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PRINCIPAL INVESTIGATOR: Robert C. Piper, Ph.D.

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Portland, Oregon 97201

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13. ABSTRACT *(Maximum 200 words)*

We have developed diagnostic assays based on the specific detection of *Plasmodium* lactate dehydrogenase (pLDH) activity. The assays have been developed into two basic formats. The first format is an Immuno-Capture pLDH assay (ICpLDH) in which active enzyme is captured and purified from whole blood with an immobilized monoclonal antibody. Enzyme activity is then measured colorimetrically using special substrates specific for pLDH. The second format is an immuno-chromatographic test which measures pLDH protein. The immuno-chromatographic test is in a "dip-stick" format and has trademarked as Opti**MAL**®. The analytical sensitivity of both tests is between 0.001 and .0001% parasitemia. Furthermore, using the panel of monoclonal antibodies, this assay can not only detect but differentiate between *P. falciparum* and non *P. falciparum* malaria. We have conducted over 7 field studies and found that both assays had clinical sensitivity of ~200 parasites/µl. Most importantly, we find that with either the quantitative ICpLDH assay or the qualitative Opti**MAL**® assay, pLDH levels were coincident with parasite levels determined by microscopy thus making it possible to predict the success of chemotherapy based on pLDH levels.

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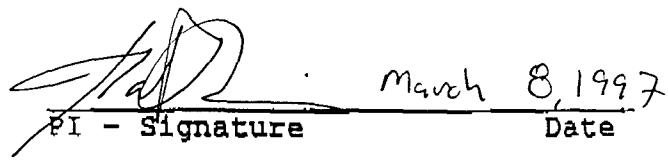

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INTRODUCTION

Overview

The research program was initiated to develop, refine and further format a diagnostic assay for malaria. All of these assays were based on the detection of the malarial enzyme Lactate Dehydrogenase (pLDH for *Plasmodium* Lactate Dehydrogenase). Previous studies have indicated that specific measurement of pLDH in blood samples could serve as an effective method for detecting the parasite in the blood stream and thus diagnosing malaria.
Before the current SBIR Phase II was granted, Flow Inc. had developed methods to measure pLDH activity using LDH substrates that were specific for pLDH and did not react with human LDH. This method was successfully developed into a product (MalStat™) that can be used to measure drug-susceptibility of *Plasmodium* strains in *in vitro* cultures. Our preliminary data at that time showed that pLDH levels correlated well with parasite infection making it attractive to design better methods of detecting pLDH as a means of diagnosing malaria. More importantly, we found early on that pLDH levels appeared to correlate with parasitemia thus providing the possibility to monitor parasitemia by monitoring pLDH levels in the blood stream. This latter feature would thus allow the detection of drug-resistant infections since parasitemia and also pLDH levels would remain in the blood despite anti-malarial therapy in drug-resistant infections. Our proposed scope of work was:

1) Design and format a simple, rapid, and sensitive "wet" (ELISA-like) method for assaying pLDH activity.
2) Promote this basic design to detect and even possibly differentiate the 4 species of malarial parasites (*P. falciparum, P. vivax, P. malariae,* and *P. ovalae*)
3) Subject the new test format to a rigorous "test of principle" to demonstrate that pLDH is an accurate indicator of malarial infection.
4) Subject the new test format to a rigorous "test of principle" to demonstrate whether pLDH can be used to monitor therapy.
5) Format a test kit that can be used to measure pLDH activity in the clinic that has practical and commercial application.
6) Produce a prototype dry "dip-stick" format that can measure pLDH levels in "far forward" conditions that utilizes little to no equipment.

We report that we have not only met these goals but have exceeded the scope of this grant since we have developed both a "wet" (ELISA-like) and a dry dipstick format and have rigorously tested them in a variety of field studies around the world. We have found an excellent correlation of pLDH levels with parasitemia and find that both methods can be used to accurately assess the efficacy of drug treatment in patients with malaria. Furthermore, our test kits are now entering in the final stages of commercialization and we are happy to report that they are being sold in limited supply currently.

The Global Disease

Malaria is one the world's most prevalent diseases. Current estimates by the World Health Organization predict over 200 million cases of malaria annually. The number of clinical cases exceeds 150 million with approximately 2-3 million deaths per year. Most of these victims are infants and young children. Over half the world's population lives in malaria's areas.

