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Comparison of a Formulated Transport Medium and EDTA Anticoagulant in Kenyan Field Isolates from Maseno Division against a Panel of Antimalarials Using SYBR Green I technique

Scolastica C. Korir
Department of Medical Microbiology, Maseno University, P.O. Box Private Bag, Maseno

Abstract
In vitro drug sensitivity relies on the growth of plasmodium in the presence of the antimalarials. These fresh isolates brought in for assay from collection sites normally not near the laboratory require a medium to keep the parasites viable in order to produce results which are a representation of the field situation in terms of drug susceptibility. This study validated the use of a formulated transport medium (TM) by assaying 322 *Plasmodium falciparum* field isolates from patients visiting Chulaimbo sub county hospital in Maseno division, for 50% inhibitory concentrations (IC_{50}) against four antimalarials using SYBR Green 1 *in vitro* assay. These samples were transported using a formulated transport medium (TM) and the conventional EDTA anticoagulant tubes to act as control. The similarity which was detected with no significant difference ($P > 0.05$) observed in the medians inhibitory concentration 50 (IC_{50}) between samples in the EDTA anticoagulant and TM warrants its continued use. However, dihydroartemisinin (DHA) which showed to have a correlation significance ($p<0.05$) with most of the drugs used, calls for more research to expound on this finding.

INTRODUCTION

In Kenya, malaria continues to cause significant morbidity and mortality, and is often a location where drug resistance emerges in Africa. The development of *Plasmodium. falci parum* resistance to chloroquine diphosphate (CQ), and later sulfadoxine-pyrimethamine, are well described (Mwai *et al.,* 2009; Khan *et al.,* 1997). This warrants continued *in vitro* drug IC_{50} monitoring of *P. falciparum* field isolates, which is becoming more practical and safer, as more reliable assays like SYBR Green I become established, combined with simpler field isolate processing such as "immediate *ex vivo*" (IEV) (Akala *et al.,* 2011).SYBR green 1 *in vitro* technique is the latest technique which is convenient and can be used in a large parasite density and produces rapid reproducible results (Rengarajan *et al.,* 2002). Its measure of sensitivity depends on the growth of parasites subjected to antimalarials in a medium.

Lack of *in vitro* plasmodial growth has been attributed to; the effects of a markedly diminished viability of all parasite populations within the same isolate leading to death of the parasites before reaching the laboratory (Basco, 2004), very low starting parasitaemia due to poor survival rates of the parasites from the field (Bacon *et al.,* 2007), or presence of trace amounts of antimalarial drugs or intake of traditional medicine undetected by urine test (Basco, 2003). Low yield of the *in vitro* assay was also observed in a Nigerian study and may have been due to suboptimal storage and transportation conditions of parasitized blood or due to the presence of traces of the drug in the sample rather than limitations of the technical approach (Issaka *et al.,* 2013). So to enhance the immediate survival and later growth of these parasites, there is need for a proper transition from the natural host (human) to the artificial medium which will subsequently be used for transportation to the laboratory.

Apart from the medium the parasites are exposed to, parasites growth rates sometimes depend on the source of the serum used. For instance, diet, medication consumed, the state of health, and the immune status of a donor can inadvertently affect growth rates and modify the 50% inhibitory concentration results (ofulla *et al.,* 1994). The presence of trace amounts of antimalarial drugs or intake of traditional medicine undetected by urine test for commonly used antimalarial drugs may partially explain why some fresh isolates do not grow *in vitro* (Basco, 2003). Furthermore, serum may contain unknown quantities of drug-binding proteins that can skew IC_{50} values. For maximum plasmodial growth, it is important eliminating all these host immune components before test, so as to render maximum growth of the parasites in the antimalarials, thus, making it reliable especially for fresh clinical isolates meant for epidemiological studies (Russell *et al.,* 2003).

