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Original Research Article

Susceptibility profile of the population to artemether, lumefantrine, dihydroartemisinin and piperaquine for samples transported in a formulated transport media and ethylenediamine tetraacetic acid anticoagulants: a study conducted in Maseno area, Western Kenya

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ABSTRACT

Background: *In vitro* sensitivity testing is one of the preferred methods of measuring susceptibility and undertaking surveillance of antimalarial drug efficacy. This method is able to measure *Plasmodium falciparum* susceptibility to several antimalarial drugs simultaneously, away from the influence of host immune related factors. The technique is being transitioned to *ex vivo* requiring tests on fresh sample. Such *in vitro* studies of field *P. falciparum* have been attributed to diminished viability as they transition from host ecosystem to lab conditions due to lack of a proper medium to stabilize the parasites. It is therefore imperative to calibrate the sample stabilization media to reduce artificial effects to the assay. This study was meant to evaluate the effect of a formulated transport medium (TM) on viability of *Plasmodium*. The study assayed standard clones (W2 and 3D7), field isolates and compared the results with other published findings to come up with antimalarial susceptibility profile of the region.
Methods: Blood samples were collected from 322 assenting individuals from Maseno division visiting Chulaimbo Sub county hospital and confirmed positive for malaria. Each sample, split in to EDTA versus TM was analysed for susceptibility to artemether (ART), lumefantrine (LUM), dihydroartemisinin (DHA) and piperaquine (PPQ) using malaria SYBR green assay. IC_{50} was determined for each sample between TM and EDTA using dose response curves.
Results: Results showed that the IC_{50} values of the field isolates in EDTA were higher although not significant ($p=0.99$, 0.74, 0.68, 0.82 for ART, LUM, DHA and PPQ respectively) than those in the TM. Among the clones, PPQ was the only drug with a high significant IC_{50} decrease ($p<0.001$) in TM for the W2 and a moderately significant decrease ($p=0.028$) in EDTA for 3D7 clone.
Conclusions: Lower IC_{50} values recorded by the field isolates against the antimalarials were indicative of their high susceptibility to the drugs.

Keywords: EDTA, Susceptibility, Sensitivity, Ex vivo, Invitro, Surveillance

INTRODUCTION

The major contributory factor to antimalarial resistance has been documented to have been contributed by the inappropriate supply of antimalarials due to unregulated market, self-medication and underdosing.[1] Resistance to antimalarials, and lately reduced susceptibility to the commonly used artemisinin combination drugs at the Kenyan coast and other African countries, indicate an urgent need for surveillance to monitor sensitivity profiles of antimalarial drugs across Africa.[2] To accommodate this reality, a faster, effective, less expensive, and high through put means of screening the activities of drugs against a variety of malarial parasites would greatly assist in drug surveillance, pharmacoepidemiology and preclinical drug development. Currently, available methods are costly and

highly dependent on culturing of parasites prior to drug tests. Culturing alters the composition of the parasite since evolution is continuous, therefore the characteristics of the initial genotype will not be shown.

Challenges associated with the assessment of antimalarial drug resistance in *vivo* led to the introduction of a number of *in vitro* tests for the measurement of antimalarial drug susceptibility in the late 1970s.[3] *In vitro* tests avoid many of the confounding factors which influence in vivo tests by removing parasites from the host and placing them into a controlled experimental environment. Thus may be a more objective method to detect drug resistance since it eliminate these host factors, including reinfections, immunity, pharamacodynamics, and pharmacokinetics.[4] *In vitro* techniques allow multiple tests to be performed on isolates, several drugs to be assessed simultaneously, and experimental drugs to be tested.[5] There are many *in vitro* testing techniques endorsed by the World Antimalarial Drug Resistance Network in 2007: The World Health Organization (WHO) microtest, the radioisotope hypoxanthine incorporation, *Plasmodium* lactate dehydrogenase immune detection.[6-8] Lately, histidine-rich protein II assays and SYBR Green I.[9,10]

These *in vitro* drug assays in the field are preferred and perfomed on fresh isolates immediately after collection due to simplicity to perform than culture adapted.[11,12] However, blood samples containing *P. falciparum* parasites for testing may be stored at collection sites for days or transported long distances to the laboratory before cultivation, a factor which could result in reduced viability during subsequent culture adaptation process.[12] It has been reported that fresh isolates may not grow well initially in an *in vitro* culture, and some parasites eventually fail to establish during culture because of problems of adapting and surviving in vitro culture conditions.[13] It is important to come up with a medium which can keep the parasites energized and viable while reaching the laboratory to enhance immediate establishment of drug tests.