The disease itself is caused by a protozoan (*Plasmodium sp.*) that invades human red blood cells. The parasite is transmitted by many species of *Anopheles* mosquito. Once introduced into the blood stream by the female *Anopheles* mosquito, the newly released sporozoite form of the parasite invades the liver. During the next ~2 weeks the intracellular hypnozoite form of the parasite undergoes vast metabolic changes and emerges as the merizoite form ready to invade human red blood cells. Once it invades the red blood cell the parasite changes to the early "ring" trophozoite, the late trophozoite, and finally divides within the blood cell forming the Schizont form. Once the blood cell ruptures, new merizoites are released now capable of **invading new red blood cells.** It is during this blood borne stage of the infection that infected patients become ill.

Four species of *Plasmodium* infect humans. *P. falciparum* accounts for ~85 % of the world's malaria. *P. falciparum* is the most virulent species of malaria since it can cause complications such as cerebral malaria, pulmonary malaria, and renal failure. The next most abundant species is *P. vivax* which accounts for ~10-12% of the world's malaria. The two other species of malaria, *P. ovalae* and *P. malariae*, are relatively rare.

Almost 85% of the world's malaria **occurs** in sub-saharan Africa. The vast majority of these cases are *P. falciparum* malaria. Malaria is also prevalent in Southeast Asia, India, South and Central America. In these latter regions, *P. falciparum* and *P. vivax* each account for ~ half of the cases of malaria.

Today , the threat of malaria is severe because of the emergence of drug-resistant strains of the parasite. *P. falciparum* now shows widespread resistence to chloroquine and mefloquine which used to be effective and inexpensive provolactic and **therapeutic chemotherapies.** Currently, isolates in Southeast Asia there are strains reported to show resistance to quinine and artemeter, two of the most effective antimalarial drugs that have been developed. Other **species** of malaria have not been so tenacious, however, *P. vivax* has now become **resistant** to chloroquine in many areas.

Malaria has proven to be a particularly difficult disease to control in the developing world. Factors such as the lack of good public and private health, mosquito vectors resistance to insecticides, and parasites resistant to drugs all have contributed to the reemergence of the disease. Furthermore, with increased travel, intervention and involvement of the US and Europe in the developing world, malaria now poses an ominous threat to the people living in the developed world.

Better diagnostics can help solve the threat of malaria. The more a diagnostic procedure is rapid, accurate, and widely used , the more judicious and effective treatments may be administered. The availability of rapid, simple, and specific diagnostic tools will make a major contribution in the overall strategy to control malaria. Currently, however, the access to effective diagnosis is limited in much of the developing world. Microscopy remains the standard, most cost-effective method. It is, however, very labor intensive, requires a well-functioning, high quality microscope, and is performed well only by highly skilled personnel. Beyond the central clinics and wherever microscopy is unavailable, diagnosis of malaria is usually based on a patient's symptoms.

pLDH and the Glycolytic Cycle

Blood stage *Plasmodium* parasites rely exclusively on glycolysis for metabolic energy. One of the most abundant enzymes in the glycolytic cascade is the enzyme Lactate DeHydrogenase (LDH) that functions at the last step in the glycolytic pathway. LDH is a soluble cytosolic enzyme that catalyses the reduction of NADH to NAD+ by converting Pyruvate to Lactate.

As an abundant soluble tetrameric enzyme, the Lactate DeHydrogenase produced by the *Plasmodium* parasite makes an ideal target protein to detect to thus serve as a diagnostic indicator for malaria. Flow Inc. has characterized both biochemical and antigenic differences between *Plasmodium* LDH (pLDH) and human LDH (hLDH) that in turn allow the unambiguous detection of pLDH in the blood of malaria infected patients.

There are four species of *Plasmodium* that infect humans. Extensive studies have shown that each of these species expresses a unique pLDH isoform. Within a given strain of *Plasmodium*, only a single isoform is expressed, unlike in humans where 6 holoenzyme isoforms are expressed. Within the species of *P. falciparum*, two pLDH isoforms have been characterized.

We have purified, cloned and expressed the pLDH isoform from the WRAIR D6 strain of *P. falciparum*. Bacterial produced recombinant pLDH is biochemically identical to bonafide pLDH isolated from infected erythrocytes grown *in vitro*. In the conversion of Pyruvate to Lactate, pLDH shows a much lower Km for pyruvate than human LDH isoforms. Unlike the human LDH, pLDH is not inhibited by pyruvate/NAD+ complexes . In the catalysis of Lactate to Pyruvate, pLDH has a remarkable biochemical difference as it can efficiently use the coenzyme analog 3-acetyl pyridine dinucleotide ($APAD^+$) in place of NAD^+ . Human LDH does not readily use $APAD^+$ and thus the activity of pLDH can be measured specifically with L-Lactate and $APAD^+$. This difference can be accounted for by a high Kcat of pLDH for APAD+ and L-Lactate since the Km of pLDH for $APAD^+$ is very similar to that of human LDH isoforms. In contrast to human LDH, pLDH is also not severely inhibited by high concentrations of L-Lactate in the conversion of L-Lactate to Pyruvate. Thus, activity assays that can specifically measure pLDH activity in solutions containing human LDH contain $APAD^+$ and high concentrations of L-Lactate.