In that regard, this study employed the use of a formulated transport media (TM) with an aim of trying to improve on the viability of parasites during transportation to the lab for further cultivation. Infected blood samples were asceptically put in the tubes with TM and transported to the laboratory alongside with the samples put in the EDTA anticoagulant tubes. In the laboratory, these samples (in TM and EDTA) were washed twice using complete medium by centrifugation as a simple pretest procedure to remove antimalarial immune plasma, white blood cells, and residual drugs which may be present in the test blood, before subjecting it to the drugs. The IC_{50} results obtained were compared with the IC_{50} results obtained from samples collected using the EDTA coated tubes, an anticoagulant which has the advantages of a lack of inhibitory action on DNA allowing the best

preservation of cellular components and morphology of blood cells (Reardon *et al.,* 1993), thus best for hematologic and biochemical investigations (Basco, 2004). This study was carried out in order to shed light on the importance of a medium with nutrients which can promote better transition and adaptation to enhance immediate survival, prolonged survival and viability of the plasmodium parasites once removed from the human host.

Subjects and methods

Protocol, sites, and subjects

This study was approved by Jaramogi Oginga Odinga teaching and referral hospital Ethics and review committee (JOOTRH ERC) accreditation No. 01713.Participating clinical center was Chulaimbo sub county hospital located in Maseno division of Kisumu County. Training of the medical staffs especially those at the laboratory on the inclusion and exclusion criteria for subjects' selection was done. Subjects attending outpatient clinic, who were at least 2years of age and having met the inclusion criteria (mono infection of *p.falciparum* with parasitaemia of between 1000 to 200,000asexual parasites /ul of blood) were invited to participate .Informed consent was obtained from adults subject (>18years of age) or legal guardians for subjects <18years of age.

Sample collection and preparation

Consented subjects provided 4ml of blood for transport to the laboratory. 2ml was collected in to a plain vacutainer tubes with 2ml of the formulated transport medium while the other 2ml was drawn into an EDTA coated vacutainer tubes. Both tubes were placed at 4^0c until transported to the lab at the close of the day to begin immediate ex vivo (IEV) drug IC_{50} testing described below. SYBR Green 1based *in vitro* IC_{50} drug sensitivity described below was used to test each *P.falciparum* field isolate transported in the two anticoagulants against a panel of 4 antimalarials supplied as Artemether (ART), Lumefantrine (LU), Dihydroartemisinin (DHA) and piperaquine (PPQ). The test drugs were obtained from Kenya Medical Research Institute (KEMRI).To prepare the test drugs, solutions at 1mg/ml were prepared in 5ml 100% dimethyl sulfoxide for ART, LU, DHA and PPQ. Further dilutions were in complete RPMI1640 media to the desired starting concentrations followed by two fold serial dilutions to generate 10 concentrations for IC_{50} testing (Mbaisi *et al.,* 2004). The concentration ranges in ng/ml from highest to the lowest were; ART (200 to 0.0976), LU (200 to 0.0976), DHA (200 to 0.0976) and PPQ (500 to 0.2441). The drug solutions were prepared and used immediately or stored at -80^0c for not longer than 1 month before use. Basic and complete RPMI 1640 culture media, the later with glucose and hypoxanthine enrichment were prepared as described (Johnson *et al.,* 2007). *Plasmodium falciparum* isolates processed IEV were placed into assay within 8 hours of phlebotomy, without culture adaptation. These samples were first washed in RPMI 1640 by centrifugation at 2500revolutions per minute for 3minutes for three consecutive times. Blood samples with > 1% parasitemia were adjusted to1% parasitemia at 2% hematocrit using uninfected O+ erythrocytes, and those with ≤ 1% parasitemia were used unadjusted at 2% hematocrit. Transfer of parasite sample and antimalarial drug aliquots in complete RPMI 1640 drug aliquots onto 96-well microculture plates and addition of lysis buffer after 72 hours incubation was done as previously described (Johnson *et al.,* 2007).The plates were then placed at room temperature in the dark, for 5–15 minutes. Parasite replication inhibition was quantified and the IC50 for each drug calculated by an equation generating a sigmoidal concentration response curve (variable slope), with log transformed drug concentrations on the *X* axis and relative fluorescent units (RFUs) on the *Y* axis (Graphpad Prism for Windows, version 5.0; Graphpad Software, Inc., San Diego, CA). (Bacon *et al.,* 2007).

