 1992); however, the stability, resistance indices and molecular mechanisms remained undetermined. 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 determined the ED₉₀, ED₅₀ and ED₅₀ of AQ against the sensitive P. berghei ANKA. The ED₉₀, ED₅₀, ED₅₀ were 0.95, 4.29 and 5.05mg/kg/day, respectively. We adopted the ramp up approach which employs the sequential increase in the drug pressure. The 5.05mg/kg drug concentration was the starting drug pressure dose and administered once percentage parasitaemia rose to 2–7%. At the onset, average parasitaemia reached 2–7% on day 3–4 post-infection, after which mice received 5.05mg/kg of AQ. Figure 2 shows parasite responses

<table>
<thead>
<tr>
<th>PCR amplifying profiles</th>
<th>Temperature (°C)/Time (min)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pbcrt</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>98°C, 30 secs</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98°C, 10 secs</td>
</tr>
<tr>
<td>Annealing Temperature</td>
<td>50°C, 15 secs</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C, 30 secs</td>
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<tr>
<td>Primer (Forward &amp; reverse)</td>
<td>2.5µM each</td>
</tr>
<tr>
<td>MgCl₂ (mM)</td>
<td>1.5</td>
</tr>
<tr>
<td>Cycles</td>
<td>30</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C, 2 min</td>
</tr>
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</table>

Table 2
The means of expression levels of each gene from three independent experiments and from triplicate assays obtained from
Table 2. Oligonucleotide sequences used in the qPCR assays. The oligos were utilised to measure the transcriptional level profiles of *Plasmodium berghei* multidrug resistance gene 1 (*Pbmdr1*), *Plasmodium berghei* V-type H+ pumping pyrophosphatase (*Pbvp2*), *Plasmodium berghei* Ca++/H+ antiporter (*Pbcvx1*), *Plasmodium berghei* sodium hydrogen exchanger (*Pbnhe1*) genes with *Plasmodium berghei* β-actin I gene (*Pbβ-actin I*) as housekeeping using Maxima SYBR Green chemistry in qPCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5' - 3')</th>
<th>Position</th>
<th>Tm</th>
</tr>
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<tbody>
<tr>
<td><em>Pbmdr1</em> - Forward</td>
<td>ACGGTAGTGGCTTCAATGGA</td>
<td>917-936</td>
<td>54.2</td>
</tr>
<tr>
<td><em>Pbmdr1</em> - Reverse</td>
<td>CTGTCGACAGCCTGGTTTCTG</td>
<td>1082-1062</td>
<td>54.7</td>
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<tr>
<td><em>Pbnhe1</em> - Forward</td>
<td>TGGAAGATTTTAGGCTACC</td>
<td>2022-2045</td>
<td>54.0</td>
</tr>
<tr>
<td><em>Pbnhe1</em> - Reverse</td>
<td>GCTAGGCCGATTTGTTAGAG</td>
<td>2202-2180</td>
<td>55.3</td>
</tr>
<tr>
<td><em>Pbvp2</em> - Forward</td>
<td>TGACGCAGGAATACACAGC</td>
<td>1449-1469</td>
<td>55.2</td>
</tr>
<tr>
<td><em>Pbvp2</em> - Reverse</td>
<td>GTCGTACTTTGGCATACTGCTG</td>
<td>1558-1535</td>
<td>56.5</td>
</tr>
<tr>
<td><em>Pbcvx1</em> - Forward</td>
<td>TCAAATTGCTCTTTTTGTTGTACCAA</td>
<td>1101-1126</td>
<td>57.9</td>
</tr>
<tr>
<td><em>Pbcvx1</em> - Reverse</td>
<td>ACACCTTCTAGCCAATTCTTACC</td>
<td>1265-1240</td>
<td>57.1</td>
</tr>
<tr>
<td><em>Pbβ-actin I</em> - Forward</td>
<td>CAGCAATGTATGTGATCAAGC</td>
<td>392-416</td>
<td>56.8</td>
</tr>
<tr>
<td><em>Pbβ-actin I</em> - Reverse</td>
<td>CATGGGGTAATGCATATCCTACA</td>
<td>523-498</td>
<td>58.9</td>
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</table>

