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In-vitro activity of artemether, lumefantrine, dihydroartemisinin and piperaquine against 3D7 and W2 *Plasmodium falciparum* reference clones in a formulated transport medium and EDTA

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ABSTRACT
Introduction: *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. However, factors such as mixed infection of drug resistant and sensitive parasites occurring in most field isolates can influence drug test outcome because they have different drug susceptibilities, thus there is a need of using clones which have identified these drug resistant sub populations to prevent treatment failures, misidentification of a populations susceptibility profile and also in any evaluation procedure so as to provide accurate test results. **Objective:** This study validated the use of a formulated transport medium (TM) by assaying clones which have been culture adapted in the lab for 50% inhibitory concentrations (IC_{50}) against four antimalarials using SYBR Green 1 *in vitro* assay. **Method:** These clones (3D7 and W2) were revived and culture adapted for assay. Prior to subjecting them to drugs, they were exposed to 2ml formulated TM and the conventional EDTA anticoagulant in 5ml vacutainer tubes to act as control. **Results:** Both clones gave successful assays with moderate RFU values of between 2000 to 4000 parasites obtained by 3D7clone while W2 had RFU values as high as 20,000 parasites. However, their curves converged into discernible dose-response curves. Similarity 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 however, samples in EDTA generally had higher IC_{50} values. **Conclusion:** This study concludes that the formulated TM holds promise for future use.

Introduction

In Kenya Malaria is still the leading cause of morbidity and mortality (Kenya Ministry of Public Health and Sanitation, 2009). The disease accounts for 30 percent of Kenya's outpatient visits, 19 percent of hospital admissions, two to three percent of inpatient deaths, and is a leading cause of death among children under the age of five (Kenya Ministry of Public Health and Sanitation 2011). Specifically, in Western Kenya, 32% of all deaths are associated with Malaria, It causes 22% deaths among children with severe malaria, out of which 10% occur in hospitals and 13% outside hospitals within 8 weeks after admission. Also, treatment of malaria with chloroquine in the region was associated with 33% case fatality rate compared to 11% for children treated with more effective regime (Jane *et al*., 1996). A variety of drugs have been used to treat malaria in Kenya over the years, including quinine and related compounds such as chloroquine, antifolate combination drugs, antibiotics, and artemisinin compounds. Efforts to control the spread of the disease, however, are hampered by a growing trend of resistance to antimalarial medications. This is a great concern to the Kenyan government, whose goal is to decrease morbidity and mortality due to malaria by 30 percent by 2017 (Kenya Ministry of Public Health and Sanitation 2011). It is predicted that clinical infections and death will begin to increase due to rapid spread of drug resistance parasites (Hastings and Alessandro, 2000).

In addition to resistance to antimalarial combination therapies, there is also a concern with *Plasmodium* mixed infections. A mixed infection is defined as an infection with more than one type of species or genotype of *Plasmodium* (Ferdig and Su, 2000). Other groups have noted that a number of malarial infections are heterogeneous in their composition (Lorenzetti *et al.*, 2008). Mixed infections of different genotypes are highly prevalent in malaria-endemic areas, particularly in Africa and Southeast Asia (Kobbe *et al.*, 2006). In fact, almost all infections occurring in nature are mixed. Although highly understudied, the implications of a mixed infection are profound. They can cause a relapse as a result of emergence of the resistant subpopulation of parasites after the sensitive subpopulation has been eradicated by drug therapy. The existence of a resistant population may be a result of both divergent evolution, where parasites have acquired resistance mechanisms, and/or two cohabitating parasites when the individual is infected (Hyde, 2002). This phenomenon has been observed in areas of malaria endemicity in Africa and Southeast Asia where the mixed-infection prevalence is as high as 30% (Mayxay *et al.*, 2004). However, there has been conflicting evidence as to the true frequency of *Plasmodium* mixed infections (Richie, 1988). Furthermore, this problem is confounded by the inability to properly identify and differentiate *Plasmodium* mixed infections. This phenomenon is possibly due to the assay's detection limit, where susceptibility of one population ceases to affect the profile of the other subpopulation, effectively masking the existence of that subpopulation. At this low parasite subpopulation level, dominance of a resistance mechanism may not be high enough in the population and may result in the appearance of only one phenotype. The reverse is also possible, where a large susceptible subpopulation may influence the apparent phenotype of the whole population. Most naturally occurring malaria infections are composed of mixed subpopulations with different drug susceptibilities (Postigo *et al.*, 1998). It is likely that mixed infections of both drug sensitive and resistant parasites in a patient will produce test readings not representative of a typical resistant or sensitive parasite. Thus, there is a need of using clones which have identified these drug resistant sub populations to prevent treatment failures, misidentification of a populations susceptibility profile (Zimmerman *et al.*, 2004) and also in any evaluation procedure so as

to provide accurate test results. This study validated the use of a formulated TM by assaying *P. falciparum* reference clones. W2 considered as chloroquine resistant, mefloquine sensitive and artemisinin sensitive and 3D7 considered as chloroquine sensitive and mefloquine sensitive clones were used.

