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PARASITE LACTATE DEHYDROGENASE ASSAY FOR THE DETERMINATION OF ANTIMALARIAL DRUG SUSCEPTIBILITY OF KENYAN FIELD ISOLATES
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ABSTRACT

Background: **Researchers have reported that parasite lactate dehydrogenase p(LDH) could be used to determine chemo-sensitivity of plasmodia to compounds with known or presumed antimalarial activities.**
Objective: **To determine the drug sensitivity profiles of field adopted malaria isolates from Kisumu using p(LDH) instead of hapoxanthine assay.**
Design: **Prospective field and laboratory study.**
Setting: **Walter Reed, KEMRI malaria laboratory (Nairobi) and Kisumu District Hospital.**
Subjects: **Twelve field laboratory adopted isolates from Kisumu, five laboratory adopted isolates from other regions in Africa and three reference strains from Walter Reed army Institute of Research, Washington, DC.**
Results: **The p(LDH) enzyme assay was successfully used to measure the IC50 of six antimalarial drugs, chloroquine, quinine, mefloquine, dehydroartemisinin, atovaquone and halofantrine but was not successful with the four other antimalarial drugs, doxycycline, azithromycin, pyrimethamine and sulphadoxine. The Kisumu isolates tested were resistant to chloroquine and mefloquine but sensitive to quinine and the new antimalarial drugs, atovaquone, halofantrine and dehydroartemisinin.**
Conclusion: **The non-radioactive p(LDH) can be used for the determination of drug sensitivity to Kenyan field isolates. It is more suited for use in a resource limited environment and may lead to more judicious prescription of new and more expensive antimalarial drugs and mitigate against the rapid spread of multi-drug resistant parasites in the East African region.**

INTRODUCTION

The unique ability of plasmodial lactate dehydrogenase p(LDH) to utilise 3-acetyl pyridine dinucleotide (APAD) in lieu of NAD as a co-enzyme in the conversion of pyruvate to lactate, led to the development of a biochemical assay for the detection of plasmodial parasitaemia (1,2). It was further shown that this assay could be used for the determination of the chemo-sensitivity of plasmodia to compounds with known or presumed antimalarial activities (1,3). Delay in the institution of effective chemotherapy can lead to higher complication rates and prolonged hospital stay (4). One of the ways to reduce this cycle is the rational use of antimalarial drugs and routine sensitivity testing must be an integral part of any such antimalarial drug-use programme.

The determination of parasite chemosensitivity to antimalarial compounds is presently determined by a variation of two basic techniques the schizont inhibition and the ^{3}H-hypoxanthine uptake microtest (5). The former requires microscopy for scoring and is not suitable for high throughout due to observer bias and fatigue. On the other hand, hypoxanthine uptake technique requires radio labelled materials, is expensive and cumbersome for routine clinical laboratory use. In contrast, p(LDH) assay is a non-radio labelled biochemical microtest with objective end-points determined with an ELISA reader (1).

In vitro studies in Kenya revealed that significantly more isolates were sensitive to amodiaquine compared to chloroquine (6). Hence, amodiaquine was recommended to replace chloroquine in Kenya because of its demonstrated efficacy, reduced therapeutic toxicity and low cost in the face of chloroquine resistance (7,8). Fansidar was recommended to remain a reasonable choice for treatment of blood slide - confirmed 4-aminoquinoline resistant malaria, and quinine to be retained for severe or complicated malaria before new drugs are made affordable and available (9). Comparative studies have shown that dehydroartemisinin predictably give faster parasite and fever clearance than other antimalarial drugs (10). Successful trials have also been carried out with atovaquone, a hydroxynaphthoquinone,

anti-parasitic drug for the treatment of malaria, toxoplasmosis, and *neumocystis carinii* pneumonia (11). This study was undertaken to assess the usefulness of the p(LDH) assay for the determination of *in vitro* drug sensitivity of Kenyan isolates against these six antimalarial drugs.

MATERIALS AND METHODS

Parasite isolates used in the study included, KS140, KS041, KS021, KS155, KS157, KS211, KS012, KS031, KS168, KS047, KS063 and KS193 which were collected from malaria patients in Kisumu, Kenya. Each isolate was transported to the laboratory using transport media (5). Other isolates Som A6 (Somali strain), M24 (Mombasa strain), FCB were donated by the Wellcome Trust Research Laboratories, Kenya. KS39 and S104 were culture adopted *P. falciparum* isolates stored in liquid nitrogen (KEMRI malaria laboratory; Immunology section). These isolates, identified as *P. falciparum* were cultivated using standard techniques (1). Parasitaemias were assessed using both thin and thick smear procedures as well as p(LDH) and tritiated hypoxanthine uptake. When parasitaemias were above 1%, drug tests were set up after diluting the cultures to initial parasitaemia of 0.2% or 0.4% depending on the drug to be tested and the incubation period for the drug (48 or 66 hours).