Figure 2. Log2 average parasitaemia of *Plasmodium berghei* ANKA during the selection of amodiaquine resistance. The growth profiles of the parasites from the untreated control group and amodiaquine treated group at the different passage stages and the different drug concentrations during the selection of the amodiaquine resistant parasites.

to AQ at the different passages and the different drug concentrations during the selection of drug-resistant parasites. On average, recovery of the parasites from the treated donor mouse was on day seven post-infection. Based on parasite growth at different passages, the drug pressure dose was increased by a factor of ED<sub>99</sub> at different passage levels. Within the first twelve passages, administration of single 5mg/kg of AQ, after attaining >2% parasitaemia, cleared the parasite to below detectable levels by microscopy. The parasite density of >2% parasitaemia was attained after 7–10 days; therefore, the same drug pressure dose was administered for the first twelve passages. From the 13<sup>th</sup> 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 the ED<sub>99</sub> (equivalent to 2.5mg/kg) after every two passages up to the 20<sup>th</sup> passage. From the 20<sup>th</sup> passage, we increased the drug pressure dose sequentially by a factor of 2 of the ED<sub>99</sub> (equivalent to 5mg/kg) after every two passages. By the 36<sup>th</sup> passage, the drug pressure dose had risen to 50mg/kg. The 50mg/kg dose was fifty and ten times higher than the ED<sub>50</sub> and ED<sub>99</sub> of the parent line respectively. When we quantified the ED<sub>50</sub> and ED<sub>99</sub> in the 4DT, we expected higher indices of resistance.
Surprisingly the $I_{50}$ and $I_{90}$ were only twelve and four 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 5.86mg/kg and 18.22mg/kg respectively. Similarly, the ED$_{50}$ and ED$_{90}$ values after ten drug-free passages corresponded to 8.05mg/kg and 20.34mg/kg respectively (Table 3a). We then tested drug response of the 36th passage AQ resistant line (AQR$_{36}$th), drug-sensitive parent line (AQ_S), and drug-free AQ resistant line (DF_AQR) at 2.5mg/kg and 20mg/kg of AQ. As expected, 2.5mg/kg was active against the AQ_S with 68%. However, the same concentration yielded a mere 12.5% and 31% activity against the AQR$_{36}$th and DF_AQR respectively (Figure 3). On increasing the drug concentration to 20mg/kg, we recorded a 96% and 83% activity against the AQR$_{36}$th and DF_AQR. Our data indicate that the AQR parasite line retained an index of resistance after the ten passages in the absence of the drug and freeze-thawing process. We thus concluded that stable-AQ resistant *P. berghei* parasite line was successfully selected and the resistance mechanisms are probably encoded in the cell genome.

<table>
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<th>Table 3. (A) The 50% and 90% Effective Dose (ED$<em>{50}$ and ED$</em>{90}$) in mg/kg/day of amodiaquine resistant Plasmodium berghei ANKA line at different passage levels showing a sharp rise in ED$<em>{50}$ in comparison to the steady but slow increase in ED$</em>{90}$. Index of resistance at 50% ($I_{50}$) and 90% ($I_{90}$) from the ratio of ED$<em>{50}$ or ED$</em>{90}$ of the resistant line with ED$<em>{50}$ or ED$</em>{90}$ of sensitive line respectively. The effective dose was measured in the 4-Day suppressive Test using at least four different drug concentrations and at least four Swiss mice per dose. (B) Cross-resistance profiles of the amodiaquine resistant <em>Plasmodium berghei</em> ANKA line and sensitive parent line as measured in the 4-Day suppressive Test using at least four different drug concentrations and at least four Swiss mice per drug concentration. The Index of resistance ($I_{90}$) calculated from the ratio of ED$_{90}$ of the resistant line to that of the sensitive parent line.</th>
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<td><strong>TABLE 3A</strong></td>
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<tr>
<td>36th</td>
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<tr>
<td>Stability after freezing for one month</td>
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<td>Stability results after ten passages in the absence of the drug</td>
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<td><strong>TABLE 3B</strong></td>
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<tr>
<td>Primaquine</td>
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<td>Artemether</td>
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<td>Chloroquine</td>
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<td>DEAQ</td>
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Using Student’s t-test the differences between the sensitive parental line and amodiaquine resistant line were significant