Materials and methods

Ethical consideration

This study was approved by Jaramogi Oginga Odinga teaching and referral hospital Ethics and review committee (JOOTRH ERC).

Sample preparation

These are clones which were kept in liquid nitrogen and needed to be culture adapted for the experiment. First the isolates were removed from liquid nitrogen and indicated in the nitrogen freezer log sheet, and then the vials were left to thaw in the incubator. Once the content liquefied it was transferred aseptically in to a 15ml centrifuge tube noting the volume. Slowly 1/5volume of 12% solution Nacl was added while swirling the tube. It was allowed to stand at room temperature for 5 minutes. 9 volume of 1.6%Nacl solution was added to it and was centrifuged at 1500rpm for 3minutes. Supernatant was aspirated before another 9volume of 0.9%Nacl and 0.2% dextrose solution were added. This was mixed gently and centrifuged at 1500rpm for 3min, and then supernatant was aspirated. It was resuspended in culture medium by adding 4.5ml of 10% complete medium and 0.5ml of washed Rbcs for a 5ml culture. Then the culture was gassed and placed in an incubator at 37^0c to grow. Medium was being changed after every 48 hours while gassing the bottles. Parasitaemia was checked three times weekly until the parasitaemia was 3-8%.

Once the clones were ready for assay they were subjected to EDTA and the formulated TM for at least 6hours before they were washed in RPMI 1640 by centrifugation at 2500 revolutions per minute for 3minutes for three consecutive times. 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 IC_{50} 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).

Results

Reference *Plasmodium falciparum* clones were assayed against a panel of antimalarials namely atermether, lumefantrine, Dihydroartemisinin and piperaquine.W2 considered as chloroquine resistant, mefloquine sensitive and artemisinin sensitive and 3D7 considered as chloroquine sensitive and mefloquine sensitive were used. Both clones were revived from liquid nitrogen and cultured for *in vitro* adaptation. Prior to exposing them to the respective drugs, they were subjected to EDTA and the formulated TM in a 5ml vacutainer tubes for the same duration as the field isolates and washed using complete medium. Both clones gave successful assays in 95% of the plates in both EDTA and TM. Moderate RFU values of between 2000 to 4000 parasites were obtained by 3D7clone while W2 had RFU values as high as 20,000 parasites. However, as shown in figure 1 below, their curves converged into a discernible dose-response curve for IC_{50} determinations with most/all of the points of the 10 standard dilutions included in the sigmoidal curve, for 90% of the plates and recording a reliability value (r^2) of almost 1 (to be specific 0.9999).

For W2 clone the IC_{50} values in ng/ml for the samples in EDTA were all higher than their respective IC_{50} values in TM as shown in table 1 below .The same table continues to show that there was no significant difference in IC_{50} values in samples treated with LUM (32.61 vs 29.32 ng/ml, p=0.369) and DHA (18.22 vs 14.93 ng/ml, P=0.2084). However, a high significant decrease in IC_{50} values for samples in TM was recorded when they were subjected to ART (3.118 vs1.873 ng/ml, p<0.001) and PPQ (18.93 vs 11.41 ng/ml, p=<0.001). ART emerged to have the best activity with the least IC_{50} values for samples in both EDTA and TM thus coinciding with the fact that W2 is artemisinin sensitive. On the other hand, LUM which is the first line partner drug to ART in treatment of uncomplicated malarial had the highest IC_{50} values in EDTA (32.61ng/ml) and TM (29.32ng/ml). As seen in figure 2 below, samples in both categories had the lowest individual IC_{50} of 20 and 21 ng/ml.

Compared to W2, 3D7 clone generally showed reduced IC_{50} values for all the antimalarials for samples in EDTA and TM. Unlike W2, samples in EDTA had lower IC_{50} values than samples in TM as shown in table 2. However, there was no significant difference for samples exposed to ART (1.667 vs 2.353 ng/ml, p= 0.255) and LUM (22.27 vs 9.905 ng/ml, p=0.3377) in the two categories, while there was a high significant decrease for samples in TM when they were treated with DHA (2.853 vs 10.03 ng/ml, p= 0.009) and PPQ (1.181 vs 13 ng/ml, p=0.028). Notably, in both clones, PPQ is the only drug with a persistent significant difference in IC_{50} values in the two anticoagulants. PPQ and ART had close IC_{50} ranges between the minimum and the maximum values as shown in the figures 3 below.