Statistical analysis

Data were analysed by using Mann-Whitney test; a non parametric test which doesn't assume a gaussian distribution, to compare the medians IC_{50} values of antimalarials in the two categories (EDTA and TM). Pearson correlation coefficient was also used to determine the r value which gave the possible correlation of the logarithmic values of IC_{50} between the antimalarials in each category. Data were analysed using GraphPad Prism 5.00 for Windows; GraphPad Software, San Diego, CA, and Sigma Plot version 10; Systat Software, Inc., San Jose, CA).

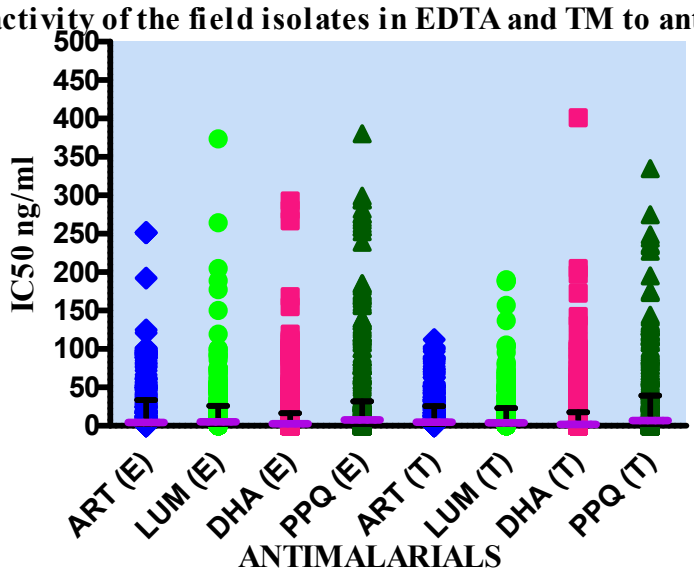
Results

We assayed 322 *P. falciparum* field isolates from Chulaimbo sub county hospital against four panels of drugs namely ART, LUM, DHA and PPQ. These samples were brought in the EDTA anticoagulant and plain tubes with formulated TM from the hospital which is 20minutes drive to the laboratory. A successful assay result (defined as dose concentration relationship across the 10 drug dilutions for one or all drugs per field isolate) occurred in more than 50% of all the isolates in EDTA and TM. 70% of the assays had parasitaemia >0.5%. Few plates gave high relative fluorescent units (RFU) for samples in both groups while more plates gave modarate RFU of between 4000-8000 parasites or even lower. However, their curves converged to create IC_{50} dose

respose curves apart from few which didn't and thus were excluded. From the *in vitro* assay (table 1), the median IC_{50} values in ng/ml obtained from the 322 samples transported using the EDTA anticoagulant were higher in three drugs (LU 5.409, DHA 2.567, and PPQ 7.887) than the median IC_{50} values obtained from the same samples in the TM (LU 4.007, DHA 1.691, and PPQ 6.911). While the median IC_{50} for ART in TM emerged higher than in EDTA with values of 4.887 and 4.275 respectively (table 1). However, this table further shows that the individual activity of these drugs didn't differ significantly ($P<0.05$) for the two categories. Using Mann Whitney test, there was no significant difference ($P = 0.9873$) between the median IC_{50} values of samples exposed to ART in both EDTA and TM. Similarly, we observed no significant difference ($P = 0.7448$) in the samples subjected to LU when the two groups were compared. The least median IC_{50} values were experienced by samples treated with DHA (2.567 and 1.6911 for EDTA and TM respectively) while the highest IC_{50} values were experienced with the samples exposed to PPQ with medians of 7.887 and 6.911 for EDTA and TM respectively. Nevertheless, the activity of both antimalarials didn't differ significantly with $p=0.6839$ and $p= 0.8153$ for DHA and PPQ respectively. Comparison using their mean IC_{50} values gave no significant different ($p>0.05$) results for the drugs in both categories except for ART which showed a big significant decrease ($p=0.0056$) in mean IC_{50} for samples in the TM. The IC_{50} ranges for all the drugs were generally in the upper half of the IC_{50} range as shown in figure 1 below.