`p < 0.01;`                  
`$^*p < 0.001;`                  
`$^\dagger p < 0.0001.`
Amodiaquine resistance associated with cross-resistance to CQ, LM, PMQ, PQ and ATM

The selection of stable AQ resistant parasites allowed us to study whether AQ resistance also reduced the susceptibility of other antimalarial drugs (Dataset 2). Using dilution cloned parasite, we determined the ED$_{90}$ of PQ, LM, PMQ and ATM against both the AQ sensitive (AQS) and AQR. To our surprise, the AQR yielded moderate and slight resistance to ATM (I$_{90}$ = 10.2) and PMQ (I$_{90}$ = 5.8) respectively. Interestingly, the AQR had a lower resistance level to PQ (I$_{90}$ = 2.2-fold) when compared with LM (I$_{90}$ = 3.5-fold), despite PQ and AQ belonging to the same chemical class of 4-aminoquinoline and LM belonging to the different chemical class of the aryl-alcohols (Table 3b). Our results mean that the AQR also acquired 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, and chemically related to AQ, thus may share some resistance mechanisms. Indeed, selection of the 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-amin quinoline) and LM (an aryl-alcohol) to share specific mechanisms with 4-amino quinoline-based on the similarity in the modes of action. However, the high cross-resistance levels for ATM (I$_{90}$ = 10fold) is entirely surprising. Artemether is mechanistically and chemically unrelated to AQ (Robert et al., 2001; Tilley et al., 2016). Amodiaquine inhibits heme polymerization within the digestive vacuole, thus killing the parasite by the accumulation of toxic heme (O’Neill et al., 2006). Artemisinins has multiple targets, for instance, 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 an 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 drug accumulation within the cells. To date, the combination of ATM/LM (Coartem®), dihydroartemisinin/PQ (Artekin®) and ASN/AQ are the drugs of choice in many sub-Saharan African countries (WHO, 2016).

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 Coarsucam™ would compromise the efficacy of Artekin® and Coartem®. However, so far studies in P. falciparum do not indicate a correlation between the decrease in AQ and artemisinin activity (Borrmann et al., 2013; Nsoyba et al., 2010).