Studying drug resistance in *Plasmodium falciparum* requires accurate measurement of parasite response to a drug. Factors such as mixed infection of drug resistant and sensitive parasites can influence drug test outcome.[13] The parasites of *P. falciparum*, as well as those of other plasmodial species, exhibit a high degree of biological and pathological diversity between strains with respect to a number of important characteristics, such as pathogenicity, responses to drug treatment, and transmissibility by mosquitoes.[14]

Further, each *Plasmodium* species comprise a number of distinct strains which are autonomous, have stable biological entities and distinguishable by clinical, epidemiological or other features.[15] In addition, any strain infecting a host at any one time may often comprise of several distinct populations. As a result, considerable diversity could be demonstrated in the parasites present at a given geographical location.[16]

The exact nature of the mechanisms underlying most of the parasite's variable characteristics includes pathogenicity and transmissibility by mosquitoes. Therefore, to guide on the range of sensitivities and reduce such variations, it is important to assay reference clones since they have reduced biological variability due to the absence of resistant and sensitive subpopulations of the parasites.[13] Clones 3D7 and W2 are standard reference clones for *P. falciparum* which are susceptible and resistant to antimalarials respectively among other clones.

METHODS

Study site

The study included population residing within Maseno division and visiting Chulaimbo Sub District Hospital.

Study population

Patients who visited Chulaimbo Sub District Hospital presenting with symptoms of malaria were eligible for the study if they met the inclusion criteria. Children below 6 months were not recruited for the study because they are vulnerable in management, they are mostly under 5 kg and the drugs as per WHO regulations are administered from 5 kg and above. These individuals still have passive immunity from the mothers which can affect their response to drugs.[17]

Patients testing positive for malaria upon testing by microscopy were requested to participate in the study. Consent for patients who were under eighteen years of age was signed by their guardians. Consenting/assenting individuals were requested to provide 4 ml of blood samples for the study. Results obtained from the processed pre-culture thick and thin films formed the basis for the criteria.

Reviving of the W2 and 3D7 clones

These were clones which were kept in liquid nitrogen and needed to be recultured for the experiment. Thawing procedure was done as follows; 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 at 37 ^{0}C. Once the content liquefied it was transferred aseptically in to a 15 ml centrifuge tube noting the volume. Slowly 1/5 volume of 12% solution NaCl was added while swirling the tube. It was allowed to stand at room temperature for 5 minutes. Nine volumes of 1.6% NaCl solution was added to it and was centrifuged at 1500 rpm for 3 minutes. Supernatant was aspirated before another nine volumes of 0.9% NaCl supplemented with 0.2% dextrose solution were added. This was mixed gently and centrifuged at 1500 rpm for 3 min, and then supernatant was aspirated. It was resuspended in culture medium by adding 4.5 ml of 10% complete medium and 0.5 ml of washed RBCs for a 5 ml culture. Then the culture was gassed and placed in an incubator at 37 ^{0}C. Medium

was changed after every 48 hours while gassing the bottles. Parasitaemia was checked three times weekly until 3-8% was obtained.[7] They were then subjected to EDTA and formulated TM in 5 ml vacutainer tubes and transported to the laboratory while kept at 4 ^{0}C in a cool box with ice packs.

Procedure for the in vitro assay

On arrival of the field isolates and clones in EDTA and TM to the laboratory, the following stepwise procedures were undertaken in processing samples for culturing.