A pearsons correlation coefficient analysis of the antimalarials was perfomed separately for the samples which were transported in EDTA and those in the TM. For the samples in the EDTA tube (table 2), the results showed that there was no correlation significance ($p=0.3699$, 0.2916 and 0.6364 respectively) between ART and LU, ART and PPQ and also between LUM and PPQ as indicated by their r values 0.04967, 0.0262 and 0.0584 respectively. We observed a correlation significance ($p=0.0259$, 0.0122 and 0.0203) of Artemisinin derivatives ART and DHA, LUM and DHA, and DHA and PPQ with high r values of 0.1230, 0.1382 and 0.1281 respectively. Among the samples in the TM, correlation significance ($p<0.05$) was recorded by ART and LUM, ART and PPQ and LUM and DHA while the rest showed no correlation significance $p>0.05$ with their r values lower than 0.1 as shown in table 2 below

Figure 1: *In vitro* activities of ART, LUM, DHA and PPQ against *Plasmodium falciparum* field isolates transported in EDTA and in TM



The abbreviations E and T represent samples in EDTA and in Transport medium respectively. The medians IC_{50} (purple bars) with their interquartile ranges in black for all the drugs

Table 1: Drug susceptibility testing of *Plasmodium falciparum* isolates in EDTA and TM against a panel of antimalarials

Median IC_{50} in ng/ml for isolates				
Isolates in EDTA			Isolates in (Transport medium) TM	P values of the medians using Mann - whitney test (P)
	Drugs	Median	Median	
1	Artemether	4.275	4.887	0.9873
2	Lumefatrine	5.409	4.007	0.7448
3	Diydroartemisinin	2.567	1.691	0.6839
4	Piperaquine	7.887	6.911	0.8153

Table 2: Correlation of *in vitro* responses of antimalarials drugs against field isolates of *Plasmodium falciparum* in EDTA and TM

Isolates in EDTA			Isolates in (transport medium)TM		
Drug pairs	Correlation coefficient (r)	Probability P value	Drug pairs	Correlation coefficient (r)	Probability P value
ART - LUM	0.04967	0.3699	ART - LUM	0.1260	0.0225
ART - DHA	0.123	0.0259	ART - DHA	0.09014	0.1032
ART - PPQ	0.0262	0.6364	ART - PPQ	0.1834	0.0008
LUM - DHA	0.1382	0.0122	LUM - DHA	0.1229	0.0261
LUM - PPQ	0.0584	0.2916	LUM - PPQ	0.0758	0.1709
DHA - PPQ	0.1281	0.0203	DHA - PPQ	0.0372	0.5024

The correlation coefficient was calculated by Pearson's correlation coefficient of log IC_{50} values. Probability refers to the significance level of the test ($p<0.05$). ART- Artemether, LUM - Lumefantrine, DHA - Dihydroartemisinin and PPQ - Piperaquine

DISCUSSION

In vitro drug assays in the field are best perfomed on fresh isolates immediately after collection (Basco, 2004) and it's simpler to perform than culture adapted as others have observed (Akala *et al.,* 2011; Bacon *et al.,* 2007). It also reduces concerns on the selection of dominant clones and loss of minor subpopulations (Liu *et al.,* 2008) hence consequent misidentification of a population's susceptibility profile (Mbaisi *et al.,* 2004). However, blood samples containing *P. falciparum* parasites for testing may be stored at collection sites for days transported long distances to the laboratory before cultivation (Akala *et al.,* 2011), a factor which could cause failure of growth attributed to a prolonged time between the times when samples were collected and when they were tested (Bacon *et al.,* 2007) hence reducing the parasites viability to even zero (Basco, 2004). Availability of a medium would provide a proper transition from the natural host to an artificial medium and would keep the parasites viable to the laboratory for cultivation. This is the first study to report a direct comparison between two anticoagulants, evaluating the possible influence of the formulated transport medium (TM) on maintaining parasite viability during storage at 4°C against the conventional EDTA anticoagulant. Among the assays, the upper half of the IC_{50} was notably higher for all the drugs in both categories, this may reflect greater biological variability among the field isolates with the presence of sub populations of drug resistant and sensitive parasites (Jefari *et al.,* 2004).This factor addressed the fact that some plates produced assays whose curves didn't calculate an IC_{50} because the points could not converge to create an ideal IC_{50} dose response curve due to the presence of a subpopulation in low proportions (10%), as also evident a study (CO-EM *et al.,* 2009).