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

To investigate the possible resistance mechanisms, we first interrogated for polymorphisms in two drug resistance transporters in the malaria parasite, the Pbcrt and Pbmdr1. The two transporters directly mediate and modulate susceptibility to quinoline-based drugs in P. falciparum. Our study focused on...
the whole coding regions of these two genes. To date, several studies have demonstrated the association between 4-amino-quinoline resistance and the mutations in \textit{mdr1} gene, changes in expression profiles and copy number variation in \textit{the mdr1} gene (Borges \textit{et al.}, 2011; Duraisingh \& Cowman, 2005; Dhingra \textit{et al.}, 2017). The single nucleotide polymorphism (SNP) in \textit{Pfcr} (codon 76) associates with CQ and AQ resistance in \textit{P. falciparum} (Ecker \textit{et al.}, 2012; Fidock \textit{et al.}, 2000; Ochong \textit{et al.}, 2003). Studies in the rodent malaria \textit{Plasmodium chabaudi}, however, found no association between \textit{ct} and CQ resistance (Afonso \textit{et al.}, 2006; Hunt \textit{et al.}, 2004), suggesting that other genes may mediate CQ and the 4-aminoquinoline resistance. Recent studies also identified potential \textit{ct} background mutations; Ile356Thr and Asn326Ser that associate with artemisinin resistance (Miotto \textit{et al.}, 2015). In the present study, the nucleotide codons corresponding to amino acid position 76, 326 and 356 of the \textit{PbCrt} protein were found not to harbour any mutation in AQ resistant line (compared to the sensitive line). However, we observed a substitution mutation (A -> C 284) in the nucleotide sequence of the AQR, that resulted in a His95Pro mutation in the \textit{PbCrt} protein. The His95Pro mutation localises within the second transmembrane domain close to the food vacuole compartment suggesting that the mutation could play a role in drug transport. However, the functional role and biological consequence of His95Pro mutation in AQ resistance require further investigation. We then extended our study to the \textit{mdr1} transporter. Mutations at positions 86, 184, 1034, 1042, and 1246 of the \textit{Pfmdr1} mediate and modulate CQ, LM and mefloquine resistance (Ecket \textit{et al.}, 2012; Price \textit{et al.}, 1999; Price \textit{et al.}, 2004; Sisowath \textit{et al.}, 2005). Similarly, our recent investigation using LM and PQ resistant \textit{P. berghei} parasite found no polymorphisms in \textit{ct} and \textit{mdr1} genes (Kiboi \textit{et al.}, 2014). Sequencing of the whole coding region of the \textit{mdr1} from AQR and the AQS did not reveal any sequence variation. The presence of a novel mutation (His95Pro) in the \textit{ct} gene coupled by the absence of hitherto known mutations within the \textit{ct} and \textit{mdr1} genes suggest that the malaria parasite may develop resistance by the acquisition of mutation in other positions of the proteins. Indeed, the addition of C101F mutation in the \textit{ct} gene of the CQ resistant \textit{P. falciparum} conferred high resistance to PQ but generated a reciprocal susceptibility to AQ, quinine and ATM (Dhingra \textit{et al.}, 2017). The specific introduction of the His95Pro mutation using CRISPR/Cas9 approach would provide additional insights on the role of the mutation in mediating AQ resistance as well as the quinoline drugs.

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 a 10-fold difference. Recent reports have validated \textit{Kelch13} propeller domain, Met476Ile, Tyr493His, Arg539Thr, Ile543Thr and Cys580Tyr mutations as markers for artemisinin resistance (Miotto \textit{et al.}, 2015; Straimer \textit{et al.}, 2015). We hypothesised that \textit{PbKelch13} might possess SNPs, and thus mediate this cross-resistance. Our data showed no mutation in \textit{the PbKelch13} domain, thus AQ and ATM resistance observed \textit{in vivo} is not associated with SNPs in the \textit{Kelch13} domain. We focused our study on \textit{Kelch13}. However other genes such as \textit{TCTP}, \textit{SERCA} and \textit{PI3P} that associate with artemisinins action or resistance in \textit{P. falciparum} (Eckstein-Ludwig \textit{et al.}, 2003) may also associate with our selected AQ resistant line. As the index of resistance to ATM (\textit{I}$_{\text{ATM}}$ = 10.2) was double that of AQ (\textit{I}$_{\text{AQ}}$ = 4.2) indicate that AQ and ATM could share some resistance mechanisms in \textit{P. berghei}. Thus, these AQ resistant lines could be used to define these shared mechanisms, and some of them may be \textit{TCTP}, \textit{SERCA} and \textit{PI3P} or other unknown genes.