Washing of the blood samples

Samples brought in vacutainer tubes containing EDTA and TM in a cool box were transferred aseptically to a 15 ml tubes for centrifugation inside the biosafety cabinet hood to avoid contamination. The content in these tubes were labeled accordingly. Washing was done using 10% complete medium by centrifugation at a speed of 2500 revolutions per minute (rpm) for 3 minutes prior to removal of the supernatant, for three successive times. The resulting pellet was ready for loading into drugs predosed plates.

Coating of the microtitre plates with drugs

Coating of the mother plates

To make the in vitro antimalarial drug sensitivity test plates, sterile 96 well flat-bottom microtitre plates were dosed with decreasing concentrations of test grade antimalarial drugs (artemether, dihydroartemisinis, lumefantrine, and piperaquine). This was done by introducing 300 ml of the diluted drugs in their final concentration to each well in the first column, with every two consecutive rows (A and B, C and D, E and F, G and H) having the same drugs hence every concentration of drugs was done in duplicate, while the rest of the columns had 150 ml of CMS. 150 ml of the content in the first column was picked using the 200 µl fixed volume eppendorf pipette and a disposable sterile tip and was serially diluted across the 10 columns of the plate, excluding the last 2 columns which acted as controls. This gave rise to a mother plate which was used to make 10 daughter plates for parasites culture. Several of these mother plates were made and kept at -65 ^{0}C to -80 ^{0}C for 1-2 weeks and could be freeze thawed only twice for experimental use.

Coating of the daughter plates

To make a daughter plate, 12.5 µl of the content of each well in the mother plate were aseptically transferred to the wells of the sterile microtitre plates using the 200 µl fixed volume eppendorf pipette and disposable sterile tips. Coating was done starting with the control wells (columns 12 and 11) and following an increasing order of concentrations, ending with column 1. New sterile disposable tips were fitted to the eppendorf pipette and the next daughter plate was set up in exactly the same way, and so on until the entire scheduled daughter plates were dosed. These plates were kept at -20 ^{0}C and prior to use in drug screens they were thawed in 35-37 ^{0}C culture incubator for 1-2 hours.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
A												
B												
C												
D												
E												
F												
G												
H												

Figure 1: Layout of the microtitre plate.

Antimalarial drugs assay procedure

Giemsa stained thin blood smears were again prepared for every sample (clone and the field isolates) to confirm the presence of parasites and to calculate the % parasitaemia. Calculated amount of washed blood was picked using a 200 µl fixed volume Eppendorf pipette and a disposable sterile tip and mixed in a trough with 10.3 ml of 10% complete medium enough to dose the 96 well microtitre plate. Blood samples with >1% parasitemia were adjusted to 1% parasitemia at 2% hematocrit, by adding prepared fresh RBCs and those with <1% parasitemia were used unadjusted at 2% haematocrit. About 100 µl of the samples collected in the normal EDTA vacutainer tube and that in the TM tubes were transferred to the pre-coated drug plates separately. Dosing was done starting with the control wells in column 11 and 12 and following an increasing order of concentrations, ending with wells in column 1. The lids were replaced on the microtitre plates and contents labeled accordingly. The plates were shaken gently, without lifting it from the work bench, so that the drugs would be homogeneously suspended in the assay components.

The plates were placed in tight ziplock bags with humidity provided by wet paper towels and gassed with mixed gas of 90% N, 5% CO_2 and 5% O_2. After which they were incubated in 37 ^{0}C for 72 hours to allow growth of the parasites. To facilitate reading of results, the plates were dried in the hood overnight then kept in tightly sealed ziplock bags until they were taken to KEMRI for reading. At KEMRI, the plates were rehydrated by adding 100 µl of lysis buffer containing SYBR Green I (1 X the final concentration) to each well of the plate and were mixed gently. The plates were then incubated for an overnight stay at room temperature in the dark. Relative fluorescence

units (RFU) per well were examined using the Tecan Genios Plus flourimetre (Tecan US, Inc., Durham, NC).