Using pearsons correlation coefficient, there was no correlation significance (P>0.05) between the two anticoagulants as indicated by their r values shown in table 3 and 4 below. However, no significant difference (P>0.05) was observed between the individual activities of drugs as illustrated earlier in tables 1 and 2.

Discussion

Among the assays of the reference clones, in both anticoagulants, the IC_{50} range between the individual minimum and maximum values were small, this reflects reduced biological variability due to the absence of resistant and sensitive subpopulations of parasites, therefore, they were able to produce distinct IC_{50} dose response curves with most points of the standard dilutions include in the sigmoidal curve. This is in accordance with a study which argued that a pure parasite population either resistant or sensitive to a drug, will produce a curve with a sharp inhibition of parasite growth at a relatively narrow range of drug concentration, whereas a mixed population with a minor resistant population of 10% or higher will produce a flattened curve or a double-hump curve, reflecting two parasite populations with different IC_{50}.(Liu *et al.,* 2008). Nonetheless, it has been reported that among the field isolates, most naturally occurring malaria infections are composed of mixed subpopulations with different drug susceptibilities (Postigo *et al.,*1998, Zimmerman *et al.,* 2004) but a large susceptible or resistant subpopulation may influence the apparent phenotype of the whole population (Co-em *et al.,* 2009), a factor relevant to the current finding. On the other hand, W2 considered chloroquine resistant demonstrated an aspect of 'resistance' in all the drugs by having elevated IC_{50} values in all the drugs except for ART since its considered artemisinin sensitive.

The high significant decrease in the medians for the W2 samples exposed to ART and PPQ and also a slight significant difference for DHA and PPQ in the 3D7 samples would have been affected by the selection of subpopulations in a clinical sample during initial culturing and isolation of the clones, since it has been reported that when clinical strains are culture adapted, they result in possible loss of the minor subpopulation and consequent misidentification of the population's susceptibility profile (Basco and Lebras, 1994, Basco and Lebras, 1990). Also in line with a finding which was concerned that during culture adaptation there could be selection of a dorminant clone (Liu *et al.,* 2008). Anyway, other studies have also shown that even with culture adaptation, the minor subpopulation is still detectable (Mbaisi *et al.,* 2004).

Additionally, the PPQ activities of 3D7 and W2 in both anticoagulants obtained in the current study were in line with the PPQ activities of 8.3 and 16 ng/ml in chloroquine sensitive and chloroquine resistant clones obtained by Vennerstrom *et al.,* (1992). It was further observed that PPQ and LUM among the clones had the least activity, and this could have been attributed by the fact that the drugs are longer acting partner drugs to artemisinin derivatives with much longer half life, of 3-4 days and 3-4 weeks for LUM and PPQ respectively (German and Aweeka, 2008) thus, in subsequent infections they are exposed to sub-therapeutic concentrations of these drugs, facilitating selection of parasites.

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Figure 1: Example of sigmoidal curves generated by clones indicating RFU values against the concentration of a drug.

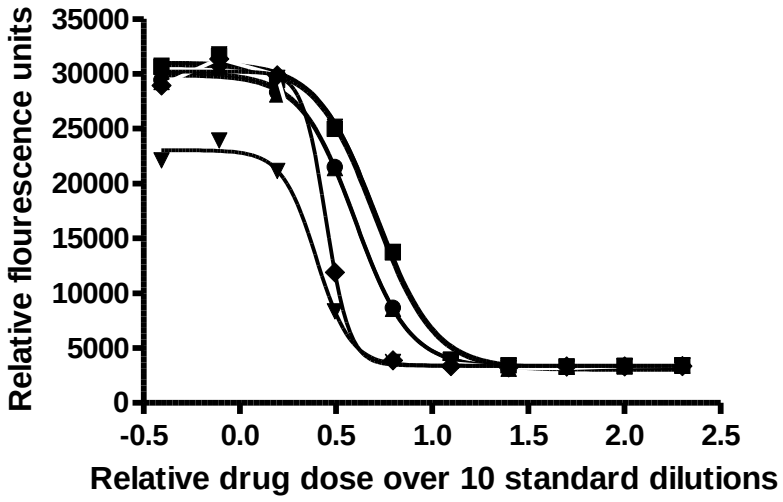
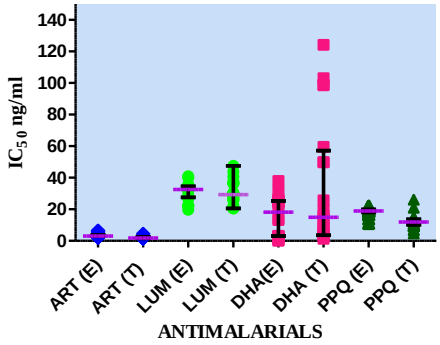


