Published in final edited form as:
Exp Parasitol. 2014 December ; 147: 23–32. doi:10.1016/j.exppara.2014.10.008.

Piperaquine and Lumefantrine Resistance in *Plasmodium berghei* ANKA associated with Increased Expression of Ca^{2+}/H^{+} antiporter and Glutathione Associated Enzymes

Daniel Kiboi[a,b,*], **Beatrice Irungu**[b], **Jennifer Orwa**[b], **Luna Kamau**[c], **Lynette Isabella Ochola-Oyier**[d], **Joseph Ngángá**[a], and **Alexis Nzila**[e]

[a]Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, P.O Box 62000, 00200, Nairobi, Kenya

[b]KEMRI-Centre for Traditional Medicine and Drug Research, P.O Box 54840-00200, Nairobi, Kenya

[c]KEMRI-Centre for Biotechnology Research and Development, P.O Box 54840-00200, Nairobi, Kenya

[d]KEMRI-Wellcome Trust Research Programme, P.O Box 230-80108, Kilifi, Kenya

[e]Department of Chemistry, King Fahd University of Petroleum and Minerals, P.O Box 468, Dharan, 31261, Kingdom of Saudi Arabia

Abstract

We investigated the mechanisms of resistance of two antimalarial drugs piperaquine (PQ) and lumefantrine (LM) using the rodent parasite *Plasmodium berghei* as a surrogate of the human parasite, *Plasmodium falciparum*. We analysed the whole coding sequence of *Plasmodium berghei chloroquine resistance transporter (Pbcrt*) and *Plasmodium berghei multidrug resistance gene 1*(*Pbmdr-1*) for polymorphisms, these genes are associated with quinoline resistance in *Plasmodium falciparum*. No polymorphic changes were detected in the coding sequences of *Pbcrt* and *Pbmdr1* or in the mRNA transcript levels of *Pbmdr1*. However, our data demonstrated that PQ and LM resistance is achieved by multiple mechanisms that include elevated mRNA transcript levels of V-type H+ pumping pyrophosphatase (*vp2*), Ca^{2+}/H^{+} antiporter (*vcx1*), gamma glutamylcysteine synthetase (*ggcs*) and glutathione-S-transferase (*gst*) genes, mechanisms also known to contribute to chloroquine resistance in *P. falciparum* and rodent malaria parasites. The increase in *ggcs* and *gst* transcript levels was accompanied by high glutathione (GSH) levels and elevated activity of glutathione-S-transferase (GST) enzyme. Taken together, these results demonstrate that *Pbcrt* and *Pbmdr1* are not associated with PQ and LM resistance in *P. berghei* ANKA, while *vp2*, *vcx1*, *ggcs* and *gst* may mediate resistance directly or modulate functional mutations in other unknown genes.

[*]**Corresponding author:** Address: Kenya Medical Research Institute (KEMRI)/Centre for Traditional Medicine and Drug Research, P.O Box 54840-00200 Nairobi, Kenya. Tel: 254 (020) 2722541 Ext 2250, dmuthui@jkuat.ac.ke (D. Kiboi).

Ethics Approval

The study and all animal work were conducted according to national and international guidelines. The study was approved by the National KEMRI scientific and ethical review Committees and the Animal Care and Use Committee

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Keywords

Malaria; *Plasmodium berghei*; Resistance; Piperaquine; Lumefantrine

1 Introduction

Chemotherapy remains central in the control of malaria. However, the rapid emergence and spread of resistance still hinders malaria control through the use of drugs. To minimize the loss of drugs to resistance World Health Organization (WHO) recommends the use artemisinin based combination therapies (ACT), combinations of the short acting artemisinin derivative and a long half-life partner drug (WHO, 2010). Today, the combination, lumefantrine (LM) and artemether (ATM), is the first line malaria treatment in many African countries, including Kenya (Ogutu *et al.,* 2014), but there is a concern that resistance to LM will be selected relatively quickly due to mismatched pharmacokinetics between partner drugs (Sisowath *et al.,* 2009; Mwai *et al.,* 2012). An alternative combination treatment for malaria is piperaquine (PQ) and dihydroartemisinin (DHA), the second line treatment in Kenya (Ogutu *et al.,* 2014). In both combinations, an artemisinin derivative is partnered with a drug against which resistance may arise relatively quickly, especially in high malaria transmission settings.

The initial use of PQ in China, a bisquinoline antimalarial drug chemically related to chloroquine (CQ) and other 4-aminoquinolines, was thought to herald a new dawn for malaria chemotherapy. Due to the high potency and tolerability of PQ, the drug supplanted CQ as the first line regimen in China (Davis *et al.,* 2005). However, extensive and indiscriminate use for treatment and prophylaxis in China led to the emergence of resistance in *Plasmodium falciparum* and the subsequent withdrawal of PQ as a monotherapy (Davis *et al.,* 2005). The other artemisinin derivative partner drug, LM belongs to the arylalcohol group of antimalarials structurally similar to mefloquine (MQ), halofantrine (HF) and quinine (QN) (Schlitzer, 2008). LM is effective against CQ resistant parasites with studies on resistance mechanisms indicating an inverse correlation with CQ and AQ resistance in *P. falciparum* (Mwai *et al.,* 2009a).

To date, the mechanism of action and resistance markers for LM and PQ are poorly understood. Emergence of LM and PQ resistance seems to involve a complex network of genes. The mechanism of action of PQ and LM is predicted to be similar to that of CQ (Raynes, 1999; Tarning, 2007; Mwai *et al.,* 2009a), which binds to ferriprotoporphyrin-IX (heme) in the digestive vacuole inhibiting polymerization of toxic heme into non-toxic hemozoin and consequently killing the parasite (Biagini *et al.,* 2003; Robert *et al.,* 2001). Thus, these drugs may share some resistance mechanisms. For instance, CQ resistance in *P. falciparum* is known to be associated with the mutation at codon 76 [Lys76Thr] in the *chloroquine resistance transporter (Pfcrt)* gene (Fidock *et al.,* 2000; Cooper *et al.,* 2005), and interestingly, the selection of Lys76 (wild-type allele) of *Pfcrt* has been associated with LM reduced susceptibility (Mwai *et al.,* 2009b). Likewise, point mutations, increased transcript levels and increased copy numbers of the *Plasmodium falciparum multidrug resistance gene 1 (Pfmdr1)* have been associated with LM resistance in *P. falciparum* (Sidhu *et al.,* 2006;

Sisowath *et al.,* 2005; Sisowath *et al.,* 2007) and modulation of CQ resistance (Reed *et al.,* 2000). Whole genome hybridization studies of PQ resistant *P. falciparum* revealed a single nucleotide polymorphism (SNP) in the *pfcrt* gene and copy number reduction in a 82kb region in chromosome 5 that included *pfmdr1* (Eastman *et al.,* 2011). The SNP in the *crt* gene and the amplification were however retained with loss of PQ resistant phenotypes (Eastman *et al.,* 2011). Therefore, response to LM and PQ may potentially involve *crt* and *mdr1* but also additional genes.

Resistance mechanisms are accompanied by compensatory and modulatory changes within the same or different genes (Jiang *et al.,* 2008). For instance, studies on adaptive modification accompanying altered functionality of *crt* identified the V-type H^+ pumping pyrophosphatase 2 (*vp2*) and Ca^{2+}/H^+ antiporter (*vcx1*) as genes associated with modifying drug resistance (Jiang *et al.,* 2008). Furthermore, researchers have linked glutathione (GSH) pools to drug resistance in malaria parasites (Ginsburg *et al.,* 1999; Meierjohann *et al.,* 2002; He *et al.,* 2009; Patzewitz *et al.*, 2013). For instance, *P. berghei* and *P. falciparum* lines resistant to CQ revealed increased levels of both GSH and GSH-associated enzyme activity relative to their sensitive counterparts (Dubois, et al., 1995; Meierjohann, et al., 2002). In recent findings CQ resistance in *P. chabaudi* was also associated with an increase in GST and GSH levels (He *et al.,* 2009). An increase in gamma glutamylcysteine synthetase (*ggcs*) mRNA levels was linked with CQ and mefloquine (MQ) resistance in *P. berghei* (Perez-Rosado *et al.,* 2002).

Due to the limitations and complexity of selecting resistance in *P. falciparum in vitro* (Nzila & Mwai, 2010), the malaria parasite that infects rodent models have been used to study the genetic organization of drug resistance *in vivo* (Hunt *et al.,* 2004a; Hunt *et al.,* 2004b; Hunt *et al.,* 2010; Carlton *et al.,* 2001; Martinelli *et al.,* 2011). Gervais *et al.* (1999) demonstrated an overexpression of *mdr1* in MQ resistant *P. berghei* lines, the gene associated with MQ resistance in *P. falciparum* and *P. chabaudi* (Cravo *et al.,* 2003). Recently, two mutations in a novel gene deubiquitinating enzyme 1 (*ubp1*) were associated with artesunate (ASN) and CQ resistance in *P. chabaudi* (Hunt *et al.,* 2007). Although *ubp1* remains to be confirmed as a gene involved in ASN or CQ resistance in *P. falciparum*, several studies have focused on this gene as a possible ASN resistance marker (Chavchich *et al.,* 2010; Hunt *et al.,* 2007; Rodrigues *et al.,* 2010). In addition, the *mdr-2* gene was recently mapped as a new resistance marker for sulfadoxine–pyrimethamine resistance in *P. chabaudi* (Martinelli *et al.,* 2011).

In this study, we examined the resistance mechanisms to PQ and LM using the malaria parasite that infects rodents, *P. berghei* ANKA as a surrogate of the human parasite, *Plasmodium falciparum.* We previously selected stable PQ and LM resistant *P. berghei* ANKA lines through continuous drug pressure (Kiboi *et al.*, 2009), further phenotypic analysis established that LM and PQ resistant lines were also resistant to mechanistically and chemically related and unrelated drugs such as DHA, CQ, MQ and primaquine (PMQ), thus are multidrug resistant phenotypes (Kiboi *et al.,* 2009; Langat *et al.,* 2012).

This study is based on two assumptions; first, the mode of PQ and LM action is similar to that of CQ and they also share mechanisms of resistance, therefore the orthologs of *Pfcrt* and *Pfmdr1* in *P. berghei* may also carry mutations that confer PQ and LM resistance. Secondly,

acquisition of PQ and LM resistance is augmented by increased transcript of modulatory and compensatory genes associated with quinoline drug transporters. We first cloned the resistant lines and interogated for sequence variation in *Pbmdr1* and *Pb*crt genes by PCR amplification and sequencing. We then investigated changes in expression of resistance compensating and modulating genes by measuring the relative amounts of mRNA of *Pbvp2, Pbvcx1*, *Pbggcs* and glutathione-S transferase (*gst*) in the resistant clones and their sensitive progenitors. Finally, to assess whether changes in *gst* and *ggcs* transcript levels are concomitant with changes in gene product, we measured GSH levels and activities of glutathione metabolism enzymes; glutathione-s-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR).

2 Material and Methods

2.1 Parasites, Host and Compounds

Two drug sensitive parasite lines *of P. berghei* ANKA, denoted PQ sensitive (PQ^S) (MRA-865, MR4, ATCC® Manassas, Virginia) and LM sensitive (LM^S) (MRA-868, MR4, ATCC® Manassas, Virginia) were used as reference parental lines. Stable PQ resistant (PQ^r) and LM resistant (LM^r) *P. berghei* ANKA previously submitted to drug selection pressure were used (Kiboi *et al.,* 2009).

Male Swiss albino mice weighing 20±2g out-bred at KEMRI, Animal house Nairobi, were used for the study. The animals were housed in the animal house in standard polypropylene (hard plastic) cages and fed on commercial rodent food and water *ad libitum*. PQ or LM were freshly prepared by dissolving it in a solvent consisting of 70% Tween-80 (d=1.08g/ml) and 30% ethanol (d=0.81g/ml) and subsequently diluted 10 fold with double distilled water.

2.2 Dilution cloning of resistant parasite

To generate genetically homogenous resistant parasites, three different generations from PQ^r and three generations from LM^r parasite lines (Table 1) were dilution cloned based on the protocol by Janse *et al.* (2004). Briefly, a mouse with parasitaemia between 0.5 and 1% was selected as a donor mouse. 5μl of infected blood was collected from the tail 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 diluted blood. The cell suspension was then diluted further with 1×PBS to an estimated final concentration of 0.5 parasites/0.2ml PBS. Twenty mice were intravenously injected each with 0.2ml/mouse of infected blood. Cloning was deemed successful when 20-50% of the inoculated mice became positive and showed a parasitaemia of between 0.3-1 percent at day 8 post infection.