RESULTS

IC_{50} obtained from each of the drugs against the clones and field isolates in both EDTA and TM were compared. Further comparison was done with the results of other published studies. Summaries of the correlates are shown in figures 4.6-4.9. In the correlation, 3D7 and W2 clones were used to guide on the range of sensitivity of the field isolates with mixed strains.

When the IC_{50} values of all the samples exposed to ART were compared in Table 1, it was observed that in EDTA, the isolate had highest IC_{50} values compared to all the other samples tested (4.275 ng/ml for the isolate, 1.667 ng/ml for 3D7 clone and 3.118 ng/ml for W2 clone). This was also observed in samples in the TM (4.887 ng/ml for the isolate, 2.35 ng/ml for 3D7 clone and 1.87 ng/ml for W2 clone). 3D7 the chloroquine sensitive strain had least IC50 values in both media (1.667 ng/ml in EDTA and 2.353 ng/ml in TM). The W2 (CQ resistant and AR sensitive) IC_{50} values in ART were considered sensitive when compared to the results of other drugs tested. It was further observed that the IC_{50} ranges among the field isolates were higher than the clones despite comparable medians. Their IC_{50} were not tight with most samples above the median.

Table 1: Response of *P. falciparum* samples in EDTA and TM against artemether.

Sample	N	Median IC_{50} (ng/ml) for samples, (median (IQR)	
		EDTA	TM
Isolate	322	4.275 (0.1169-251.6)	4.887 (0.226-112.1)
3D7	22	1.667 (0.131-9.09)	2.353 (0.182-28.2)
W2	26	3.118 (1.985-6.455)	1.873 (1.514-4.403)

EDTA: Ethylene diamine tetra acetate, TM: transport medium, n: number of samples, IQR: interquartile range

When the samples were exposed to LUM (Table 2), the IC_{50} range for the isolates both in EDTA and TM were larger than for the clones, however they have remained the most sensitive with the least IC_{50} values in EDTA (5.409 ng/ml for the isolate versus 22.27 ng/ml and 32.61 ng/ml for the clones) and in TM (4.007 ng/ml for the isolate versus 9.905 ng/ml and 29.32 ng/ml for the clones). More than 50% of the isolates in both categories presented with individual IC_{50} values which were lower than the median IC_{50} values of the resistant W2 clone in EDTA (32.61 ng/ml) and TM (29.32 ng/ml). W2 clone was the least sensitive with the highest IC_{50} values (32.61 ng/ml in EDTA and 29.32 ng/ml in TM) while 3D7 clone had moderate IC_{50} values (22.27 ng/ml in EDTA and 9.905 ng/ml in TM). Remarkably, the IC_{50} values obtained by W2 in LUM were the highest values in the whole study, recording the minimum individual IC_{50} values in both anticoagulants to be above 20 ng/ml.

Table 2: Response of *P. falciparum* samples in EDTA and TM against lumefantrine.

Sample	N	Median IC_{50} (ng/ml) for samples, (median (IQR)	
		EDTA	TM
Isolate	322	5.409 (0.198-373.7)	4.007 (0.103-190.1)
3D7	24	22.27 (0.417-48.1)	29.905 (1.078-41.62)
W2	24	32.61 (20-40.9)	29.32 (20.56-47.6)

EDTA: Ethylene diamine tetra acetate, TM: transport medium, n: number of samples, IQR: interquartile range

Further, when the samples were exposed to DHA (Table 3), the isolate become the most sensitive sample to DHA which is a derivative of artemisinin especially in the TM (isolate=1.69 ng/ml, 3D7=6.229 ng/ml and W2=14.93 ng/ml). W2 considered artemisinin sensitive had the highest values of IC_{50} to DHA compared to the isolates (1.69 and 14.93 ng/ml in TM versus 2.57 and 7.941 ng/ml in EDTA) an implication that the samples from this region are still sensitive to the drug. Meanwhile, the 3D7 clone considered a sensitive strain has got comparable results with the isolate especially the samples in the EDTA anticoagulant (isolate=2.57 ng/ml, 3D7=2.85 ng/ml and W2=18.22 ng/ml). Unlike other clones in other drugs, it was noted that there was variability in response to DHA among the clones with most IC_{50} generally on the upper interquartile range especially for the 3D7 clone. However, isolates like those in other drugs had higher IC_{50} interquartile ranges depicting variability.