The reagents used in this study for p(LDH) analysis were obtained from Sigma (St. Louis, Mo) and included lithium lactate, trizma buffer, triton X-100, nitroblue tetrazolium (NBT), phenazine ethosulphate (PES), 3- acetyl pyridine dinucleotide (APAD) and nucleic acid(NAD). The malstat reagent, which is a formulation for parasite LDH detection, was obtained from flow, Inc. (Portland, OR.) and used as outlined earlier (1). The RPMI 1640 medium was obtained from Amersham International (Buckinghamshire, U.K). The pre-dosed microtitre drug test plates were ordered from WHO regional office, Manila (The Philippines) and included, chloroquine, amodiaquine, quinine, pyrimethamine and sulfadoxine. Later, drug plates were prepared in KEMRI laboratories using WHO standard procedures for the drugs, chloroquine, mefloquine, quinine, dehydroartemisinin, atovaquone, halofantrine, doxycycline, azythromycin, pyrimethamine and sulfadoxine.

Sensitivity assays: All assays were performed in triplicates. Eight-fold 1 in 2 serial dilutions of drugs were distributed into a 96-well microtitre plates in which fresh and infected erythrocytes were added to give a volume of 200 µl well and initial parasitaemia of 0.2% or 0.4% depending on the time of incubation (48 or 66 hours) and the drugs for testing. Control wells with uninfected erythrocytes and infected erythrocytes in medium without drugs were always included in each plate to monitor parasite growth, 100µl of prepared culture medium was mixed with an equal amount of the starting concentration of the drug. A two fold dilution was carried out up to well number ten, and the remaining medium discarded. The 96 well drug plates were divided into three chambers to allow for multiple comparative studies, that is: chamber l (A, B, C) for hypoxanthine studies; chamber 2 (D, E, F) for p(LDH) studies while the last chamber(G,H) was for microscopy. Plates were incubated at 37°C for 24 hours in a humidified chamber in the gas mixture, following which 10 µl per well 0.5 µci of ^{3}H hypoxanthine was added to the correct wells (5) and the trays were incubated for a further 24 hours for the hypoxanthine assay. The 48-hour isotopic microtest was performed according to the modified method (5).

Measurement of drug sensitivity profiles for used and candidate antimalarial drugs using parasite lactate dehydrogenase p(LDH) assay: The parasite lactate dehydrogenase assay was performed as a 48 hour test without ^{3}H-hypoxanthine addition. The fast acting drugs were set at 0.2% initial parasitaemia for 48 hours. These included chloroquine, starting concentration 100ng/ ml, mefloquine 100ng/ml, halofantrine, l0ng/ml, quinine, 500ng/ml, dehydroartemisinin, l0ng/ml and atovaquone, l0ng/ml. The slow acting drugs were set at 0.4 % initial parasitaemia, for 66 hours. These included doxycycline 1000ng/ml, azythromycin 10,000 ng/ml, sulfadoxine 10,000ng/ml and pyrimethamine 250 ng/ml. At the end of the incubation period, 50 µl of 1% parasitised red blood cells of each isolate were put in 96 well microtitre plates (incubated for 20 minutes with 100µl of malstat reagent). Equal volumes of nitroblue tetrazolium (NBT) and phenazine ethosulphate (PES) were mixed and 10µl of fresh mixture added to each well and incubated in the dark for 20 minutes. The plate was read at optical density 650nm wavelength after the reaction was stopped, with 5% acetic acid (1). The blue formazan product was evaluated by end point analysis at 650 nm using ELISA reader (Molecular Devices Thermomax 7). The p(LDH) data was then analysed by log-logit method using softmax nine software that directly extrapolated the titration data of the drugs (1). The IC50 values were expressed in nanograms per mililitre (ng/ml) of test culture. The p(LDH) assay was run for each isolate with or without the drug in the 96-well culture plates. The reference strains with known drug sensitivity profiles for chloroquine and mefloquine D6 (chloroquine sensitivity, mefloquine resistant) and W2 (chloroquine resistant, mefloquine sensitive) served as controls for the wells with or without drug assays (1).

Statistical analysis: Analysis of variance (ANOVA) was used to compare the IC50 values between drugs and between isolates. Means were separated using the SNK test. All tests were carried out at 5% level of significance.