To further understand the AQ and ATM resistance in AQR, we focused on the \textit{ubp1} gene. The acquisition of V739F and V770F mutations in the conserved C-terminal region of the \textit{ubp1} is associated with artesunate resistance in \textit{P. chabaudi} (Hunt \textit{et al.}, 2010). Similarly, Tyr835Ly and Ser836Gln mutations occurred in both LM and PQ resistant \textit{P. berghei} (unpublished data: Kiboi, Irungu, Orwa, Kamau, Ochola-Ojier, Ng’ang’a and Nzila). In our current study, the analysis of the sequence fragments flanking 739, 770, 834 and 835 positions of the \textit{PbUBP1} protein revealed no amino acid changes in the selected AQR. Studies in \textit{P. falciparum in vitro} also found no association between artemisinin resistance and mutation in \textit{ubp1} (Chavichich \textit{et al.}, 2010); however, analysis of field \textit{P. falciparum} isolates from Western Kenya associated \textit{Pfubp1} Glu1528Asp mutation with tolerance to artesinin (Henriques \textit{et al.}, 2015). 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 \textit{Pbmdr1}, \textit{Pbhe1}, \textit{Pbvp2} and \textit{Pbcvx1} associated with AQ resistance**

To further probe other probable mechanisms of AQ resistance, we hypothesised that essential transporters or ion exchangers, \textit{Pbmdr1}, \textit{Pbhe1}, \textit{Pbvp2} and \textit{Pbcvx1} could mediate AQ resistance via altered mRNA transcript levels (Dataset 3). The results show that the mRNA transcript of \textit{Pbmdr1} and \textit{Pbvp2} were elevated 3.0fold (p<0.0001) and 2.3fold (p<0.0001), respectively (Figure 4). Concerning the \textit{Pbhe1} and \textit{Pbcvx1}, the AQR had a significantly high amount of \textit{Pbhe1} mRNA transcripts of 2.6fold compared to the AQS (p<0.0001), and similar results were recorded on \textit{Pbcvx1}, 1.7fold (p<0.001) (Figure 4). Therefore, high \textit{mdr1}, \textit{vp2}, \textit{cvx1} and \textit{nhel} transcript level associated with AQ resistance. The overexpression of \textit{mdr1} is a marker for \textit{P. falciparum} resistant to MQ, AQ, CQ and ATM (Borges \textit{et al.}, 2011; Gonzales \textit{et al.}, 2008). However, the amplification of \textit{mdr1} gene was not linked with CQ and PQ resistance in \textit{P. falciparum} (Sidhu \textit{et al.}, 2006; Witkowski \textit{et al.}, 2017), suggesting a complex regulation of the resistance mechanisms for the quinoline related drugs. Also, the \textit{mdr1} regulates transcription of other drug resistance genes (Gonzales \textit{et al.}, 2008; Jiang \textit{et al.}, 2008). For instance, augmenting CQ resistance in parasites harbouring \textit{Pfcr} K76T mutation (Fidock \textit{et al.}, 2000). Here, we show that \textit{mdr1} overexpression may play a direct role in mediating AQ resistance.

Two genes, \textit{vp2} and \textit{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 the mutation in drug resistance genes) in a mutated PfCRT protein (Jiang et al., 2008). In a recent report, the PQ resistance was associated with a high vp2 and cvx1 expression in P. berghei, though there was no mutation in the PfCRT gene (Kiboi et al., 2014). The AQ resistant line carried a His95Pro mutation in PbCRT protein. Thus, the elevation of vp2 and cvx1 may compensate for this mutation, as it has previously reported with the Lys76Thr crt mutation in P. falciparum (Jiang et al., 2008). To date, the proffered mode of action for CQ, AQ and PQ is the inhibition of heme polymerisation 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 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 PfCRT and Pfmdr1 (Nkrumah et al., 2009). Since the nhe1 to regulates the Na+ and H+ exchange, this ion exchanger may also contribute to the resistance in AQ parasite lines.

In conclusion, we provide essential evidence about AQ resistance in P. berghei ANKA. First, the emergence of AQ resistance led to the loss of susceptibility to ATM, PMQ, LM, PQ and CQ; thus, the AQ resistant parasite is a “multi-drug” resistant parasite. Second, a 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 augment the resistance levels and confer a 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 the 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, 2018a).


Competing interests
No competing interests were disclosed.
Grant information
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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Richard T. Eastman
National Library of Medicine & NIH, Bethesda, MD, USA

The authors have significantly improved the manuscript, and the additional data further supports their hypothesis that the observed mutation in PbCRT may modulate amodiaquine susceptibility.