Figure 2: *In vitro* distribution of W2 IC_{50} values for samples in EDTA and TM



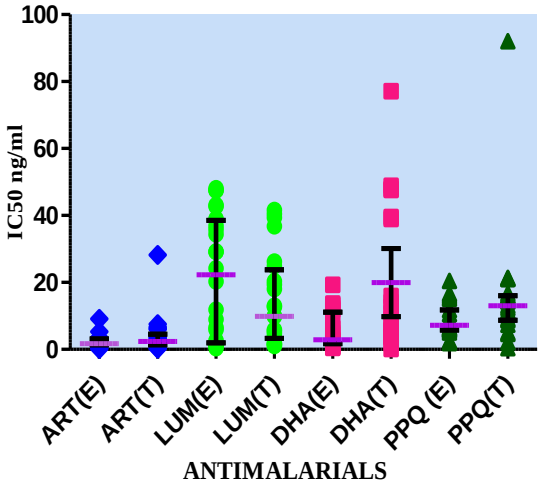
The abbreviations E and T represent samples in EDTA and in Transport medium respectively. The medians IC_{50} (purple bars) with their interquartile ranges in black for all the drugs

Table 1: Drug susceptibility testing of W2 reference clone in EDTA and TM against a panel of antimalarials

		Median IC_{50} in ng/ml for W2 *P. falciparum* reference clone transported in:				
		EDTA		**TM**		
DRUGS	**n**	**median**	**IQR**	**median**	**IQR**	**p-value**
Artemether (ART)	26	3.118	1.985-6.455	1.873	1.514-4.403	<0.001
Lumefatrine (LUM)	24	32.61	20 - 40.9	29.32	20.56-47.6	0.3697
Diydroartemisinin (DHA)	20	18.22	0.106-37.92	14.93	1.283-124.2	0.2084
Piperaquine (PPQ)	26	18.93	10.92-22.06	11.41	4.68-26.11	<0.001

Comparison of the medians using Mann - whitney test
Key: TM= Transport medium**,** n= number of samples, IQR=interquartile range, p = significance level of the test

Figure 3*: In vitro* distribution of 3D7 IC_{50} values for samples in EDTA and 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 2:

Drug susceptibility testing of 3D7 reference clone in EDTA and TM against a panel of antimalarials

		Median IC_{50} in ng/ml for 3D7 *P. falciparum* reference clone transported in:				
		EDTA		**TM**		
DRUGS	**n**	**median**	**IQR**	**median**	**IQR**	**p-value**
Artemether (ART)	22	1.667	0.131-9.09	2.353	0.1824-28.2	0.2549
Lumefatrine (LUM)	24	22.27	0.417 - 48.1	9.905	1.078-41.62	0.3377
Diydroartemisinin (DHA)	20	2.853	0.441-19.3	10.03	0.166-77.09	0.0098
Piperaquine (PPQ)	23	7.181	1.88 - 20.45	13	0.374 - 92	0.028

Comparison of the medians using Mann - whitney test
Key: TM= Transport medium**,** n= number of samples, IQR=interquartile range, p = significance level of the test

Table 3: Correlations between EDTA and TM: results for W2 reference clone

Sample in EDTA vs TM subjected to:	**Correlation coefficient (r**	**Probability P value**
ART-ART	-0.0110	0.9611
LUM-LUM	-0.277	0.1901
DHA-DHA	-0.2577	0.2727
PPQ-PPQ	-0.1487	0.4983

Table 4: Correlations between EDTA and TM: results for 3D7 reference clone

Sample in EDTA subjected to:	**Correlation coefficient (r)**	**Probability P value**
ART-ART	0.03275	0.8765
LUM-LUM	0.156	0.4771
DHA-DHA	-0.021	0.9297
PPQ-PPQ	-0.3025	0.1416

Correlation of the activity of antimalarials using Pearson's correlation coefficient of log IC_{50} values.
Key; P= significance level of the test, ART= Artemether, LUM= Lumefantrine, DHA= Dihydroartemisinin and PPQ= Piperaquine