Patient blood samples usually have very different parasitemia, and most drug assays are sensitive to variation in parasitemia. (Liu *et al.,* 2008). Parasitaemia estimation in any *in vitro* assay is highly dependent on microscopy therefore variation occurs depending on the expertise of the microscopist in the laboratory. If the starting parasitaemia in an assay is mistakably high then much infected parasites would be found in each well of the plate leading to an increase in the absorption of drugs, (Ritchie *et al.,* 1996) consequently increasing the IC_{50} levels obtained (Geary *et al.,* 1990).This explains the high levels of RFU when curves were plotted against the drug concentration in the present study. The vice versa is true such that a very low starting parasitaemia may lead to a lack of growth and less IC_{50} values as reported by Bacon (Bacon *et al.,*2007). Increasing the number of infected erythrocytes leads to higher drug uptake (Basco, 2004) such that there is need for an increased concentration of drugs to inhibit growth when numbers of parasites per volume were inoculated (Duraisinngh *et al.,* 1999; Gluzman *et al.,* 1987). A cambodian study also confirmed that parasitaemia level is inversely proportional to the IC_{50} values (Suwanna *et al.,* 2013), these previous studies justified the high IC_{50} values obtained with some plates which consequently raised the mean IC_{50} especially for PPQ in the current study for samples in both EDTA and TM since both used the same amount of parasitaemia.

DHA was expectedly the most active drug with the least median IC_{50} values (2.567 and 1.691 in EDTA and TM respectively) and ranges in both EDTA and TM samples which still wasn't significant. This could be attributed by the fact that they are few if any reduced susceptibility cases of the drug in the region and generally in Kenya, this is in line with a study conducted at the Kenyan coast and emerged the most active drug too (Mwai *et al.,*2009). Higher IC_{50} values (7.887 in EDTA and 6.911 in TM samples) experienced by PPQ in our data were unlikely to represent resistance but probably methodological variability, since these values obtained were way below the IC_{50} values from previous reports from malaria endemic areas (Basco *et al.,* 2003; Basco *et al.,* 2007 and Mwai *et al.,* 2009). In contrary with other studies which have found an IC_{50} LUM activity of 30nm for more than 95% of isolates (Mayxay *et al.,* 2007; Parola *et al.,* 2007; Paradines *et al.,* 2006), our study generated only 24 and 20% from samples in EDTA and TM respectively, a clear indication that the activity of the drug which is used as a partner to ART for treatment of uncomplicated malaria cases remains high in the region. Encouragingly, our ART IC_{50} data which were 4.27 and 4.88 in EDTA and TM respectively were comparable with those found earlier for clinical isolates collected in Cameroon and other African countries where their IC_{50} values were ranging between 3.46 and 5.66nm (Basco and LeBras, 1993; Basco and Ringwald, 2003 and Issaka *et al.,* 2013)

The inoculum size used in *in vitro* testing influences the measured *in vitro* susceptibility to antimalarials, therefore resistance can be overestimated when inoculum effects aren't considered. On the other hand, occurrences of cross resistance between antimalarials can also be falsely determined in some cases (Duraisinngh *et al.,* 1999). Positive correlation ($P<0.05$) was observed between DHA and all the tested drugs (ART, LUM and PPQ) for samples in EDTA, unlike the same samples in TM where DHA had a correlation significance ($P=0.0261$, $r=0.1229$) with LUM only while others weren't. The variation in the cross resistance of DHA in the samples in EDTA and TM would have been attributed by the inoculum effect. However, further research need to be conducted to rule out possible cross resistance between these drugs in the region. Although, a Kenyan coast study which found a positive correlation between DHA and LUM, and also between LUM and PPQ (Mwai *et al.,* 1999) were in line with the results of the current study.