For the experiments assessing the stability of the amodiaquine resistance, both after freezing of the parasite line and passage in the absence of drug selection pressure, there is a notable reduction in the ED50 values, but maintenance of the ED90 levels. Is this difference significant (would be helpful for statistical analysis to be conducted for all comparisons). In addition, discussion (with possible inclusion of a dose response supplemental figure) would be helpful to understand this altered drug response phenotype (alteration in the slope of the amodiaquine dose response after removal of drug pressure). As this may suggest two independent genetic/epigenetic elements contributing to the observed amodiaquine resistance, where one is stable through the freezing/thawing process and serial passage without drug selection pressure and the other reverts without maintained selection pressure.

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.

Axel Martinelli
Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

Reviewer Report 13 June 2018

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I am pleased to see that the authors addressed my main concern regarding the stability of the resistant phenotype by passaging the resistant line in the absence of drug selection and confirmed the acquisition of stable AQ resistance.

There are still occasional small grammar mistakes in the main text, although I do take note of the fact that the written English has been considerably improved. I will let the editor decide whether this minor issue requires further action or not.

I noticed the authors ordered their references alphabetically rather than chronologically (i.e. from oldest to most recent) when citing them in the main text. I am used to the latter format, but if the alphabetical format is according to the journal guidelines. I have no further comments.

The His95Pro mutation may play a role, but until other mutations can be excluded by WGS and the role is verified by transfection studies, I would refrain from asserting that it is associated with AQ resistance (although it does make a plausible candidate). Thus I would change the concluding remark:

"Second, a novel His95Pro mutation in *PbCRT* is associated with AQ resistance:

to

"Second, a novel His95Pro mutation in *PbCRT* may be associated with AQ resistance"

**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.

David A. Fidock
Department of Microbiology and Immunology, Columbia University Medical Center (CUMC), New York, NY, USA

I am satisfied with their revision and support the indexing of this report, which provides interesting data on an important topic in antimalarial chemotherapy

**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.
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).
lower IC50 and IC90 values after the freeze/thaw suggest some instability in the drug-resistance phenotype.

5. As the drug dose response is sigmoidal 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, Kenya Medical Research Institute (KEMRI)/Wellcome Trust, Collaborative Research Program, Kilifi, 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 *Pbmbr1*, *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 *Pbmbr1* 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 ED$_{50}$ and ED$_{90}$ using the standard 4DT test. Figure 3 illustrates this data on the stability of the mutant parasites. Table 3a also contains the new computed ED$_{90}$ and indices of resistance.

5. We consistently used the linear regression analysis in estimation of the ED$_{50}$ and ED$_{90}$. Since, the linear regression would provide a good estimate, we presume our results on the ED$_{50}$ and ED$_{90}$ would correlate well across the different assays we conducted.

**Competing Interests:** No competing interests were disclosed.
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 ED$_{50}$ and I$_{50}$ 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 IC$_{50}$ 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 form 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,** Kenya Medical Research Institute (KEMRI)/Wellcome Trust, Collaborative Research Program, Kilifi, 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 *Pbcr1* 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 (ED$_{50}$ and ED$_{90}$) 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 ED$_{50}$ and ED$_{90}$ 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 ED$_{90}$ for primaquine and lumefantrine against the sensitive parent parasite. We have included the new data on ED$_{90}$ 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
Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

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 ED$_{50}$ 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.

References

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.

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**Author Response 17 Jul 2017**

Daniel Kiboi, Kenya Medical Research Institute (KEMRI)/Wellcome Trust, Collaborative Research Program, Kilifi, 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.

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**Author Response 23 May 2018**

Daniel Kiboi, Kenya Medical Research Institute (KEMRI)/Wellcome Trust, Collaborative Research Program, Kilifi, 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 (ED$_{50}$ and ED$_{90}$) 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 ED$_{50}$ and ED$_{90}$ 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 “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.