2.3 Determination of GSH level and activity profiles of GST, GPx and GR in parasites

The infected erythrocytes were obtained from mouse whole blood, which was collected by cardiac puncture into 50μl of heparin. Erythrocytes were separated from plasma by centrifugation at 600g for 10 minutes, washed two times with three volumes of PBS and counted. For measurement of GSH levels and GST, GPx and GR activities in the isolated parasites, infected erythrocytes were lysed using ammonium chloride based on the protocol

by Martin *et al.* (1971). Infected blood was diluted to a suspension of 10^9parasites/ml. The GSH levels and the activities of GST, GPx and GR in isolated parasites were measured by enzyme kinetics. The rate of increase of the reaction product or decrease of the substrate is directly proportional to the enzyme activity in the sample. GSH was determined in the isolated parasite using glutathione assay kit (Sigma-Aldrich, Saint Louis, Missouri). Briefly, 50μl aliquot of isolated parasites was added to 200μl of 5% Sulfosalisylic acid solution. The solution was voltexed, left on ice for 15 minutes and then centrifuged at 10,000 *x g* for 10 minutes. The supernatant was measured for calculation of glutathione levels. The GST, GR and GPx activities were measured using commercial kits (Sigma-Aldrich, Saint Louis, Missouri). Briefly, the isolated parasites were first subjected to five freeze-thaw cycles (Meierjohann *et al.*, 2002). After centrifugation of the suspension for 15 minutes at 10,000 *x g,* the supernatant was collected for determination of GST, GR and GPx activities.

2.4 Drug sensitivity Profiles Tests

To assess the resistance profile of individual clones generated by dilution cloning, the fastest growing clone in each generation (27^{th}, 40^{th} and 60^{th} for PQ^r) and (40^{th}, 48^{th} and 60^{th} for LM^r) was selected and evaluated for its response to PQ or LM in the 4-Day Suppressive Protocol as per Fidock *et al.*, (2004). Briefly for each clone selected, mice were infected intraperitoneally with 1×10^6 parasites/mouse. Oral treatment of drug was initiated on day 0, (4 h post-infection) and continued for four days, (24, 48 and 72 h post-infection). Parasite density was estimated microscopically (×100) on day 4 (96 h) post parasite inoculation using thin blood films made from tail blood. Parasite growth was then followed for at least 15 days post-infection to assess the recrudescence of the parasites after cessation of drug treatment.

2.5 DNA extraction, PCR and Sequencing

Parasite DNA was extracted by first removing mouse white blood cells through successive filtration of infected blood using Plasmodipur filters, (Euro-Diagnostica). Briefly, packed cells were re-suspended in 5 volumes of cold (4ºC) 1× erythrocyte Lysis buffer (ammonium chloride solution) for 15-30 minutes, before spinning at 2000rpm for 8 minutes to obtain the parasite pellet. Genomic DNA was extracted using the commercially available QiAamp DNA Blood Kit (Qiagen).

To amplify *Pbcrt* (PBANKA_121950) and *Pbmdr1* (PBANKA_123780), 1μl of genomic DNA from each sample was used as template in 25μl PCR reactions. The other reagents $MgCl_2$, dNTPs, forward and reverse primers, Dream Taq Polymerase (Thermo-Scientific) and cycling conditions were optimized accordingly as shown in Table 2a. PCR products were analysed in 1% agarose gel, purified using GeneJet™ PCR purification kit (Thermo scientific™) and then sequenced based in BigDye v3.1 using a 3730xlsequencer. The primers used for sequencing the genes are shown in Table 2b and 2c. Contigs were assembled using Lasergene 11 Core Suite, the DNA sequences and the predicted amino acid sequences were analysed using CLUSTAL W available in EBI website (www.ebi.ac.uk) and searched by BLAST© software available at the NCBI Website and PlasmoDB version 11.0 (EuPathDB, 2013).

2.6 Analysis of *Pbmdr1, Pbvp2, Pbggcs, Pbgst* and *Pbvcx1* transcription

To quantify mRNA transcripts of *Pbmdr1*, *Pbvp2* (PBANKA_132050), *Pbvcx1* (PBANKA_010230), *Pbggcs* (PBANKA_081980) and *Pbgst* (PBANKA_102390) genes, fresh parasite pellets were prepared and total RNA was extracted from at least 1×10^6 parasites based on High Pure RNA extraction kit (Roche™). The RNA was immediately used for cDNA synthesis. The first-strand cDNA synthesis was performed in a final volume of 20µl using Transcriptor First Strand cDNA synthesis kit (Roche™) and oligo-dT as primers, briefly 5µg of total RNA, 1µl of oligo-dT (2.5µM) and water were mixed with 4µl of Transcriptor Reverse Transcriptase buffer (5×), 0.5 µl RNase Inhibitor (40U/µl), 2 µl of dNTPs (10mM) and 0.5 µl of Transcriptor Reverse Transcriptase (20U/µl) was added. The RT reaction mix was incubated at 50°C for 60 min, then at 85°C for 5 min and finally chilled on ice. The cDNA was used as template for RT-PCR assays or stored at -15 to -20°C for longer period.

2.7 Quantitative RT-PCR Assays

Real-Time PCR assays were designed to evaluate the levels of *Pbmdr1*, *Pbvp2*, *Pbvcx1*, *Pbggcs* and *Pbgst* RNA transcripts relative to those of *Pbβ-actin* (PBANKA_145930), a housekeeping gene. Oligonucleotides and TaqMan™ probes (Table 3) were designed to run PCR reactions for the genes in the same plate (using similar cycling conditions). cDNA samples, primers and probes were added to FastStart Essential DNA probes Master (Master (Roche™) according to the manufacturer's instructions. The PCR amplification was done as per the following conditions: 95°C for 4 minutes; followed by denaturation at 95°C for 15sec and annealing/extension at 58°C for 30 sec, for 45 cycles

2.8 Statistical Analysis

The means of expression levels of each gene from three independent experiments and from triplicate assays obtained from LM^r and PQ^r were compared to LM^S and PQ^S respectively using Student's t-test; P value was set at 0.05. The relative expression level results were normalized using Pbβ-actin as the housekeeping gene using the formula $2^{-\Delta\Delta C_T}$ based on Livak and Schmittgen, 2001. The means of GSH levels, GST, GPx and GR activities obtained from five mice for each of the parasites clone were also compared using a Student's *t* test and p values less than 0.05 were considered significant.

3.0 Results

3.1 Cloning by limiting dilution of resistance phenotypes

We selected the fastest growing clone from each generation for subsequent drug sensitivity profile. The PQ^r selected clone from the 60^{th} generation was termed PQ^R60c1; the clone from 27^{th} generation, PQ^R27c1 and the clone from 40^{th} generation were termed $^{mp}PQ^Rc1$. For the LM^r parasites, the selected clone from the 60^{th} generation was termed LM^R60c1; the clone from 48^{th} generation, LM^R48c1, while the clone from the 40^{th} generation was termed $^{mp}LM^Rc1$.

We then submitted PQ^R60c1, $^{mp}PQ^Rc1$, PQ^R27c1 and PQ^S clones to PQ treatment while LM^R60c1, $^{mp}LM^Rc1$, LM^R48c1 and LM^S clones were treated with LM. Piperaquine and

LM were evaluated at 50mg/kg administered for 4 successive days in groups of five mice along the PQ^S or LM^S which received a lower dose of 12.5mg/kg for four consecutive days. As expected all eight (8) clones (6 resistant and 2 wild-type sensitive) grew well in mice in the absence of drug treatment with peak parasitaemia reaching 19.43% (PQ^S), 13.63 % (LM^S) while for resistant clones, 15.06% (PQ^R60c1), 9.70% (PQ^R27c1), 9.05% ($^{mp}PQ^R$c1), 14.61% LM^R60c1, 9.98% (LM^R48c1), 7.86% ($^{mp}LM^R$c1) Figure 1a and 1b.

Under PQ and LM treatment (12.5mg/kg), no parasites were detected in mice infected with PQ^S or LM^S parasite over the 15 day post infection (p.i.) follow up period. In the presence of drug (50mg/kg), resistant clones grew well with parasitaemia reaching 16.68 % (PQ^R60c1), 8.43 % (PQ^R27c1), 4.86 % ($^{mp}PQ^R$c1), 14.28% (LM^R60c1), 2.61% (LM^R48c1), 4.30% ($^{mp}LM^R$c1) 7 days p.i, Figure 1c and 1d. We therefore concluded that PQ^R60c1, $^{mp}PQ^R$c1, PQ^R27c1, $^{mp}LM^R$c1, LM^R48c1 and LM^R60c1 were successfully cloned. PQ^R60c1 and LM^R60c1 clones were selected for interrogation of point mutation and differential expression assay due to their high resistance level.

3.2 Evaluation of sequence variation of *Pbcrt and Pbmdr1* genes

We amplified and sequenced the whole coding region of *Pbcrt* and *Pbmdr1* in PQ^R60c1 and LM^R60c1 and their parental drug sensitive parasite, PQ^S and LM^S, respectively. The results showed the nucleotide and translated protein sequences of these genes are the same in all tested lines (drug-sensitive and drug resistant strains) (supplementary material).

3.3 Assessment of mRNA expression by quantitative Real-time PCR

To gain an insight into the potential modulation and compensatory mechanisms, we measured mRNA transcript profiles of *Pbmdr1*, *Pbvp2*, *Pbcvx1 Pbggcs* and *Pbgst*. As shown in Figure 2a, no difference in *mdr1* expression was observed between PQ^S and PQ^R60c1, with level of expression of 0.9 in PQ^R60c1 and 1.0 in PQ^s ($p=0.99$); while values for LM^S and LM^R60c1 was 1.09 and 1.0, respectively ($p=0.82$). Thus, there was no change in expression level in both LM^r and PQ^R60c1 clone. We further assessed V-type H^+ pumping pyrophosphatase, a transporter involved in regulation H^+ molecules in protozoa and plants cells (McIntosh *et al.*, 2001). The transcript level of *Pbvp2* in LM^R60c1 remained unchanged (1.10 folds, $p=0.81$). Interestingly in PQ^R60c1 clone, *Pbvp2* levels were 4 times higher compared to PQ^s ($p<0.0001$) Figure 2b. We also evaluated the transcript intensities of enzymes associated with glutathione metabolism, the levels of *Pbggcs* were significantly elevated in both PQ^R60c1 and LM^R60c1 parasites (Figure 2c). Surprisingly, the level of *Pbggcs* in LM^R60c1 was 13-fold higher compared to the LM^S ($p<0.0001$). In the PQ^R60c1 clone the level was 3 times higher ($p<0.0001$). Remarkably, the LM^R60c1 parasites showed a 26-fold change in *Pbgst* of higher levels than in the LM^S parasites ($p<0.0001$) (Figure 2d). The PQ^R60c1 contained *Pbgst* transcripts that were 4 times higher relative to sensitive parasite ($p<0.0001$). Finally, we measured the transcript amount of Ca^{2+}/H^+ mobilizing transporter, *cvx1*, and the data showed that in comparison with sensitive parasites, the difference in levels were statistically significant 1.34-fold ($p<0.002$) and 1.43-fold ($p<0.001$) in PQ^R60c1 and LM^R60c1, respectively (Figure 2e).

3.4 Assessment of GSH levels and GST, GPx and GR activities

To determine whether the increase in transcription of the *Pbggcs* and *Pbgst* was concomitant with increase in its product, we measured the relative amount of GSH and activity of GST enzyme in the different parasite clones using enzyme kinetics. We observed a significant four-fold increase in GSH levels in LM^R60c1 ($p<0.0001$) and GSH levels were three times elevated in PQ^R60c1 ($p<0.0001$) parasites (Figure 3a). Similarly the activity of GST was significantly higher in LM^R60c1 ($p<0.0001$) and PQ^r ($p<0.0001$) with a three and two- fold rise compared to sensitive progenitors, LM^s and PQ^s, respectively. Finally, we assayed the activity profiles of GR and GPx. Interestingly, GR activities in LM^R60c1 and PQ^R60c1 were two times lower compared to LM^s ($p<0.0001$) and PQ^s ($p<0.0001$) parasites, respectively. There was however no significant difference in GPx activities between LM^s and LM^R60c1 ($p<0.82$) nor PQ^s and PQ^R60c1 ($p<0.99$).

4.0 Discussion

In this study we have shown that in both LM^r and PQ^r phenotypes, *Pbcrt* is not linked with PQ, LM or CQ cross-resistance in *P. berghei* ANKA. We thus suggest the selection of PQ and LM resistance is nonspecific and not related with predicted mechanism of CQ action. Second, we suggest that CQ cross-resistance observed in LM^r and PQ^r is also nonspecific, mediated by mechanisms independent of the *crt* gene, therefore novel mutations may exist that associate with PQ, LM and also CQ cross-resistance. Indeed, studies show that PQ and LM remain active against parasites possessing the key mutation in *Pfcrt* K76T (Pascual *et al.*, 2013), indicating that PQ and LM resistance may evolve independently of mutations in the *Pfcrt* gene.