Table 3: Response of *P. falciparum* samples in EDTA and TM against dihydroartemisinin.

Sample	N	Median IC_{50} (ng/ml) for samples, (median (IQR)	
		EDTA	TM
Isolate	322	2.567(0.32-292.7)	1.691 (0.119-401.1)
3D7	20	2.853 (0.441-19.3)	6.229 (0.166-39.52.09)
W2	20	18.22 (0.106-37.92)	7.941 (1.283-25.71)

EDTA: Ethylene diamine tetra acetate, TM: transport medium, n: number of samples, IQR: interquartile range

In PPQ IC_{50} distribution in Table 4, all the clones had similar IC_{50} range, the isolates IC_{50} range were comparable despite the anticoagulants used. In EDTA W2 was the least sensitive (18.93 ng/ml) strain among them all while the isolate and 3D7 clone had comparable IC_{50} values (3D7=7.89 ng/ml versus 7.181 ng/ml). In TM the isolate

was still sensitive (3D7=12.71 ng/ml versus 6.911 ng/ml and W2=11.41 ng/ml).

Table 4: Response of *P. falciparum* samples in EDTA and TM against piperaquine.

Sample	N	Median IC_{50} (ng/ml) for samples, (median (IQR)	
		EDTA	TM
Isolate	322	7.887(0.629-380.3)	6.911 (0.516-334.8)
3D7	23	7.181 (1.88-20.45)	12.71 (0.374-21.36)
W2	26	18.93 (10.92-22.06)	11.41 (4.68-26.11)

EDTA: Ethylene diamine tetra acetate, TM: transport medium, n: number of samples, IQR: interquartile range

DISCUSSION

Artemisinin-based combination therapies (ACTs) have shown excellent efficacy and are now recommended to treat falciparum malaria in nearly all countries.[18] ACTs include potent, short acting artemisinins that rapidly reduce parasite biomass and alleviate malaria symptoms and longer acting partner drugs that improve antimalarial efficacy and reduce the risk of selection for artemisinin resistance.[19] This could explain the high sensitivity to artemisinin derivatives as compared to their partner drugs in the current study for both the field isolates and the reference clones and in another study.[20]

Artemether-lumefantrine (AL) is the most widely recommended ACT in Africa.[21] ACTs were adopted as first-line treatment for uncomplicated malaria in many African countries in 2006, including Kenya where it has shown outstanding efficacy.[22,23] Studies have also confirmed its high success rates of 90 to 95% in many areas where malaria is endemic including Kenya.[24-26] These concur with the results of LUM in the current study where the median IC_{50} values of 5.4 ng/ml in EDTA and 4 ng/ml in TM were the least ever reported.

The *in vitro* activity of LUM against field isolates from several areas where malaria is endemic has been investigated using the WHO microtest, and in all these studies LUM activity was high with IC_{50} of 15 ng/ml for more than 95% of isolates.[27-32] A similar study which was performed and observed even higher IC_{50} range and IC_{50} values of 106 ng/ml for most isolates were in agreement with the report of a Senegalese study.[33,34] Therefore, LUM is still effective in malarial treatment in the region.