In a comprehensive search for biochemical differences between human LDH and *Plasmodium* LDH, Flow Inc. discovered that the co-enzyme 3-acetyl pyridine dinucleotide (APAD+) could be used in the conversion of L-Lactate to pyruvate by pLDH but not by human LDH. With equal activity units of pLDH and any of the human LDH isoforms present in blood, pLDH utilizes $APAD^+$ >200 times more rapidly than hLDH. Other NAD^+ coenzyme analogs do not give this activity bias. Utilization of $APAD^+$ was found to be a property of all four human malarial pathogens, however, limitations in the availability of *P. vivax, P. ovalae,* and *P. malariae* infected blood has prevented the purification and finer biochemical characterization of these LDH isoforms. Partially purified preparations of these other *Plasmodium* LDH isoforms confirms that the Km, Kcat, and bias toward $APAD^+$ is similar among species specific pLDH isoforms.

BODY

Abstract

We have developed diagnostic assay based on the specific detection of *Plasmodium* lactate dehydrogenase (pLDH) activity. This procedure utilizes a panel of monoclonal antibodies that capture the parasite enzyme. The assay has been developed into two basic formats. The first format is an Immuno-Capture pLDH assay (ICpLDH) in which active enzyme is captured and purified from whole blood with an immobilized monoclonal antibody. Enzyme activity is then measured colorimetrically using special substrates specific for pLDH. The second format is an immuno-chromatographic test which measures pLDH protein. The immuno-chromatographic test is in a "dip-stick" format and has trademarked as Opti**MAL**®.
Using known amounts of recombinant pLDH as well as standardized samples from *in vitro* cultures, the analytical sensitivity of both tests is between 0.001 and .0001% parasitemia. Furthermore, using the panel of monoclonal antibodies, this assay can not only detect but differentiate between *P. falciparum* and non *P. falciparum* malaria.
Laboratory tests show convincingly that pLDH is a good marker for malarial infection. To test the utility and applicability of both assay formats we conducted over 7 field studies . We found that both assays had clinical sensitivity of ~200 parasites/µl. We also find that both assays could correctly differentiate between *falciparum* and non-*falciparum* malaria. Most importantly, we find that with either the quantitative ICpLDH assay or the qualitative Opti**MAL**® assay, pLDH levels were coincident with parasite levels determined by microscopy. Patients undergoing anti-malarial chemotherapy also showed concomitant decreases in pLDH levels thus making it possible to predict the success of chemotherapy based on pLDH levels.

Methodology

pLDH purified from *in vitro* cultures of W2 and D6 strains of *P. falciparum* maintained as previously described in previous reports. Centrifuged Red Blood Cell lysates from *in vitro* cultures were adjusted to 50 mM Tris pH 8.0 and fractionated over Cibacron Blue Chromatography using a gradient of NADH to differentially elute pLDH from contaminating human LDH isoform. Peak fractions containing pLDH activity were pooled and further fractionated by ion exchange chromatography as described previously. Purified pLDH was judged >95% pure by SDS-PAGE and Coomasie blue staining. Recombinant pLDH was expressed in XL1-Blue using the vector pTrc99 (Pharmacia) containing the pLDH open reading frame cloned from the D6 isolate of *P. falciparum* .

Purified pLDH was used to immunize a series of mice to raise monoclonal antibodies that specifically recognize pLDH. This provided the means for a more sensitive and more specific assay for pLDH. After extensive testing of many antibodies we have chosen the antibodies 6C9, 17E4, and 19G7 for use in our assays. 17E4 and 19G7 capture active enzyme without adverse affects on enzyme activity. The 6C9a antibody will capture pLDH but at high concentrations will inhibit enzyme activity. All antibodies were subcloned and prepared and purified on a large scale.

Figure 1A shows a schematic of the "wet" ICpLDH assay (Immuno-Capture pLDH). To prepare the test plates for the ICpLDH the assay, antibody coated plates are prepared by incubating the wells of polystyrene 96 well microtiter plates with solutions of the 19G7 and 17E4 antibody. Wells are washed and then blocked by incubation with a solution containing BSA for 4 hrs. Wells were then washed and dried and stored at 4°C until used.