As previously reported, PQ^r and LM^r are cross-resistant to chemically related and unrelated drugs, such as DHA, PMQ, MQ, CQ and amodiaquine (AQ) (Kiboi *et al.,* 2009; Langat *et al.,* 2012), thus are true multidrug resistant phenotypes and the *Pbmdr1* polymorphisms may be mediating PQ and LM responses and also their cross-resistance profiles. The *mdr1* gene controls drug response via two mechanisms; first by the acquisition of mutations and second by variation of copy number and expression level in drug resistant *P. falciparum* (Price *et al.,* 2004; Mwai *et al.,* 2012; Valderramos & Fidock, 2006). However, our data does not link *Pbmdr1* polymorphisms with control of either PQ^r or LM^r phenotypes, similarly; we did not associate differential transcription of *Pbmdr1* with either of the resistant phenotypes. The increase in *mdr1* transcripts controls resistance to multiple drugs in *P. chabaudi*, *P. yoelii* and in *P. falciparum* (Cravo *et al.,* 2003; Ferrer-Rodriguez *et al.*, 2004; Chavchich *et al.,* 2010; Rodrígues *et al.,* 2010). We thus suggests that *mdr1* does not control PQ or LM responses in *P. berghei* and that the multidrug resistance phenotypes observed in both PQ^r and LM^r is operated by mechanisms independent of *mdr1* gene. Therefore, other novel mechanisms may control the multidrug resistance profiles observed in PQ^r and LM^r phenotypes.

The present work supports association of *Pbggcs* with the control of both PQ^r and LM^r phenotypes. The ortholog of this gene in *P. falciparum* is closely associated with mediating and modulating AQ and CQ resistance through a tightly controlled enzymatic reaction (Ginsburg *et al.,* 1998; Patzewitz *et al.,* 2013). Gamma glutamyl cysteine synthetase is the rate limiting enzyme in GSH synthesis (Tew, 1994), therefore elevated transcription would

be accompanied by high GSH concentration. Studies have shown that high GSH concentration is directly associated with increased detoxification of the CQ via GSH-drug binding mechanisms (Ginsburg *et al.,* 1999) and CQ competitively inhibits GSH mediated detoxification of ferriprotoporphyrin (FP) (Ginsburg & Golenser, 2003). Thus, if the mechanism of LM and PQ resistance is similar to that of CQ, then two possible mechanisms may exist; first, high GSH in PQ^r and LM^r phenotypes may bind LM or PQ increasing the effective dosage and second, high GSH concentration may competitively reduce the level of free FP for LM or PQ binding thus protecting the parasite from the pro-oxidant activity of the PQ- or LM-FP complex. We have further demonstrated that high GST activity is associated with LM or PQ resistance in *P. berghei*. The GST enzyme possesses peroxidase activity (Perez-Rosado *et al.,* 2002), consequently high GST activity may be increasing GSH-LM or PQ conjugates, thus reducing drug concentration available for action.

Elevated GST activity and high GSH concentration are associated with CQ and MQ resistance in *P. berghei* and *P. chabaudi* (Perez-Rosado *et al.,* 2002; He *et al.,* 2009). As we alluded to earlier, PQ^r and LM^r phenotypes showed cross-resistance to CQ, MQ, DHA and PMQ (Kiboi *et al.,* 2009). We thus hold the opinion that increase in GST activity and high GSH levels may be one of the mechanisms mediating the cross-resistance profiles possibly through drug-GSH binding and competitive FP-GSH conjugates. It has been reported that intracellular GSH level is sustained by *de novo* biosynthesis and reduction of GSSG (Ginsburg & Golenser, 2003). Our data thus suggest that both PQ^s and LM^s parasites preserve their capacity to maintain GSH-GSSG redox status through the action of GR. This redox status in PQ^r and LM^r phenotypes however seems to be maintained by the *de novo* synthesis of GSH, possibly due to high requirements for removal of the drug and FP. Our postulations do not mean that high GSH concentration and GST activity are the sole mechanisms of LM and PQ resistance. Indeed, GSH mediated mechanisms may be a downstream process that modulates drug response in PQ^r and LM^r phenotypes, thus unknown novel gene may exist that directly mediate PQ and LM resistance. Our data however does not support resistance mechanisms that may be GSH independent.

We have also shown that increased expression levels of the *vp2* gene is associated with PQ resistance, but not with LM resistance in *P. berghei* ANKA. This gene is involved in the transport and regulation of cytoplasm pH (Jiang *et al.,* 2008), recently it was shown that the *vp2* gene was differentially expressed in LM resistant parasites (Mwai *et al.,* 2012). The PQ^r parasites are also resistant to LM (Kiboi *et al.,* 2009). We thus do not rule out its link with LM cross-resistance in PQ^r phenotypes, meaning that the increase in *vp2* transcriptional levels may be selected via a different selection procedure. This may also suggest that *vp2* may not be the key mechanism for adapting LM resistant phenotypes but perhaps serves to synergize resistance or compensate for the acquisition of deleterious mutations in resistant phenotypes. Increase in *Pbvcx1* transcript levels was associated with both PQ and LM resistance. Recently, the putative drug transporter, *Pfvcx1* in response to K76T mutation in *Pfcrt* was linked with CQ resistance or to the modulation of CQ resistance (Jiang *et al.,* 2008). Assuming *Pbvcx1* plays a similar role of modulating resistance in our PQ^r and LM^r parasites, then two scenarios exist, first is that increased expression of *vcx1* is independent of the *crt* mutation in *P. berghei* ANKA and second is that novel mutations may exist that harbingers the differential expression of *Pbvcx1.*

In conclusion, we have identified for the first time the association of *vp2* with PQ resistance and *ggcs*, *gst*, and *vcx1* increased transcript levels with PQ and LM resistance in *P. berghei* ANKA. These compensatory or modulatory genes are thought to evolve in response to polymorphisms in *Pfcrt* and *Pfmdr1* genes occurring in CQ (Jiang *et al.,* 2008) and LM resistant phenotypes (Mwai *et al.,* 2012). However, our PQ^r and LM^r phenotypes possess no polymorphism in ortholog *Pbcrt* and *Pbmdr1*. Therefore, these compensatory or modulatory mechanisms may be controlled by unknown causal gene variants. This is because the genetic background in *P. berghei* and *P. falciparum* are different thus the resistance phenotype in each species is probably mediated by different molecular markers. However, if the same mechanisms prevail in *P. falciparum* in the field, this study demonstrates that the analysis of rodent malaria parasites may provide biological insights on drug response profiles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the Director of the Kenya Medical Research Institute for permission to publish this work. This study was supported by UNICEF/UNDP/WORLDBANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR) (A90201) and the Kenyan Government through the National Commission for Science and Technology (NCST/5/003/3rd CALL PhD/165).

References

Biagini GA, O'Neill P, Nzila A, Ward S, Bray PG. Antimalarial chemotherapy young guns or back to the future. Trends in Parasitology. 2003; 19:479–487. [PubMed: 14580958]

Carlton JM-R, Hayton K, Cravo PV, Walliker D. Of mice and malaria mutants: unraveling the genetics of drug resistance using rodent malaria models. Trends in Parasitology. 2001; 17:236–241. [PubMed: 11323308]

Chavchich M, Gerena L, Peters J, Chen N, Cheng Q, Kyle DE. Role of *pfmdr1* Amplification and Expression in Induction of Resistance to Artemisinin Derivatives in *Plasmodium falciparum*. Antimicrobial Agents and Chemotherapy. 2010; 54:2455–2464. [PubMed: 20350946]

Cooper RA, Hartwig CL, Ferdig M. *pfcrt* is more than the *Plasmodium falciparum* chloroquine resistance gene: a functional and evolutionary perspective. Acta Tropica. 2005; 94:170–180. [PubMed: 15866507]

Cravo P, Carlton J, Hunt P, Bisoni L, Padua R, Walliker D. Genetics of mefloquine resistance in the rodent malaria parasite *Plasmodium chabaudi*. Antimicrobial Agents and Chemotherapy. 2003; 47:709–718. [PubMed: 12543682]

Davis T, Hung T, Sim I, Karunajeewa H, Ilett K. Piperaquine: a resurgent antimalarial drug. Drugs. 2005; 65:75–87.

Dubois LV, Platel DF, Pauly G, Tribouley-Duret J. *Plasmodium berghei*: Implication of Intracellular Glutathione and its Related Enzyme in Chloroquine Resistance in Vivo. Experimental Parasitology. 1995; 81:117–124. [PubMed: 7628559]

Eastman RT, Dharia NV, Winzeler EA, Fidock DA. Piperaquine Resistance Is Associated with a Copy Number Variation on Chromosome 5 in Drug-Pressured *Plasmodium falciparum* Parasite. Antimicrobial Agents and Chemotherapy. 2011; 55:3908–3916. [PubMed: 21576453]

EuPathDB. PlasmoDB Version 9.3. 2013. Mar 11, Retrieved March 13, 2013, from *Plasmodium* Genomics Resource: http://plasmodb.org/plasmo/

Ferrer-Rodríguez I, Perez-Rosado J, Gervais GW, Peters W, Robinson BL, Serrano AE. *Plasmodium yoelii*: Identification and partial characterization of an mdr-1 gene in artemisinin-resistant line. Journal of Parasitology. 2004; 90:152–160. [PubMed: 15040683]

Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig M, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Molecular Cell. 2000; 6:861–871. [PubMed: 11090624]

Fidock DA, Rosenthal P, Croft SL, Brun R, Nwaka S. Antimalarial Drug Discovery: Efficacy Model for Compound Screening. Nature Reviews: Drug Discovery. 2004; 3:509–520. [PubMed: 15173840]

Gervais G, Trujillo K, Robinson B, Peters W, Serrano A. *Plasmodium berghei*: identification of an mdr-like gene associated with drug resistance. Experimental Parasitology. 1999; 91:86–92. [PubMed: 9920046]

Ginsburg H, Golenser J. Glutathione is involved in the antimalarial action of chloroquine and its modulation affects drug sensitivity of human and murine species of *Plasmodium*. Redox Report. 2003; 8:276–279. [PubMed: 14962364]

Ginsburg H, Famin O, Zhang J, Krugliak M. Inhibition of Glutathione-dependent Degradation of Heme By Chloroquine and Amodiaquine as a Possible Basis for Their Antimalarial Mode of Action. Biochemical Pharmacology. 1998; 56:1305–1313. [PubMed: 9825729]

Ginsburg H, Ward SA, Bray P. An Integrated Model of Chloroquine Action. Parasitology Today. 1999; 15:357–360. [PubMed: 10461161]

He ZX, Chen LL, You JL, Qin L, Chen XP. Antiretroviral Protease Inhibitors Potentiate Chloroquine Antimalarial Activity in Malaria Parasite by Regulating Intracellular Glutathione Metabolism. Experimental Parasitology. 2009; 123:122–127. [PubMed: 19538959]

Hunt P, Afonso A, Creasey A, Culleton R, Sidhu AB, Logan J, et al. Gene encoding a deubiquitinating enzyme is mutated in artesunate and chloroquine-resistant rodent malaria parasites. Molecular Microbiology. 2007; 65:27–40. [PubMed: 17581118]

Hunt P, Cravo P, Donleavy P, Carlton J-R, Walliker D. Chloroquine resistance in *Plasmodium chabaudi*: are chloroquine-resistance transporter (*crt*) and multi-drug resistance (*mdr1*) orthologues involved. Molecular & Biochemical Parasitology. 2004b; 133:27–35. [PubMed: 14668009]

Hunt P, Martinelli A, Fawcett R, Carlton J, Carter R, Walliker D. Gene synteny and chloroquine resistance in *Plasmodium chabaudi*. Molecular and Biochemical Parasitology. 2004a; 136:157–164. [PubMed: 15478795]

Hunt P, Martinelli A, Modrzynska K, Borges S, Creasey A, Rodrigues L, et al. Experimental evolution, genetic analysis and genome re-sequencing reveal the mutation conferring artemisinin resistance in isogenic lineage of malaria parasites. BMC Genomics. 2010; 11:499. [PubMed: 20846421]

Janse C, Ramesar J, Waters A. *Plasmodium berghei*: General parasitological methods. Leiden University Medical Center, Parasitology. Leaiden Malaria Research Group; Leiden: 2004.

Jiang H, Patel J, Yi M, Mu J, Ding J, Stephens R, et al. Genome Wide Compensatory Changes Accompany Drug-Selected Mutations in the *Plasmodium falciparum crt* Gene. PLoS One. 2008; 3:e2484. [PubMed: 18575593]

Kiboi DM, Irungu BN, Langat B, Wittlin S, Brun R, Chollet J, et al. *Plasmodium berghei* ANKA: Selection of resistance to piperaquine and lumefantrine in a mouse model. Experimental Parasitology. 2009; 122:196–202. [PubMed: 19318094]

Langat B, Kiboi D, Irungu B, Kimoloi S, Nyambati V, Rukunga G. Lumefantrine-resistant and Piperaquine resistant *Plasmodium berghei* show cross resistance to Primaquine but not to Atovaquone. African Journal of Pharmacology and Therapeutics. 2012; 1:35–40.

Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT method. Methods. 2001; 25:402–408. [PubMed: 11846609]

Martin W, Finerty J, Rosenthal A. Isolation of *Plasmodium berghei* (malaria) parasites by ammonium chloride lysis of infected erythrocytes. Nature New Biology. 1971; 233:260–261. [PubMed: 5288121]

Martinelli A, Henriques G, Cravo P, Hunt P. Whole genome re-sequencing identifies a mutation in an ABC transporter (*mdr2*) in a *Plasmodium chabaudi* clone with altered susceptibility to antifolate drugs. International Journal for Parasitology. 2011; 41:165–171. [PubMed: 20858498]

McIntosh MT, Drozdowicz YM, Laroiya K, Rea PA. Two classes of plant-like vacuolar-type H+-pyrophosphatases in malaria parasites. Molecular and Biochemical Parasitology. 2001; 114:183–195. [PubMed: 11378198]

Meierjohann S, Walter RD, Muller S. Regulation of intracellular glutathione levels in erythrocytes infected with chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. Biochemical Journal. 2002; 368:761–768. [PubMed: 12225291]

Mwai L, Diriye A, Masseno V, Muriithi S, Feltwell T, Musyoki J, et al. Genome Wide Adaptations of *Plasmodium falciparum* in Response to Lumefantrine Selective Drug Pressure. PLoS One. 2012; 7:e31623. [PubMed: 22384044]

Mwai L, Kiara SM, Abdirahaman A, Pole L, Rippert A, Diriye A, et al. In vitro activities of piperaquine, lumefantrine and dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in pfcrt and pfmdr-1. Antimicrobial Agents and Chemotherapy. 2009a:5069–5073. [PubMed: 19770282]

Mwai L, Ochong E, Abdirahman A, Kiara SM, Ward S, Kokwaro G, et al. Chloroquine resistance before and after its withdrawal in Kenya. Malaria Journal. 2009b; 8:106. [PubMed: 19450282]

Nzila A, Mwai L. In vitro selection of *Plasmodium falciparum* drug resistant parasite lines. Journal of Antimicrobial Chemotherapy. 2010; 65:390–398. [PubMed: 20022938]

Ogutu B, Onyango K, Koskei N, Omondi E, Ongecha J, Otieno GA, et al. Efficacy and safety of artemether-lumefantrine and dihydroartemisinin-piperaquine in the treatment of uncomplicated *Plasmodium falciparum* malaria in Kenyan children aged less than five years: results of an open-label, randomized, single-centre study. Malaria Journal. 2014; 13:33. [PubMed: 24472156]

Pascual A, Madamet M, Bertaux L, Amalvict R, Benoit N, Travers D, et al. *In vitro* piperaquine susceptibility is not associated with the *Plasmodium falciparum* chloroquine resistance transporter gene. Malaria Journal. 2013; 12:431. [PubMed: 24274185]

Patzewitz E, Salcedo-Sora J, Wong E, Sethia S, Stocks P, Maughan SC, et al. Glutathione Transport: A New Role for *PfCRT* in Chloroquine Resistance. Antioxidants & Redox Signaling. 2013; 19:683–695. [PubMed: 23256874]

Perez-Rosado J, Gervais GW, Ferrer-Rodríguez I, Peters W, Serrano AE. *Plasmodium berghei*: analysis of the -glutamylcysteine synthetase gene in drug-resistant lines. Experimental Parasitology. 2002; 101:175–182. [PubMed: 12594957]

Price R, Uhlemann A, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet. 2004; 364:438–447. [PubMed: 15288742]

Raynes K. Bisquinoline antimalarials:their role in malaria chemotherapy. International Journal for Parasitology. 1999; 29:367–379. [PubMed: 10333319]

Reed MB, Saliba K, Caruana SR, Kirk K, Cowman AF. *Pgh1* modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature. 2000; 403:906–909. [PubMed: 10706290]

Robert A, Benoit-Vical F, Dechy-Cabaret O, Meunier B. From classical antimalarial drugs to new compounds based on the mechanism of action artemisinin. Pure and Applied Chemistry. 2001; 73:1173–1188.

Rodrigues LA, Henriques G, Borges ST, Hunt P, Sanchez CP, Martinelli A, et al. Experimental Evolution of Resistance to Artemisinin Combination Therapy Results in Amplification of the *mdr1* Gene in a Rodent Malaria Parasite. PLoS One. 2010; 5:e11593. [PubMed: 20657645]

Schlitzer M. Antimalarial drugs—what is in use and what is in the pipeline. Archiv der Pharmazie. 2008; 341:149–163. [PubMed: 18297679]

Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA. Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. Journal of Infectious Diseases. 2006; 194:528–535. [PubMed: 16845638]

Sisowath C, Ferreira P, Bustamante L, Dahlstrom S, Martensson A, Bjorkman A, et al. The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. Tropical Medicine and International Health. 2007; 12:736–742. [PubMed: 17550470]

Sisowath C, Petersen I, Veiga MI, Martensson A, Premji Z, Bjorkman A, et al. *In vivo* selection of *Plasmodium falciparum* parasites carrying the chloroquine susceptible *pfcrt* K76allele after treatment with artemether-lumefantrine in Africa. Journal of infectious Diseases. 2009; 5:750–757.

Sisowath C, Stromberg J, Martensson A, Msellem M, Obondo C, Bjorkman A, et al. *In Vivo* Selection of *Plasmodium falciparum pfmdr1* 86N Coding Alleles by Artemether-Lumefantrine (Coartem). The Journal of Infectious Diseases. 2005; 191:1014–1017. [PubMed: 15717281]

Tarning J. Piperaquine: Bioanalysis, drug metabolism and pharmacokinetics. Göteborg: Institute of Neuroscience and Physiology, Department of Pharmacology, the Sahlgrenska Academy at Göteborg University; 2007.

Tew K. Glutathione-associated Enzymes in Anticancer Drug Resistance. Cancer Research. 1994; 54:4313–4320. [PubMed: 8044778]

Valderramos S, Fidock DA. Transporters involved in resistance to antimalarial drugs. TRENDS in Pharmacological Sciences. 2006; 27:594–601. [PubMed: 16996622]

WHO. Guidelines for the treatment of malaria. 2nd Edition. Geneva: World Health Organization; 2010.

Europe PMC Funders Author Manuscripts

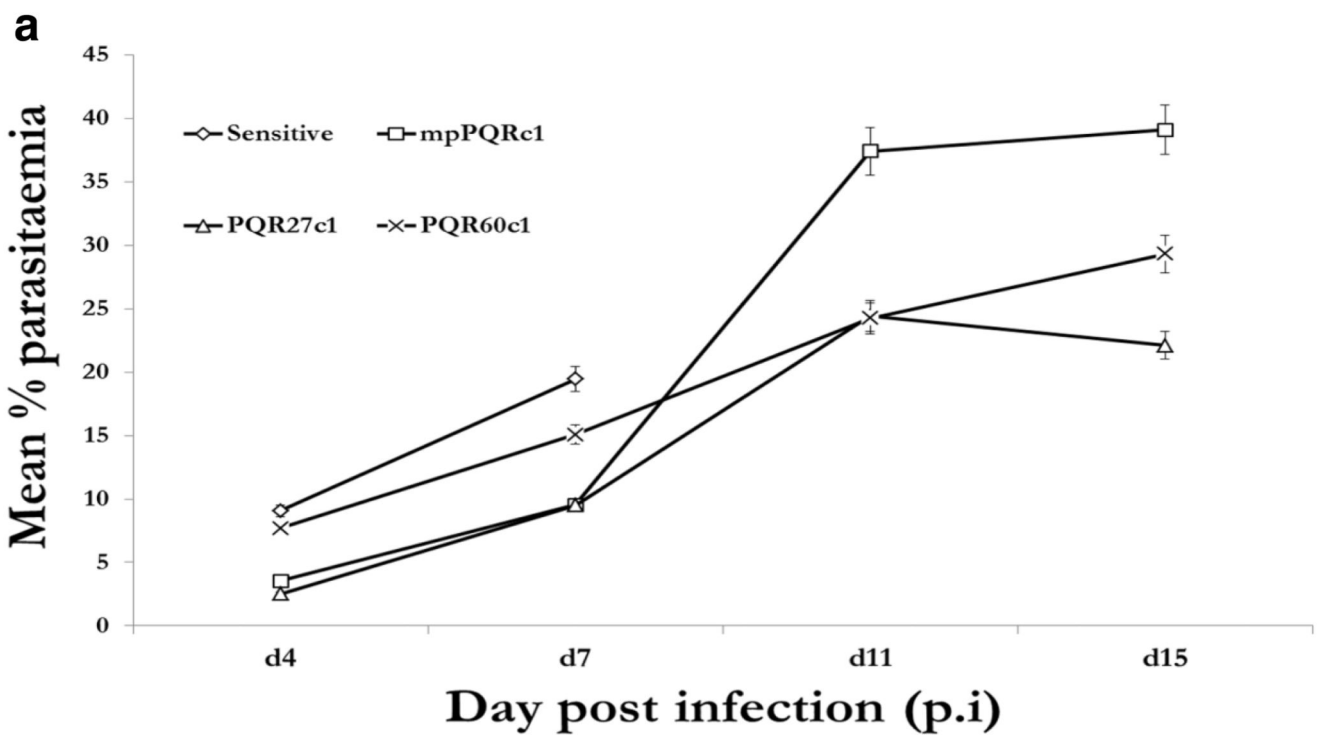

Figure 1a.

Growth profiles of the fastest growing clones obtained by dilution cloning piperaquine resistant parasite of 40th (mpPQRc1), 27th (PQR27c1) and 60th (PQR60c1) generation in absence of piperaquine in reference to the wild-type drug sensitive line. The data points obtained from an average of five mice per group.

Europe PMC Funders Author Manuscripts

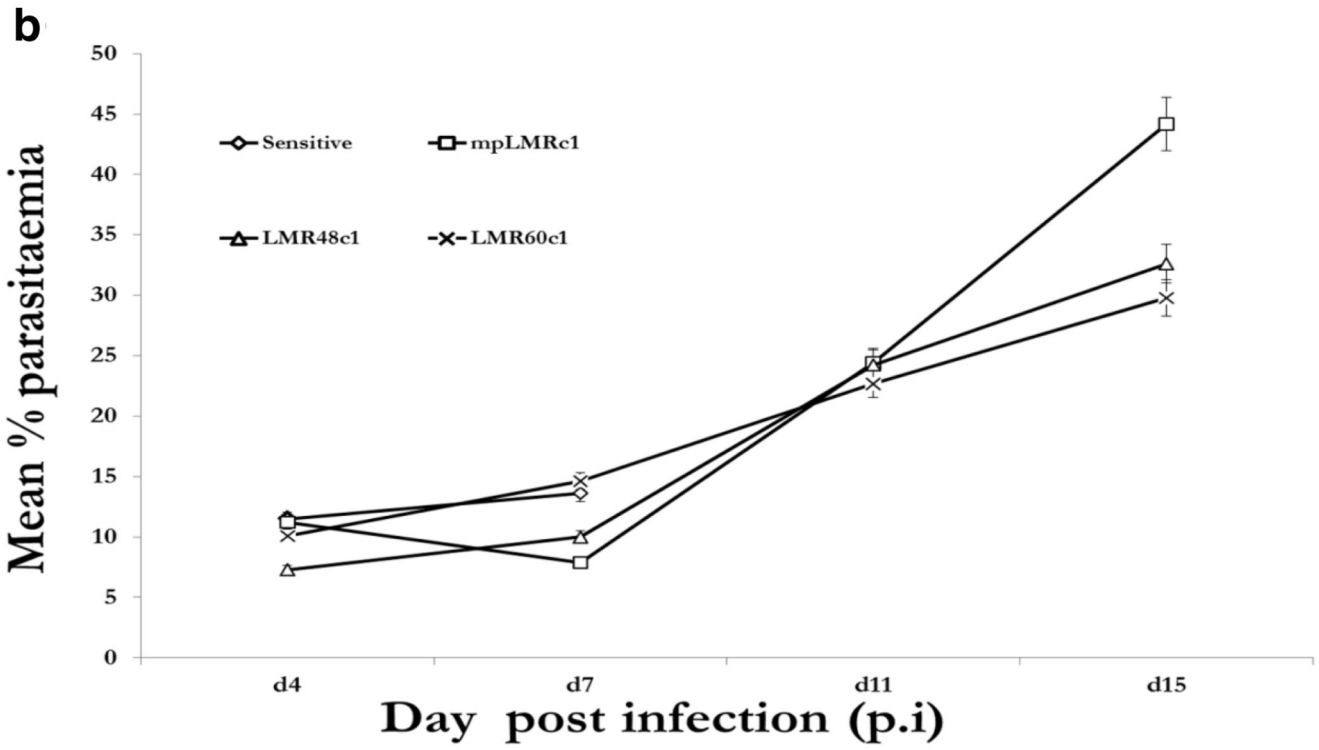

Figure 1b.