RESULTS

Table 1 shows the IC50s of the isolates for the different drugs. For chloroquine only isolate KS044 was significantly higher than the resistant standard W2, suggesting that this isolate is highly resistant to the drug. Isolates KS063 and KS155 were also resistant. Other isolates were not significantly different from the susceptible D6. All the isolates tested were sensitive to quinine though at different levels of IC50 values. The isolates showed three categories of response to the drug. Isolates, KS012, KS193, KS031, and D6 were the first group: most sensitive in decreasing order; isolates, W2, KS157, KS168, KS068, KS211, KS047 and KS044 were in group two: nearly sensitive though not significantly different from the first group; and the third group, KS155, had the highest reading though not significantly different from the second group. Isolate KS211 had the highest mean IC50, for dehydroartemisinin though this was not significantly different from KS193 and KS044. The other isolates,

including the reference strains, D6 and W2 were highly sensitive to very low doses of dehydroartemisinin. Only two field isolates and the reference strains could be replicated for atovaquone. The three isolates, KS044, KS155 and W2 were sensitive to atovaquone, though D6 gave the lowest sensitivity level with this drug.

Table 1

Mean ± S.E. IC5O (ng/ml) p(LDH) values for chloroquine (CQ), mefloquine, quinine and dehydroartemisinin for different field isolates and two reference isolates

Isolate	Mean IC50 ± SE Chloroquine	Mean IC50 ± SE Mefloquine	Mean IC50 ± SE Quinine	Mean IC50 ± SE Dehydroartemisinin
D6	6.33b ± 0.76	14.08a ± 5.54	41.33b ± 6.10	0.48c ± 0.089
KS044	67.82a ± 50.90	9.95a ± 3.44	58.45ab ± 18.70	0.56bc ± 0.29
KS063	36.65ab ± 9.95	17.88a ± 10.12	47.30ab ± 10.70	0.19c ± 0.01
KSI55	34.21ab ± 6.4	7.41a ± 1.06	87.57a ± 15.65	0.20c ± 0.095
KS00I	23.40b ± 7.80	1.90a ± 0.60	-	-
KS168	3.32b ± 0.80	5.44a ± 2.22	46.82ab ± 16.60	0.54c ± 0.29
KS157	14.96b ± 7.11	3.92a ± 2.46	44.70ab ± 7.40	0.22c ± 0.05
KS012	14.37b ± 4.40	18.07a ± 2.77	22.26b ± 5.00	0.50c ± 0.032
KS211	7.93b ± 2.74	15.09a ± 5.70	52.55ab ± 5.40	1.38a ± 0.99
KS031	7.83b ± 1.74	9.38a ± 2.32	35.68b ± 14.00	0.33c ± 0.042
KS193	4.74b ± 1.13	7.48a ± 2.09	24.80b ± 9.70	1.26ab ± 0.50
KS047	4.08b ± 0.84	8.02a ± 4.44	53.36ab ± 27.00	0.56c ± 0.17
W2	16.57b ± 2.28	9.34a ± 1.76	45.10ab ± 8.40	0.54c ± 0.12

The means within a column followed by the same letter are not significantly different (SNK, p=0.05).

Table 2

Summary of measurement of mean IC50 (in ng/ml) p(LDH) values for six antimalarial drugs

Drug	Field parasites combined	Reference strain D6	Reference strain W2
Chloroquine	19.56 ± 4.17	6.33bc ± 0.75	16.57b ± 2.27
Mefloquine	9.83bc ± 1.19	14.08b ± 5.50	9.34b ± 1.76
Quinine	45.37a ± 4.95	41.33a ± 6.10	45.1a ± 8.4
Dehydroartemisinin	0.49c ± 0.08	0.48c ± 0.09	0.54b ± 0.12
Atovaquone	0.36c ± 0.03	0.14c ± 0.04	0.33b ± 0.06
Halofantrine	1.47c ± 0.44	-	-

The means within a column followed by the same letter are not significantly different (SNK, p=0.05)

Table 3

IC50s of drugs which failed to give reproducible results by p(LDH)

Reference strain	Doxycycline (l000ng/ml)	Azithromycin (10,000ng/ml)	Pyrimethamine (250ng/ml)	Sulfadoxine (10,000ng/ml)
D6	F	10000	0.0137	6090
	8300	2900	F	F
	391	2970	267.00	F
	1690	1700	30.40	F
	1560	546	9400.00	F
	F	786	3.820	F
	2190	391	0.840	F
	2490	1800	34.500	F
W2	4490	3300	249.000	110
	F	2490	2.270	0.00013
	39500	25000	9.180	F
	19200	10800	2.280	19500
	2300	6250	0.083	F
	0.0059	3130	62.500	F
	2360	2070	0.0091	F
	F	F	F	F