Conclusion

This current study makes it explicit that the formulated transport medium holds promise for future use having shown similar IC_{50} results with no significant difference with the samples which were in the EDTA anticoagulants tubes. Variations in the positive correlations experienced by DHA is a proof of possible cross resistance between this drugs which need further research to elucidate on the finding.

References

Anderson, T. J., S. Nair, H. Qin, S. Singlam, A. Brockman, L. Paiphun, and F. Nosten. (2005). Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? *Antimicrob. Agents Chemother*. 49:2180–2188.

Bacon D.J., Latour C., Lucas C., Colina O., Ringwald P.., Picot S., (2007). Comparison of a SYBR green I-based assay with a histidine-rich protein II enzyme-linked immunosorbent assay for *in vitro* antimalarial drug efficacy testing and application to clinical isolates. *Antimicrob Agents Chemother* 51*:* 1172–1178.

Basco LK, and Le Bras J. (1993). *In vitro* activity of artemisinin derivatives against African isolates and clones of *Plasmodium falciparum*. *Am. J. Trop.Med. Hyg.* 49:301–307

Basco, L. K. (2004). Molecular epidemiology of malaria in cameroon. xx. Experimental studies on various factors of *in vitro* drug sensitivity assays using fresh isolates of *plasmodium falciparum. American Journal of Tropical Medicine and Hygiene,* 70(5), 474–480

Basco, L. K., and P. Ringwald. (2003). In vitro activities of piperaquine and other 4-aminoquinolines against clinical isolates of *Plasmodium falciparum* in Cameroon. *Antimicrob. Agents Chemother*. 47:1391–1394.

Basco, L. K., and P. Ringwald. (2007). Molecular epidemiology of malaria in Cameroon. XXIV. Trends of *in vitro* antimalarial drug responses in Yaounde, Cameroon. *Am. J. Trop. Med. Hyg.* 76:20–26.

Co EM, Dennull R.A., Reinbold, D.D., Waters, N.C., Johnson, J.D., (2009). Assessment of malaria *in vitro* drug combination screen- ing and mixed-strain infections using the malaria Sybr green I-based fluorescence assay. *Antimicrob Agents Chemother* 53*:* 2557–2563.

Duraisingh, M.T., Jones, P., Sambou, I., Seidlein, L., Pinder, M., Warhurst, D.C., (1999). Inoculum effect leads to overestimation of *in vitro* resistance for artemisinin derivatives and standard an- timalarials: a Gambian field study. *Parasitology.* 119*:* 435–440

Geary, T.G., Divo, A.D., Jensen, J.B., Zangwill, M. and Ginsburg, H. (1990). Kinetic modelling of the response of *Plasmodium falciparum* to chloroquine and its experimental testing *in vitro*. *Biochemical Pharmacology* 40, 685-691.

Gluzman, I.Y., Schlesinger, P.H. and Krogstad, D.J. (1987). Inoculum effect with chloroquine and *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy* 31; 32-36.

Jafari S, Le Bras J, Bouchaud O, Durand R, 2004. *Plasmodium falciparum* clonal population dynamics during malaria treatment. *J Infect Dis 189:* 195–203.

Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, Waters NC, (2007). Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. *Antimicrob Agents Chemother* 51*:*1926–1933.

Suwanna, C., Stuart, D. T. Chanthap, L., Kritsanai, Y., Wiriya,R.,S., Sundrakes, P. S., Jacob D.