Growth profiles of the fastest growing clones obtained by dilution cloning lumefantrine resistant parasite of 40th (mpLMRc1), 48th (LMR48c1) and 60th (LMR60c1) generation in absence of lumefantrine in reference to the wild-type drug sensitive line. The data points obtained from an average of five mice per group

Europe PMC Funders Author Manuscripts

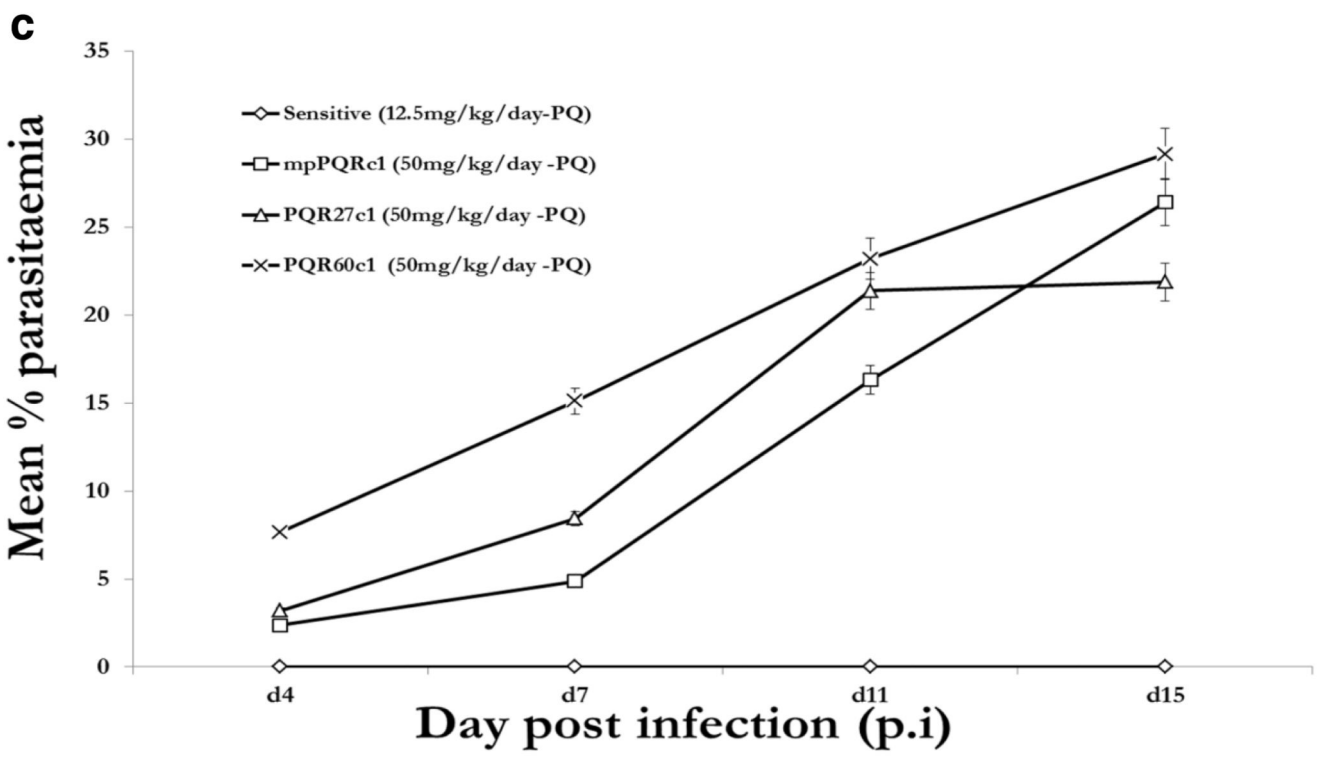

Figure 1c.

Activity profiles of piperaquine against selected dilution cloned piperaquine resistant parasites from 27th (PQR27c1), 40th (mpPQRc1) and 60th (PQR60c1) generation in reference to the wild-type drug sensitive line. The data point values were determined from an average of five mice per treatment group.

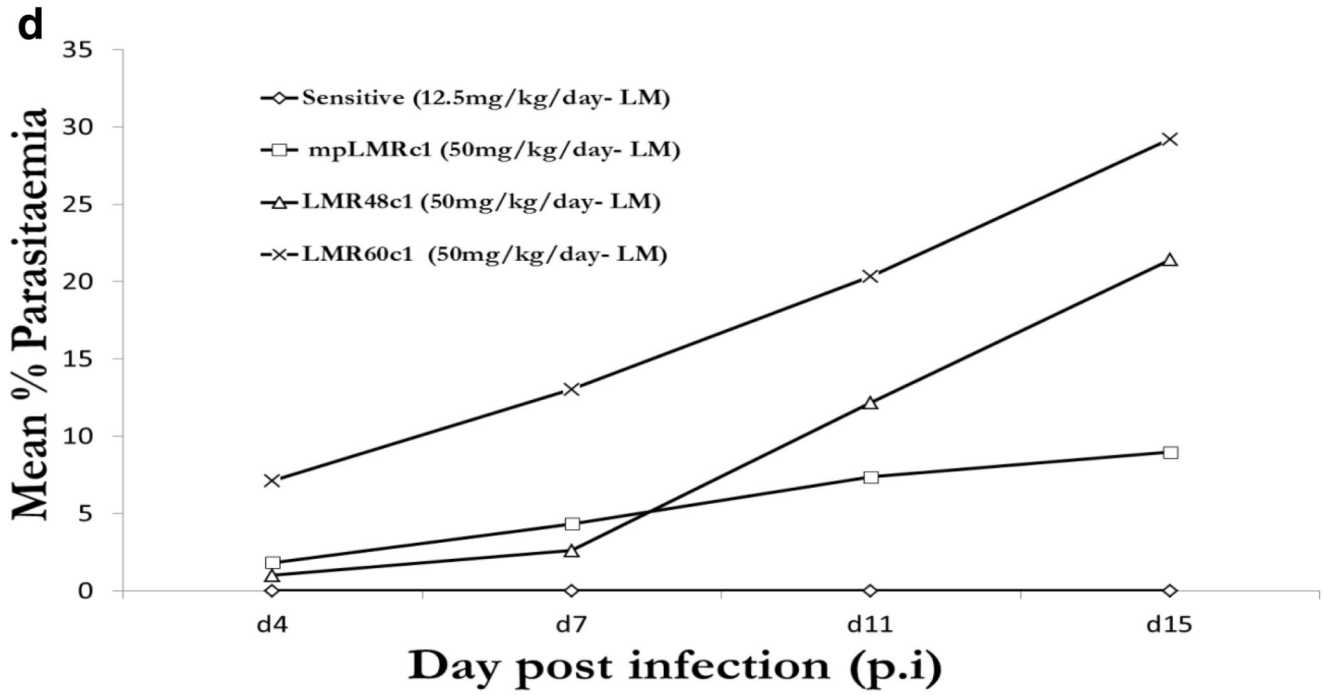

Figure 1d.

Activity profiles of lumefantrine against selected dilution cloned lumefantrine resistant parasites from 40th (mpLMRc1), 48th (LMR48c1) and 60th (LMR60c1) generation in reference to the wild-type drug sensitive line. The data point values were determined from an average of five mice per treatment group.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

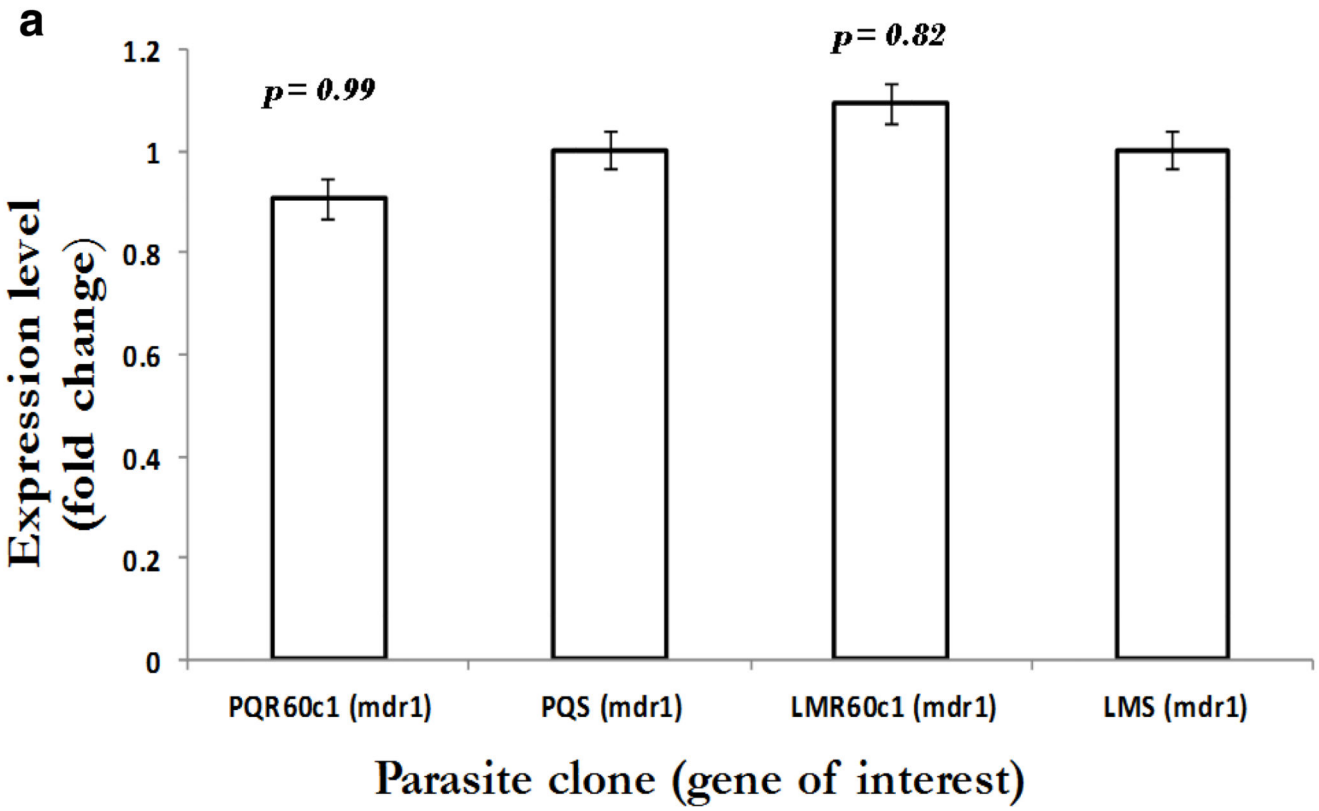

Figure 2a.

Expression profiles in multidrug resistance gene 1 (mdr1) as measured from cDNA amount derived from 5μg of total RNA isolated from piperaquine resistant (PQ^R60c1) and lumefantrine resistant (LM^R60c1) relative to their wild type drug sensitive parental clones piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The differential expression from a mean of three independent experiments were not significantly different for PQ^R60c1 (p= 0.99), for LM^R60c1 (p = 0.82) after student's t-test analysis with p-value set at 0.05.