This study also generated the sensitivity data for ART and found that the field isolates were not as sensitive as the artemisinin sensitive W2 reference clone and 3D7 clone, a worrying issue at hand, suggesting that the drug should be monitored and used rationally. In addition, despite the comparable medians the IC_{50} among the isolates were not tight with most samples above the median. This depicts a high variablility with response to the drug. Nevertheless, the interpretation of these results should also consider the weaknesses of the standard ex vivo tests and other methodological variability. But then again, this study results were also higher than the results of a previous Cameroonian study whose IC_{50} value was 3.71 ng/ml but in accordance with other studies carried out in sub Saharan Africa.[35-37]

Introduction of PPQ for use as a monotherapy in Africa is not recommended due to the rapid emergence of clinical resistance observed in China.[38-40] In that case PPQ and DHA have been chosen as partner drugs as a second line treatment of uncomplicated malaria in Kenya.[18] Our results suggest that PPQ and DHA are equally active against the field isolates as compared to the sensitive and the resistant reference clones. In a study on field isolates from Madagascar the IC_{50} of PPQ were widely dispersed ranging from 12.5 to 250 ng/ml with values of 100 ng/ml for majority (83%) of isolates.[41] By contrast, this study showed moderate level of activity of PPQ within similar range but with IC_{50} of 100 ng/ml for only 14% in EDTA and 10% in TM. However, a higher level of activity was demonstrated by a Cameroonian study, with all the isolates within a narrower range of 7.76 to 78.3 ng/ml.[42]

PPQ and LUM as partner drugs demonstrated a good activity with field isolates in this study although they showed the least activity with high median IC_{50} values compared to the artemisinin derivatives (ART and DHA) drugs. This could have been attributed by the fact that they have a longer half-life (3-5 days for LUM) and much longer (3-4 weeks for PPQ) than other ACT partner drugs.[43] This facilitates selection of parasites with reduced sensitivity to the partner drugs such that in case of subsequent infections, parasites will be exposed to sub-therapeutic concentrations of the drugs, a factor which can easily lead to reduce susceptibility and even resistance to the drugs.[44] This selection is more in PPQ which has a longer half-life than LUM. PPQ however, benefits from the activity of DHA as a partner drug which has shown excellent efficacy in Africa and even in the current study.[45-47] DHA sensitivity results were much lower than the results of W2 clone (considered AR sensitive) but comparable with other ex vivo field isolates studies.[48,49] Subsequently, PPQ resistance does not appear to be a major problem and therefore, DHA and PPQ combination has been adopted as a first line therapy in South East Asia.[18]

On the field isolates, DHA showed the best activity of 2.567 ng/ml in EDTA and 1.691 ng/ml in TM. These values were below the values of both reference clones in the current study although higher than the results obtained in a study.[50] This study generated DHA IC_{50} median of 1.29 ng/ml for the field isolates against a 1.12 ng/ml of CQ sensitive and 1.39 ng/ml of CQ resistant clones and in another study in Burkina Faso.[51] The activity of DHA demonstrated by the current study isolates was below the

cut off of 10.5 ng/ml used in a study in Congo and in the Kenyan Coast but comparable with other studies.[52-55]

A marginal statistically significant correlation observed between PPQ and DHA in EDTA samples (r=0.1281; p=0.0203) and no correlation in TM samples (r=0.0372; p=0.5024) was encouraging because at this low coefficient correlation level, *in vitro* cross-resistance is unlikely to occur. These data were in accordance to the previous reports and another study which found absence of cross resistance due to the absence of association between PPQ and genes involved in quinolone resistance.[48,56] Thus, a reassurance for sustained use of the drug which is inexpensive, safe and a highly effective treatment for uncomplicated *P. falciparum* and *P. vivax* malaria.[57,58] Besides, it offers a better post treatment prophylatic effect following therapy compared with artemether lumefantrine.[20,48,60]

Limitations

Some of the limitations faced by the study were: variation on the estimation of parasitaemia, possibility of drugs residues despite washing by centrifugation, possibility of the effects of the media the standard clones had been exposed to before subjecting them to TM.

CONCLUSION

The field isolates result show that population infected with *P. falciparum* are still responsive to the tested drugs. Importantly, the results were comparable to the CQ sensitive 3D7 clone than CQ resistant W2 clone. The study further suggests that artemisinin derivatives are still very efficacious and dihydroartemisinin piperaquine seems a valuable alternative ACT. Variations in the positive correlations experienced by the drug pairs in TM and EDTA anticoagulants is possibly due to cross resistance among them, however this requires further research to elucidate on the finding.

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