Table 2 shows the IC50 values of each drug for all the field isolates combined and the reference strains. For the field isolates combined, quinine had the highest overall IC50, which was significantly different from all others. Chloroquine was next highest, but not significantly different from mefloquine. All the other drugs tested had very low over-all IC50 values, which were not significantly different. For the reference strains, quinine had a significantly higher IC50 value than the other drugs. Both chloroquine and mefloquine were similar. Mefloquine was significantly different from the other two drugs (dehydroartemisinin and atovaquone) for D6 but not for W2. Four drugs, doxycycline, azithromycin, pyrimethamine and sulfadoxine failed to give reproducible IC50 results using the reference strains, and were therefore not tested further for p(LDH) drug sensitivity. In most experiments the drugs failed to produce a standard curve by the log-logit method (Table 3).

DISCUSSION

The results of this study clearly indicate that the sensitivity patterns of Kenyan isolates to chloroquine, mefloquine, quinine and halofantrine can be determined using the p(LDH) assay. The 50% inhibitory concentration (IC50) was defined as the concentration at which the p(LDH) produced by the parasites (positively correlated with the number of viable parasites) was inhibited by 50% as compared with the drug free control wells. Failures of some isolates to give successful p(LDH)lC50 was probably due to a low level of parasitaemia in the drug plates, making it impossible for the extrapolation of the IC50 using log-logit by soft max nine software (1,12). There were also some field isolates which failed to grow, probably due to an inherent incapacity of certain isolates to adapt to *in vitro* culture conditions (5). This observation of the limitation of p(LDH) for drug susceptibility due to low level parasitaemia was also reported in previous studies (13,14). The study also cited the major limitation of the enzymatic test as its level of sensitivity to detect malarial LDH (15).

This study was carried out at a haematocrit of 1%, and a significant LDH activity was not observed at a parasitaemia of below 1%. In the culture adapted field isolates, the initial parasitaemia of which a sigmoid curve could be plotted for LDH activity was <0.4%, the same cut-off reported earlier following *in vitro* studies. The drug sensitivity studies were carried out using haematocrit of 1%, 2% and 3%. By increasing haematocrit, the human LDH background effect was also increased making it difficult to visualise the p(LDH) readings. The results suggest that the enzymatic assay can be applied for *in vitro* drug screening using culture adapted *P. falciparum* clones (D6, W2), and also to an extent, by using culture adapted *P. falciparum* field isolates.

The IC50 values obtained for quinine, mefloquine, halofanthrine, chloroquine, dehydroartemisinin and atovaquone against *P. falciparum* were within susceptible range (16,17,10). This attests to the potential of the drug atovaquine, a broad spectrum anti-infective agent for clinical use against malaria and opportunistic infections in AIDS patients (16). An increase in sample size is likely to give a better picture of the anti-malarial drug resistant pattern in Western Kenya. These isolates were collected from Kisumu, a malaria endemic region in Western Kenya. Carrying out the same study in other parts of the country like the coastal and the highlands region is likely to give a more complete picture of the drug susceptibility pattern in the country and potentially lead to a comprehensive and rational policy for malaria treatment and prophylaxis. No study has previously reported on drug sensitivity profiles of Kenyan isolates by use of p(LDH) assay against the six antimalarial drugs tested. By incorporating the reference strains (D6 and W2) and culture adapting the field isolates the present study has provided a reference point for drug sensitivity studies for the field isolates from the surveyed population. Perhaps the difficulty associated with culture adapting the field isolates could be one possible reason why such studies were not carried out earlier. Adaptation takes an average of two to three weeks, it is expensive in time and materials to attain large numbers of parasites that can be used for the susceptibility studies. However, this is nothing compared to the cost in human life and suffering - the result of the administration of wrong drugs in the wake of drug resistance in these parts of the country. Therapy and treatment based on the susceptibility profile of the isolate saves time and money and hence minimize drug pressure against new and expensive antimalarial drugs like halofantrine, dehydroartemisinin and atovaquone. Plasmodia anti-malarial drug susceptibility is a dynamic process (18) that is dependent on various factors to include changes in drug pressure. Hence, there is need for a simple, rapid and cost effective tool like the p(LDH) assay for monitoring parasite drug susceptibility profiles. Fansidar has replaced chloroquine as the first line drug in the treatment of uncomplicated malaria in most parts of Kenya. However, our study indicates that not all Kisumu isolates are chloroquine resistant. Therefore, the availability of a cost effective screening assay for chloroquine resistance can favourably impact the clinical management of malaria in that hyper-endemic region of Kenya where it would save not only scarce healthcare resources but human life.

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