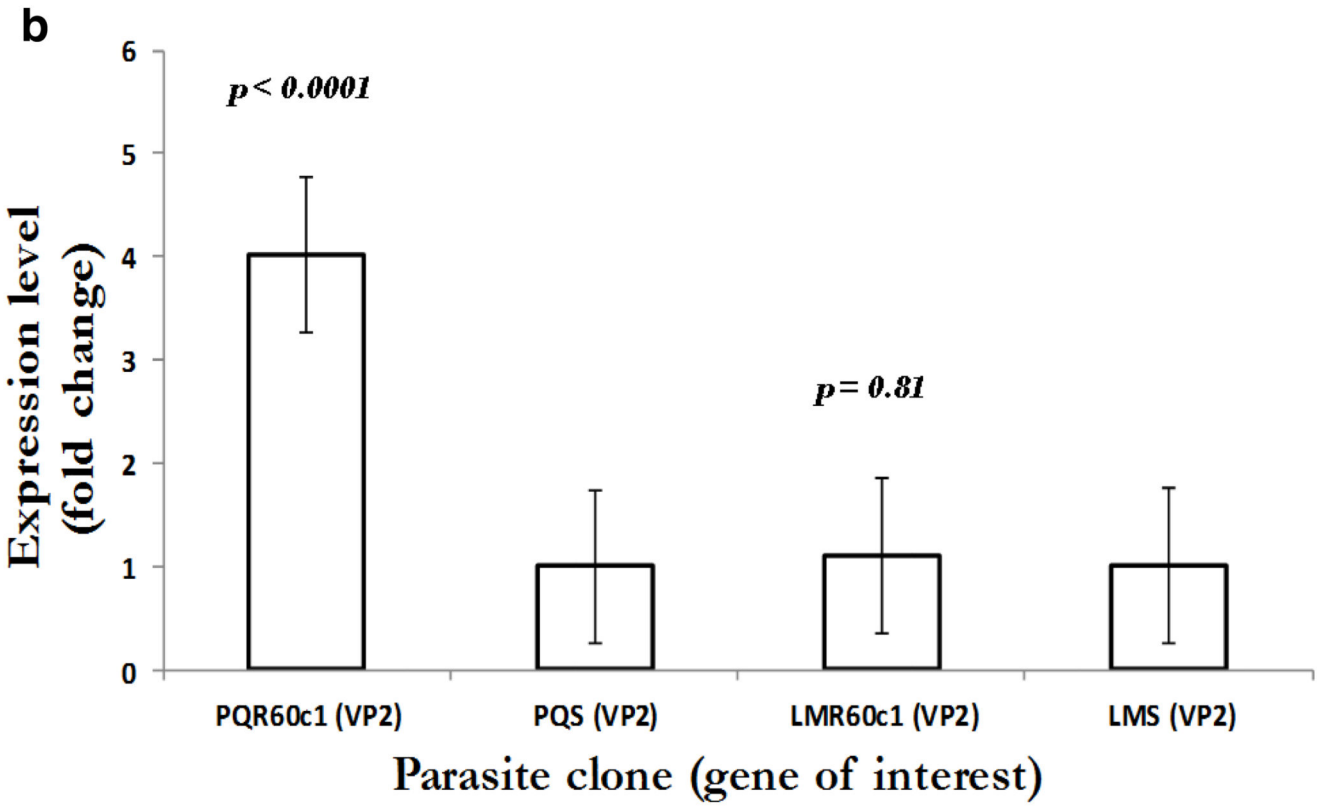

Figure 2b.

Expression profiles in V-type H+ pumping pyrophosphatase (VP2) as measured from cDNA amount derived from 5μg of total RNA isolated from piperaquine resistant (PQ^R60c1) and lumefantrine resistant (LM^R60c1) relative to their wild type drug sensitive parental clones piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The differential expression from a mean of three independent experiments showed significant difference ($p<0.0001$) in PQ^R60c1 with 4 folds increase but levels remained unchanged in LM^R60c1 ($p = 0.81$) parasite clones after student's t-test analysis with p value set at 0.05.

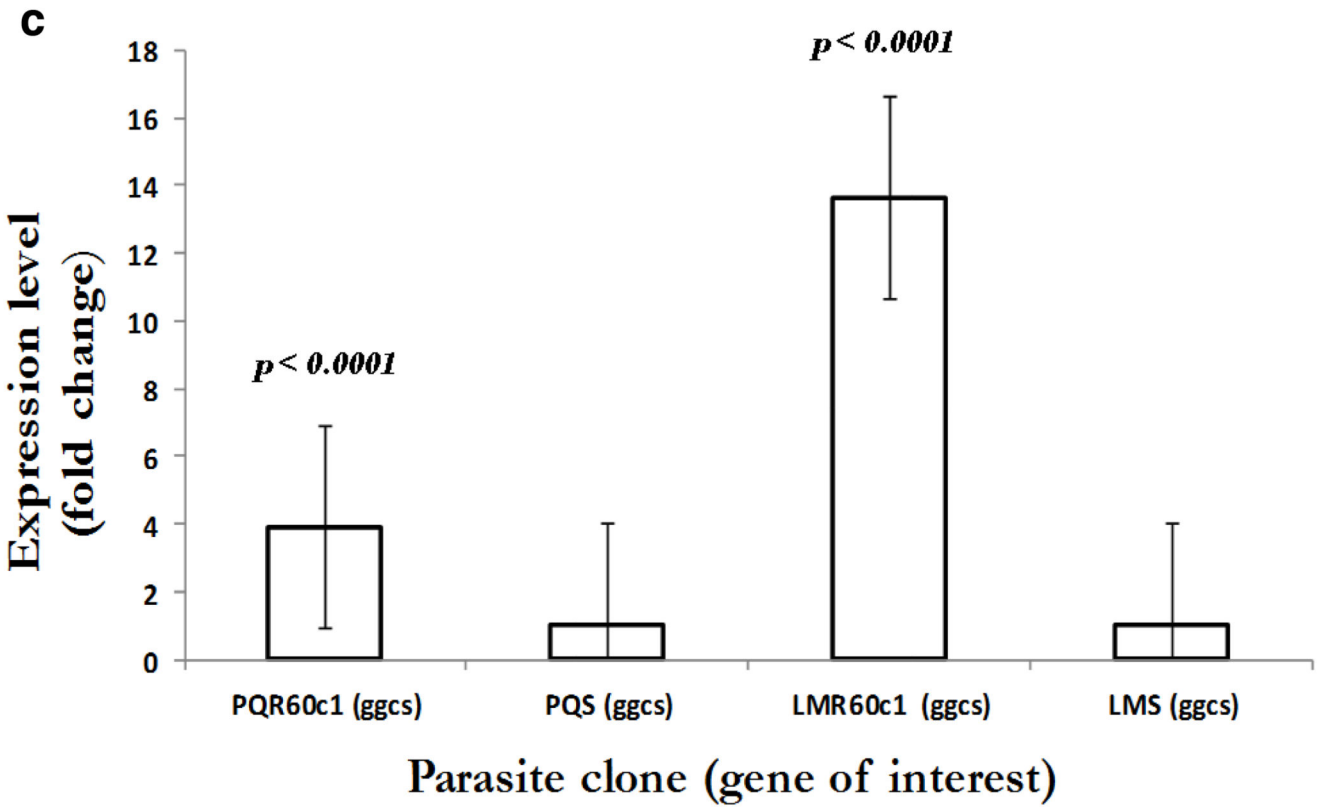

Figure 2c.

Expression profiles in gamma (γ)-glutamyl-cysteine-synthetase (ggcs) as measured from cDNA amount derived from 5μg of total RNA isolated from piperaquine resistant (PQ^R60c1) and lumefantrine resistant (LM^R60c1) relative to their wild type drug sensitive parental clones piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The differential expression from a mean of three independent experiments showed significant increase with a 3.89 and 13.65 folds times in PQ^R60c1 (p<0.0001) and LM^R60c1 (p<0.0001) respectively after student's t-test analysis with p value set at 0.05.

Europe PMC Funders Author Manuscripts

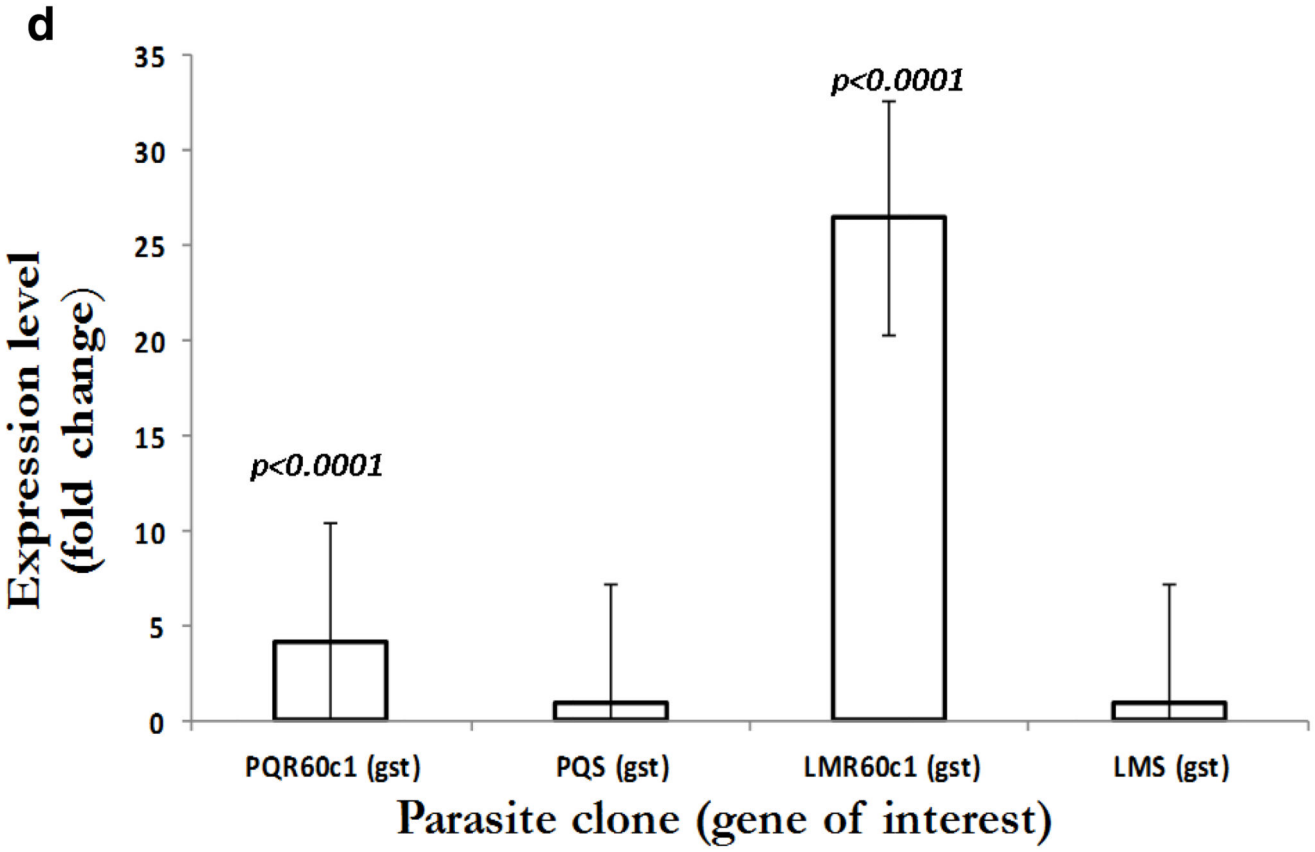

Figure 2d.

Expression profiles in glutathione S- transferase (gst) as measured from cDNA amount derived from 5μg of total RNA isolated from piperaquine resistant (PQ^R60c1) and lumefantrine resistant (LM^R60c1) relative to their wild type drug sensitive parental clones piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The differential expression from a mean of three independent experiments showed a significantly high levels of 4.17 and 26.49 folds in PQ^R60c1 (p<0.0001) and LM^R60c1 (p<0.0001) resistant clones respectively after student's t-test analysis with p value set at 0.05.

Europe PMC Funders Author Manuscripts

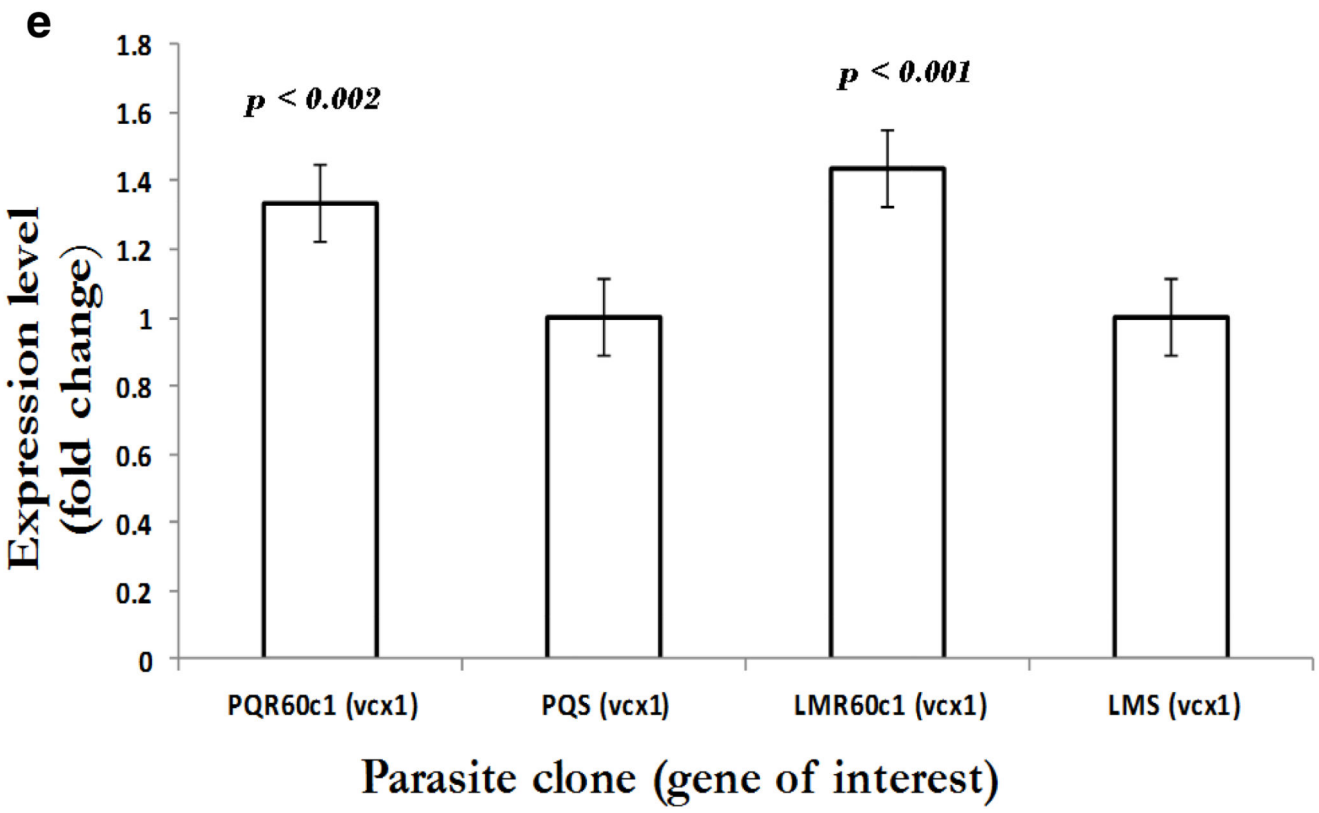

Figure 2e.

