AD NUMBER
ADB158380
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METHOD FOR EVALUATION OF DRUG RESISTANCE FOR PLASMODIUM FALCIPARUM

FINAL REPORT - PHASE I

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JULY 15, 1991

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-91-C-1015

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 15, 1991	Final Phase I 15 Jan 91 - 15 Jul 91

4. TITLE AND SUBTITLE
Method for Evaluation of Drug Resistance for Plasmodium Falciparum

5. FUNDING NUMBERS
DAMD17-91-C-1015
65502A
3P665502M802 AA 043
DA335712

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution authorized to DOD components only, under special authority of the Small Business Innovation Development Act of 1982, PL 97-219 and PL 99-443 (16 Sep 91).

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The purpose of the Phase I project was to determine the feasibility of measuring the growth of Plasmodium falciparum by an enzyme assay. The Phase I project was divided into tasks which initially were devoted to defining those conditions which most favorably allowed the measurement of the parasite derived enzyme. Subsequently the measurement of parasite growth (assessed microscopically) was correlated with the enzyme detection method and it was found that the level of enzyme produced correlated directly with microscopically determined growth of P. falciparum. The enzyme detection method was then applied as a means to measure growth of P. falciparum in a drug inhibition assay. This procedure proved to be the equivalent of other more tedious or complex assays. The final experiments conducted during this Phase I grant demonstrated that cholorquine-sensitive and chloroquine-resistant strains of P. falciparum (obtained from the Division of Experimental Therapeutics, WRAIR) could be identified as to their drug sensitivity by the enzyme assay.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Plasmodium falciparum, Drug sensitivity; Enzyme assay	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

13. Abstract (continued)

In this Phase I period we have demonstrated the feasibility of measuring the growth of P. falciparum based on an enzyme assay. This assay has the potential to supplant currently existing procedures due to its technical simplicity and shorter time period of measurement.

FINAL REPORT

Project Objectives

This contract was designed to show the feasibility of a method to more efficiently assess the drug sensitivity of Plasmodium falciparum. The method depends on the differential detection of parasite produced enzyme against a background of host produced enzyme. The basic premise is that any compound with anti-malarial activity should be detected with this enzyme based readout since any anti-malarial activity should lead to lower levels of parasite-produced enzyme in test compared to control cultures. The contract goals were to optimize the conditions for enzyme measurement and then to demonstrate that the enzyme assay could be substituted for existing methods of growth measurement of P. falciparum in vitro. The final goal of this project was to obtain recent isolates of chloroquine-sensitive and chloroquine-resistant strains of P. falciparum and demonstrate that the enzyme measurement system could be used in chloroquine sensitivity assays in vitro.

Work Carried Out and Results Obtained

During the first part of this contract period we initiated procedures to allow us to focus on the measurement of parasite derived lactate dehydrogenase (pLDH) in the format common to drug sensitivity measurement. Thus we evaluated the culture conditions used routinely for the assay of growth of Plasmodium falciparum in microtiter plates to ensure the optimal conditions for the detection of pLDH. From these initial studies we concluded that the optimal buffer system for the detection of pLDH activity consists of 0.1M Tris at pH 9.0 with 200 mM lactate, 0.1% Triton X 100.

The next task was to define suitable starting parasitemias to detect the earliest change caused by any growth inhibiting reagent. We did not exhaustively evaluate various conditions but did demonstrate that a starting parasitemia ranging from 0.1- 1.0 % was adequate for growth assessment and that significant increases in pLDH enzyme levels could be measured as early as 8-10 hrs following the initiation of the parasite culture. We also demonstrated that the enzyme assay could be performed on frozen and then thawed cultures. Thus plates could be grown for 24 or 48 hrs and either measured immediately for parasite growth by enzyme assay or the plates could be frozen for at least 2 weeks and subsequently thawed and then assayed for enzyme activity. These frozen samples yielded similar enzyme levels compared to the samples measured directly following culture. Although this finding is not of clinical relevance it does mean that samples can be frozen and assayed when convenient and that samples can be shipped to a central laboratory for analysis.

We then embarked on a series of experiments which were designed to directly demonstrate the feasibility of this assay as a means to determine antimalarial activity of a "standard" antimalarial compound. We have used chloroquine as a model compound to demonstrate the efficacy of this enzyme assay. Since this assay is dependent on the measurement of pLDH we initially had to demonstrate that chloroquine itself did not interfere with the assay method. We found that for all concentrations tested chloroquine had no apparent influence on the measurement of pLDH. Subsequently we set up a standard culture of chloroquine-sensitive P. falciparum in a 96-well microtiter plate. To some of the

wells we added various concentrations of chloroquine. At the end of a 48 hr culture period we measured total pLDH activity levels in each of the wells by our standard spectrophotometric technique in which the conversion of the coenzyme 3-acetyl nicotinamide adenine dinucleotide (APAD) to APADH is measured spectrophotometrically at 363 nm. We have previously shown that human LDH does not use APAD as a coenzyme, thus the activity of pLDH can be readily measured independent of the presence of human LDH. Collectively, the results of these experiments demonstrated that the enzyme assay could be used as an indication of parasite growth and that the antimalarial activity of chloroquine could also be measured by enzyme activity of pLDH. Compared to untreated control wells the level of pLDH in chloroquine test wells correlated directly with the antimalarial influence of each concentration of chloroquine tested in this model assay system.

We then requested and received from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, recent isolates of P. falciparum. When we used these isolates in an in vitro assay for chloroquine resistance we again found that the pLDH based enzyme assay could be used to readily identify the chloroquine resistant strain. We measured the assay at 48 hrs and read the results immediately. Thus with this method we determined drug sensitivity of P. falciparum 48 hrs following initiation of culture and within one hour following termination of the culture period. As this final report is being prepared we are also in the process of demonstrating the use of a color reaction to measure enzyme activity as an alternative to the spectrophotometric measurement system.

Estimate of Technical Feasibility

At the present time the measurement of drug sensitivity of P. falciparum requires procedures that are time-consuming and labor intensive with a readout usually achieved no sooner than 72-96 hours following initiation of the procedure. The use of pLDH as a measure of growth and metabolic activity of P. falciparum can be readily accomplished and the activity of this enzyme can be used as a substitute for other methods to determine the influence of the antimalarial, chloroquine, on the in vitro growth of P. falciparum. Thus the feasibility of the Phase I contract has been shown. More importantly however, is the observation that the use of pLDH in the development of a method for the determination of drug sensitivity of P. falciparum should lead to a procedure that can save time and reduce the manpower needs for the assay. We have been able to detect the influence of chloroquine on P. falciparum 48 hrs following the initiation of the test period. Thus, at least for chloroquine, the pLDH assay allows an earlier point for sensitivity assessment. Since the assay for pLDH is a simple reduction reaction of APAD to APADH the endpoint can be quickly measured. Since the prime purpose of this Phase I project was to demonstrate the feasibility of the approach we have not attempted to shorten the time period of this assay. However given the signal to noise signal that we measure at 48 hrs we are confident that for certain antimalarial compounds measurement can be made as early as 24 hrs following addition of the antimalarial compound.