Johnson, Douglas S. Walsh, David L. Saunders and Charlotte A Lanteri. (2013). Direct comparison of the histidine-rich protein-2 enzyme-linked immunosorbent assay (HRP-2ELISA) and malaria SYBR green I fluorescence (MSF) drug sensitivity tests in Plasmodiumfalciparum reference clones and fresh ex vivo field isolates from Cambodia. *Malaria Journal* 12:239

Khan B, Omar S, Kanyara JN, Warren-Perry M, Nyalwidhe J, Peterson DS, Wellems T, Kaniaru S, Gitonga J, Mulaa FJ, Koech DK, 1997. Antifolate drug resistance and point mutations in *Plasmodium falciparum* in Kenya. *Trans R Soc Trop Med Hyg*. 91*:* 456–460.

Mariama Issaka, Ibrahim Arzika, Julia Guillebaud, Abani Maazou, Sabine Specht, Halima Zamanka, Thierry Fandeur and Adamou Salissou.(2013). *Ex Vivo* Responses of *Plasmodium falciparum* Clinical Isolates to Conventional and New Antimalarial Drugs in Niger. *Antimicrobial Agents and Chemotherapy*. 57; 7 3415–3419.

Mayxay, M., M. Barends, A. Brockman, A. Jaidee, S. Nair, D. Sudimack, T. Pongvongsa, S. Phompida, R. Phetsouvanh, T. Anderson, N. J. White, and P. N. Newton. (2007). In vitro antimalarial drug susceptibility and *pfcrt* mutation among fresh *Plasmodium falciparum* isolates from the Lao PDR (Laos). *Am. J. Trop. Med. Hyg*. 76:245–250.

Mbaisi A, Liyala P, Eyase F, Achilla R, Akala H, Wangui J, Mwangi J, Osuna F, Alam U, Smoak BL, Davis JM, Kyle DE, Coldren RL, Mason C, Waters NC, (2004). Drug susceptibility and genetic evaluation of *Plasmodium falciparum* isolates obtained in four distinct geographical regions of Kenya. *Antimicrob Agents Chemother* 48*:* 3598–3601.

Mwai L, Kiara SM, Abdirahman A, Pole L, Rippert A, Diriye A, Bull P, Marsh K, Borrmann S, Nzila A, 2009. *In vitro* activities of piperaquine, lumefantrine, and dihydroartemisi- nin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in pfcrt and pfmdr1. *Antimicrob Agents Chemother* 53*:*5069–5073.

Mwai L, Ochong E, Abdirahman A, Kiara SM, Ward S, Kokwaro G, Sasi P, Marsh K., Borrmann S, Mackinnon M, Nzila A, (2009). Chloroquine resistance before and after its withdrawal in Kenya. *Malar J 8:* 106.

Parola, P., B. Pradines, F. Simon, M. P. Carlotti, P. Minodier, M. P. Ran- jeva, S. Badiaga, L. Bertaux, J. Delmont, M. Morillon, R. Silai, P. Brouqui, and D. Parzy. (2007). Antimalarial drug susceptibility and point mutations associated with drug resistance in 248 *Plasmodium falciparum* isolates im- ported from Comoros to Marseille, France in 2004–2006. *Am. J. Trop. Med. Hyg.* 77:431–437.

Pradines, B., P. Hovette, T. Fusai, H. L. Atanda, E. Baret, P. Cheval, J. Mosnier, A. Callec, J. Cren, R. Amalvict, J. P. Gardair, and C. Rogier. (2006). Prevalence of in vitro resistance to eleven standard or new antimalarial drugs among *Plasmodium falciparum* isolates from Pointe-Noire, Republic of the Congo. *J. Clin. Microbiol*. 44:2404–2408.

Rason MA, Randriantsoa T, Andrianantenaina H, Ratsimbasoa A, Menard D, (2008). Performance and reliability of the SYBR Green I based assay for the routine monitoring of susceptibility of *Plasmodium falciparum* clinical isolates. *Trans R Soc Trop Med Hyg* 102*:* 346–351.

Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M, (2004). Simple and inexpensive fluorescence-based technique for high-throughput *Antimalarial Agents Chemother* 48*:* 1803–1806.