Expression profiles in Ca^{2+}/H^{+} antiporter (vcx1) as measured from cDNA amount derived from 5μg of total RNA isolated from piperaquine resistant ($PQ^{R}60c1$) and lumefantrine resistant ($LM^{R}60c1$) relative to their wild type drug sensitive parental clones piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The differential expression from a mean of three independent experiments show significant difference with a 1.34 and 1.43 folds increase in expression level in $PQ^{R}60c1$ ($p<0.002$) and $LM^{R}60c1$ ($p<0.001$) respectively after student's t-test analysis with p value set at 0.05.

Europe PMC Funders Author Manuscripts

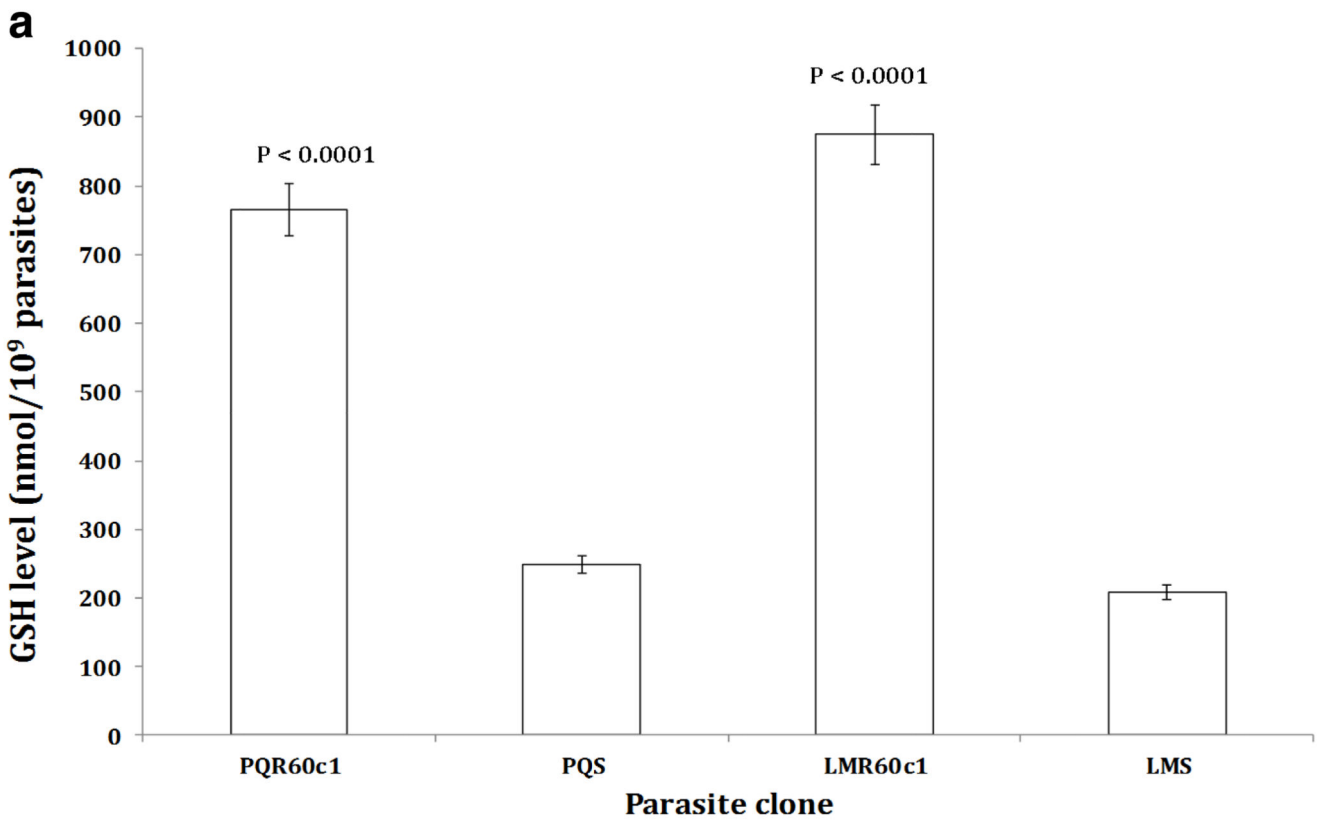

Figure 3a.

The level of intracellular glutathione (GSH) measured from isolated piperaquine resistant (PQ^R60c1) and lumefantrine resistant (LM^R60c1) relative to their wild type drug sensitive parental piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The isolated parasites were prepared to determine GSH activities as described in Section 2. The results are expressed as mean and standard errors of five mice. The GSH level was significantly higher in PQ^R60c1 ($p<0.0001$) and LM^R60c1 ($p<0.0001$) compared to PQS and LMS respectively after student's t-test analysis with p value set at 0.05.

Europe PMC Funders Author Manuscripts

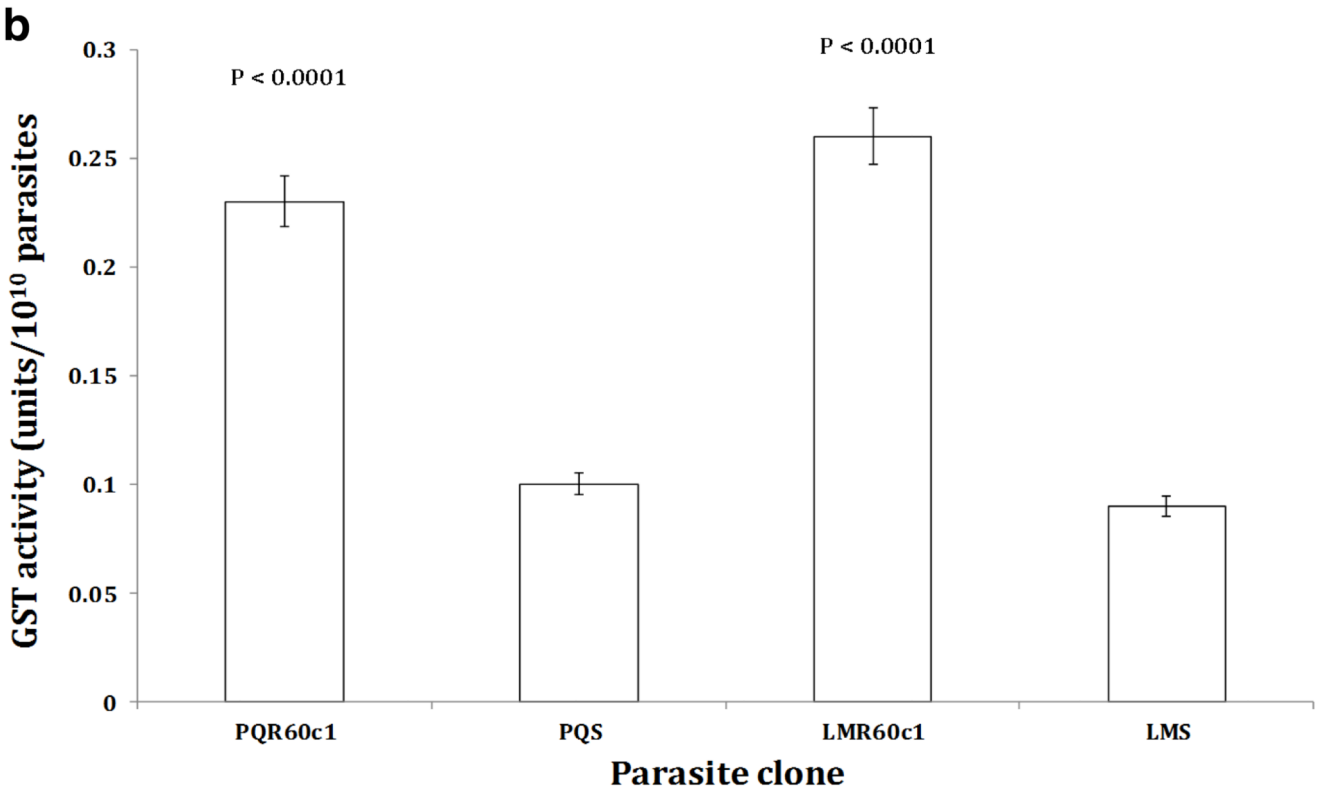

Figure 3b.

The activity profiles of glutathione-s-transferase (GST) measured from isolated piperaquine resistant (PQ^R60c1) and lumefantrine resistant (LM^R60c1) relative to their wild type drug sensitive parental piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The isolated parasites were prepared to determine GST activities as described in Section 2. The results are expressed as mean and standard errors of five mice. One unit of GST activity is defined as the amount (in μmol) of the reaction product (GS-DNB conjugate) per min. The GST activity was significantly higher in PQ^R60c1 ($p<0.0001$) and LM^R60c1 ($p<0.0001$) compared to PQS and LMS respectively after student's t-test analysis with p value set at 0.05.

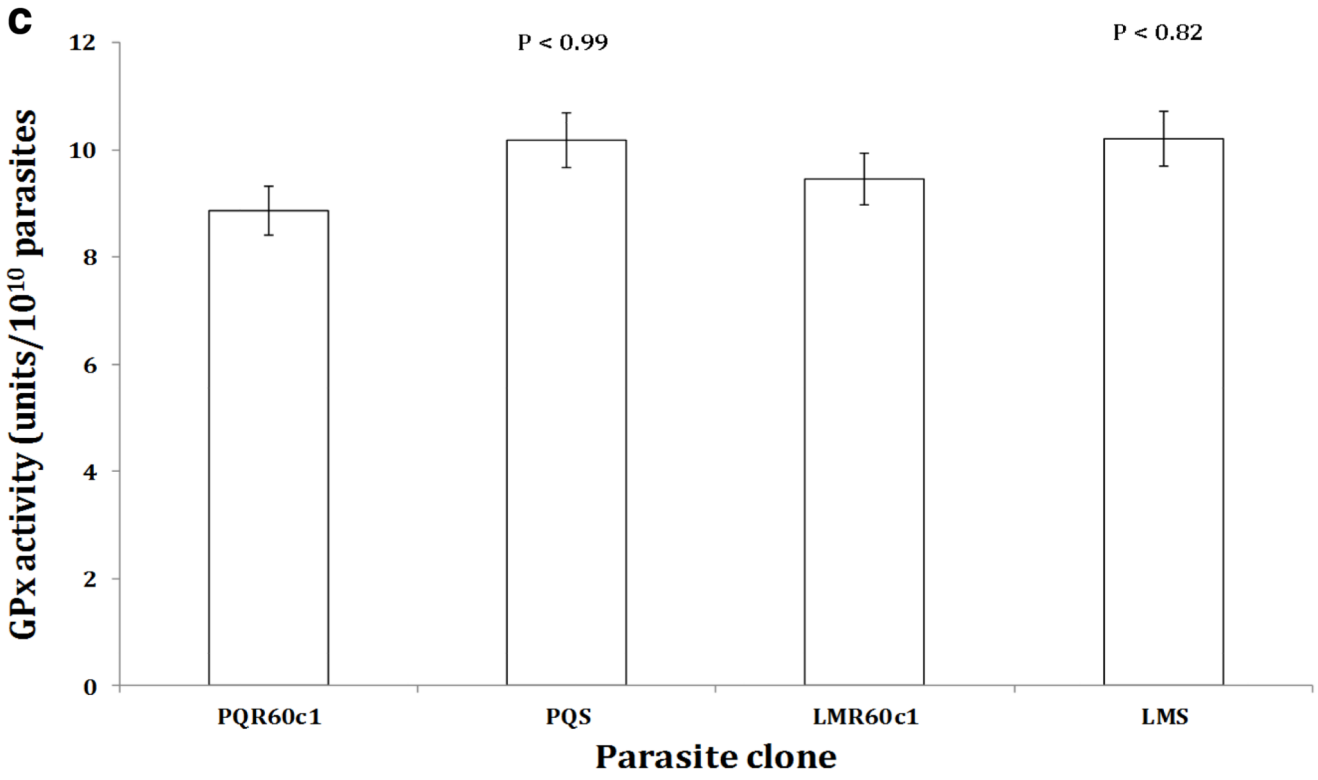

Figure 3c.

The activity profiles of glutathione peroxidase (GPx) measured from isolated piperaquine resistant (PQ^R60c1) and lumefantrine resistant (LM^R60c1) relative to their wild type drug sensitive parental piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The isolated parasites were prepared to determine GPx activities as described in Section 2. The results are expressed as mean and standard errors of five mice. One unit of GPx activity is defined as the formation of 1μmol of $NADP^+$ from NADPH per min. The GPx activity was not significantly different in PQS ($p<0.99$) and LMS ($p<0.82$) compared to PQ^R60c1 and LM^R60c1 respectively after student's t-test analysis with p value set at 0.05.

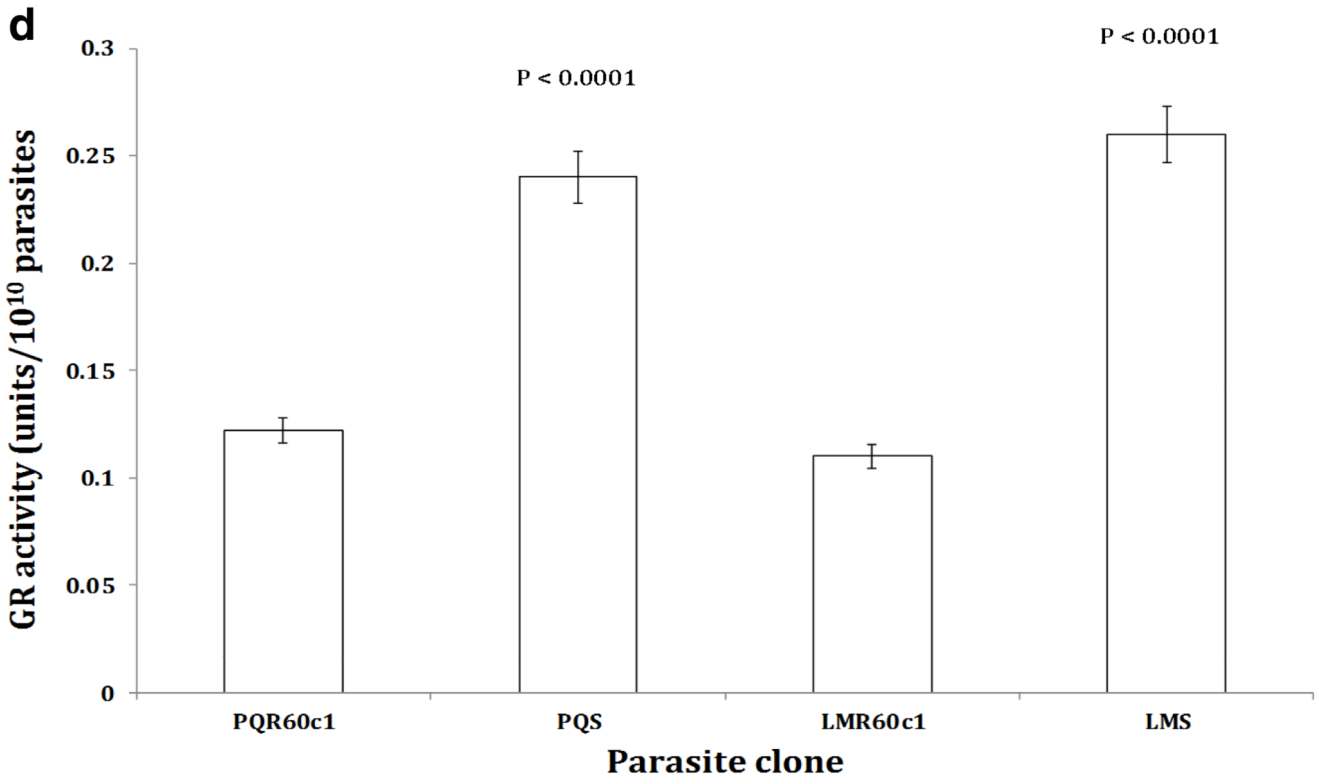

Figure 3d.

The activity profiles of glutathione reductase (GR) measured from isolated piperaquine resistant (PQ^R60c1) and lumefantrine resistant (LM^R60c1) relative to their wild type drug sensitive parental piperaquine sensitive (PQS) and lumefantrine sensitive (LMS) clones respectively. The isolated parasites were prepared to determine GPx activities as described in Section 2. The results are expressed as mean and standard errors of five mice. One unit of GR activity is defined as the reduction of 1μmol of DTNB to TNB per min. The GR activity was significantly higher in PQS (*p<0.0001*) and LMS (*p<0.0001*) compared to PQ^R60c1 and LM^R60c1 respectively after student's t-test analysis with *p* value set at 0.05.

Europe PMC Funders Author Manuscripts

Table 1

List of generations from piperaquine resistant (PQ^r) and lumefantrine resistant (LM^r), their 50% effective doses (ED_{50}) and the number of clones (number of positive mice on day 8 post infection) obtained from limited dilution experiment using 20 mice per generation with each mouse receiving 0.5 parasites

PQ^r(passage No)	**ED_{50}(mg/kg)**	**No of clones**	**LM^r (passage No)**	**ED_{50}(mg/kg)**	**No of clones**
Stability line (27^{th})	110	6	Stability line (48^{th})	116.34	8
Mosquito passed (MP) resistant line (40^{th})	25	4	Mosquito passed (MP) resistant line (40^{th})	49	6
60^{th} generation	>110	4	60^{th} generation	>116	3

Europe PMC Funders Author Manuscripts

Table 2a

Optimized condition for PCR amplification of *Pbcrt* and *Pbmdr1* genes using primer upstream of 5' and downstream of 3' untranslated region

PCR amplifying profiles	**Temperature (°C) /Time (min)**	
	Pbcrt	**Pbmdr1**
Initial denaturation	95°C, 5 min	95°C, 5 min
Denaturation	95°C, 1 min	95°C, 1 min
Annealing Temperature	50°C, 45 sec	48°C, 45 sec
Elongation	68°C, 6 min	68°C, 5 min
Primer (Forward & reverse)	1.0 pmol/µl each	1.0 pmol/µl each
Mgcl2 (mM)	2.0	1.5
dNTPs(mM)	0.2	0.2
Cycles	35	35
Final elongation	72°C, 10 min	72°C, 10 min

Europe PMC Funders Author Manuscripts

Table 2b

Primer sequences for PCR amplifying and sequencing *P. berghei Pbcrt* candidate genes

Primer Name:	**Primer Sequence (5' to 3'): PCR primers**
Pbcrt -UTR upstream	TGCTTTTCTAACTCTTGAGGACA
Pbcrt -UTR downstream	GTCTTCTAAACAACGAGCATGCT
	Pbcrt **Primer Sequence (5' to 3'): Sequencing primers**
Pbcrt 1f	TACTCCCTAATATTAGGTTACAT
Pbcrt 1r	CTGAAGTAACAAAACTATAATTTCCC
Pbcrt 2f	GGACAGCCTAATAACCAATGG
Pbcrt 2r	CGACCATAGCATTCAATCTTAGG
Pbcrt 3f	GGTTCATGTTTCTTGGATATCGG
Pbcrt 3r	GCTGGTCCTTGTATACAACTAAC
Pbcrt 4f	CCTAAGATTGAATGCTATGGTCGT
Pbcrt 4r	GTTAATTCTGCTTCGGAGTCATTG
Pbcrt 5f	TGTTAGTTGTATACAAGGACCAGC
Pbcrt 5r	TCACAAAAGGAACAAACGGTCA

Europe PMC Funders Author Manuscripts

Table 2c

Primer sequences for PCR amplifying and sequencing *P. berghei Pbmdr1* candidate genes

Primer Name:	**Primer Sequence (5' to 3'): PCR primers**
Pbmdr1-1f UTR	GTCTAAATGTTGTAATTTGTTGTCCT
Pbmdr1 r (UTR)	GACATTATCTAATTTCATCACCTTG
	Pbmdr-1: Sequencing Primer (5' to 3'):
Pbmdr1 f (UTR)	TTCACGCTATAAAAGTACAGACTA
Pbmdr1-1r	CAGTATCATTCACACTTTCTCC
Pbmdr1-2f	GTGCAACTATATCAGGAGCTTCG
Pbmdr1-2r	CACTTTCTCCACAATAACTTGCTACA
Pbmdr1-3f	GCAGCTCTATATGTAATAAAAGGGTC
Pbmdr1-3r	GTCGACAGCTGGTTTTCTG
Pbmdr1-4f	CTTTGAATTACGGTAGTGGCT
Pbmdr1-4r	TCGCTAGTTGTATTCCTCTTAGA
Pbmdr1-5f	TGGAGTAGTTAGTCAAGATCCT
Pbmdr1-5r	GTGCCTTGTTCAACTATTACAC
Pbmdr1-6f	TCAAATAGAGATCAAGAATCAACAGG
Pbmdr1-6r	GGATATAAACCACCTGCCACT
Pbmdr1-7f	GCCAAGTAAACCATCATTCTTCA
Pbmdr1-7r	TCGCGTTGTAATGGTATATGCT
Pbmdr1-8f	GGATTTTTATCGTCGCATATTAACAG
Pbmdr1-8r	TAGCTTTATCTGCATCTCCTTTGAAG
Pbmdr1-9f	TGCAATAGATTATGACAGTAAAGGGG
Pbmdr1-9r	ATCTTTCAAATCGTAGAATCGCAT
Pbmdr1-10f	CTTCAAAGGAGATGCAGATAAAGCTA
Pbmdr1-10r	GATTCAATAAATTCGTCAATAGCAGC
Pbmdr1-11f	TGCAATAGTTAACCAAGAACCAATGT
Pbmdr1-11r UTR	CAATAGCCGATTAAAAGAAAAAACGA

Table 3

Oligonucleotide primers and TaqMan probes used to assess *Pbmdr1, Pbvp2, Pbvcx1, Pbggcs,* and *Pbgst* transcription levels with *Pbβ-actin* as housekeeping

Name	Primer sequence (5′ – 3′)	Position	Tm
Pbmdr1- F	ACGGTAGTGGCTTCAATGGA	917-936	54.2
Pbmdr1- A	CTGTCGACAGCTGGTTTTCTG	1082-1062	54.7
Pbmdr1- Oligo	**FAM**–TTGCTGAATATATGAAATCGTTAGAGGCAA-**TAMRA**	1007-1036	61.9
Pbvp2 -F	TGCAGCAGGAAATACAACAGC	1449-1469	55.2
Pbvp2 -A	GTCGTACTTTTGCACTACTTGCGT	1558-1535	56.5
Pbvp2 -Oligo	**FAM**–TGCACCGAATAAGGCAAAAGCAA-**TAMRA**	1533-1511	62.3
Pbggcs -F	TGAATGCGTCGAAAAAGAAG	810-829	52.8
Pbggcs -A	CTTCGATGCCTAGCGTTTC	874-856	52.3
Pbggcs -Oligo	**FAM**–TGAATGCCAATGTGATGTTGCA-**TAMRA**	831-852	59.2
Pbgst -F	GACGCAAGAGGTAAAGCTGAAC	31-52	54.6
Pbgst -A	CGAACTATAGATTGGCTTTGAGC	230-208	54.0
Pbgst -Oligo	**FAM**–TGGTGATGCATTTGCAGAATTTAACAAT-**TAMRA**	114-141	62.3
Pb β-actin -F	CAGCAATGTATGTAGCAATTCAAGC	392-416	56.8
Pb β-actin -A	CATGGGGTAATGCATATCCTTCATAA	523-498	58.9
Pb β-actin -Oligo	**FAM**–ATTCATCAGGCCGTACAACAGGTATTGT-**TAMRA**	431-458	62.5
Pbcvx1 -F	TCAAATTGCTCTTTTTGTTGTACCAA	1101-1126	57.9
Pbcvx1 -R	ACACCTTCTAGCCAATTACTTTCACC	1265-1240	57.1
Pbcvx1-Oligo	**FAM**–CTATGACCTTAGCCTTTTCTCCTTTATCAA-**TAMRA**	1160-1189	59.8