To perform the ICpLDH assay, 200 µls of frozen blood lysate or 150 µls of unlysed blood plus 50 µls of 2% Triton X-100 are added to the test wells and allowed to incubate at 25°C for 30-60 minutes. Wells are then washed 3 times in PBS. Malstat™ supplemented with NBT and Diaphorase are added to each well and pLDH activity is monitored kinetically as an increase in absorbance at 650 nm using a Thermomax microtitre plate reader according to procedures described in previous reports. Test wells can also be scored visually since NBT is reduced to a colored product. Typical results of the ICpLDH assay are shown in Figure 2. Figure 2 also shows the specificity of the 6C9, 17E4, and 19G7 antibodies.

Figure 1B shows a schematic of the Opti**MAL**® rapid immunochromatographic assay for the detection and speciation of malaria. The Flow Inc. Opti**MAL**® assay detects the presence of the pLDH antigen in lysed whole blood. A 10 µl of fresh, frozen or dried whole blood samples (finger stick or venopuncture) collected in EDTA/ACD/heparin is added to 30 µls of Buffer A into a test well or test tube. Buffer A contains a colored bead conjugated to the pan-specific anti-pLDH antibody 6C9. The Opti**MAL**® test strip is then placed into the well and the entire sample is allowed to wick up the strip. The test strip is then moved to another test well containing 80 µls of Buffer B which is allowed to wick up the test stick and clear the hemoglobin color for proper viewing of the test result.

The Opti**MAL**® assay is designed to diagnose all forms of malaria and also differentiate between *P. falciparum* and the other three species of malaria. This differentiation is clinically relevant since the salient feature of malaria diagnosis is determined whether a malarial infection is positive or negative for *P. falciparum*. In the Flow Inc. pLDH-based dip-stick assay, there are two diagnostic zones each containing a different antibody. A monospecific antibody (17E4) is present in the bottom reaction zone which recognizes only *P. falciparum*. A second *pan*-specific antibody (19G7) is present immediately above this zone, this monoclonal antibody recognizes the pLDH isoform of *P. vivax, P. ovalae,* and *P. malariae.* A third reaction zone is present at the top of the immunochromatographic test strip where an antibody which captures the excess colloid conjugate and serves as a positive control for the assay. The colloid conjugate is coupled with a third monoclonal antibody that is *pan*-specific.

Typical test results using the Opti**MAL**® test are shown in Fig. 3. *P. falciparum* infected blood gives two test bands plus the control band. This is because the pLDH/ antibody-bead complex can be immobilized by both the *falciparum* specific anti-pLDH antibodies as well as the *pan*-specific anti-pLDH antibodies. Samples of *P. vivax* infected blood show only one test band since the pLDH/antibody-bead complex is not recognized by the *falciparum* specific antibody but is recognized by the *pan*-specific antibody. Finally, a non-infected blood sample fails to make any pLDH/antibody-bead complex and yields only the top control band due to the immobilization of the antibody-bead complex on the goat-anti-mouse reaction zone. Testing of the Opti**MAL**® test using dilutions of infected blood samples of known parasitemia showed that the test strip was capable of detecting levels of pLDH present in parasitemias of <0.001% or 50 parasites/µl.

The Interpretation of the Opti**MAL**® assay test strip is as follows:

1. POSITIVE - *P. falciparum*: One control band plus **two** test bands.
2. POSITIVE - *P. vivax*: One control band plus **one** band.
3. NEGATIVE - One control band at the top of the test strip.

To determine the dose response of the ICpLDH assay we looked at the linearity of the assay over a range of parasitemias. Red Blood cells from *in vitro* cultures of D6 *P. falciparum* were serially diluted in non infected blood to yield samples of a defined parasitemia (Figure 3).

200 µls or 20 µls of each sample were tested in the ICpLDH assay using test wells coated with either the *P. falciparum*-specific 17E4 antibody or the *pan*-specific 19G7 antibody. As shown in a log vs. log plot, detection of pLDH along the standard curve was obtained over 4 orders of magnitude from 10% parasitemia to below 0.001% parasitemia. The pLDH activity in samples of low parasitemia was not only detected by spectrophotometric measurements (Fig 3A) but also by visual inspection of the reaction plate (Fig. 3B). These data also showed that the threshold level for detection by either visual of spectrophotometric means could easily be adjusted by assaying different volumes of each sample (compare results obtained using 200 µls of blood *vs.* 20 µls of blood). These data show that the immuno-capture procedure combined with the activity assay using MalStat™ and NBT/Diaphorase is proportional to the amount of pLDH present in the sample and that this assay can measure pLDH levels in blood over a range that is pertinent to the clinical diagnosis of malaria.

FIGURE 1.

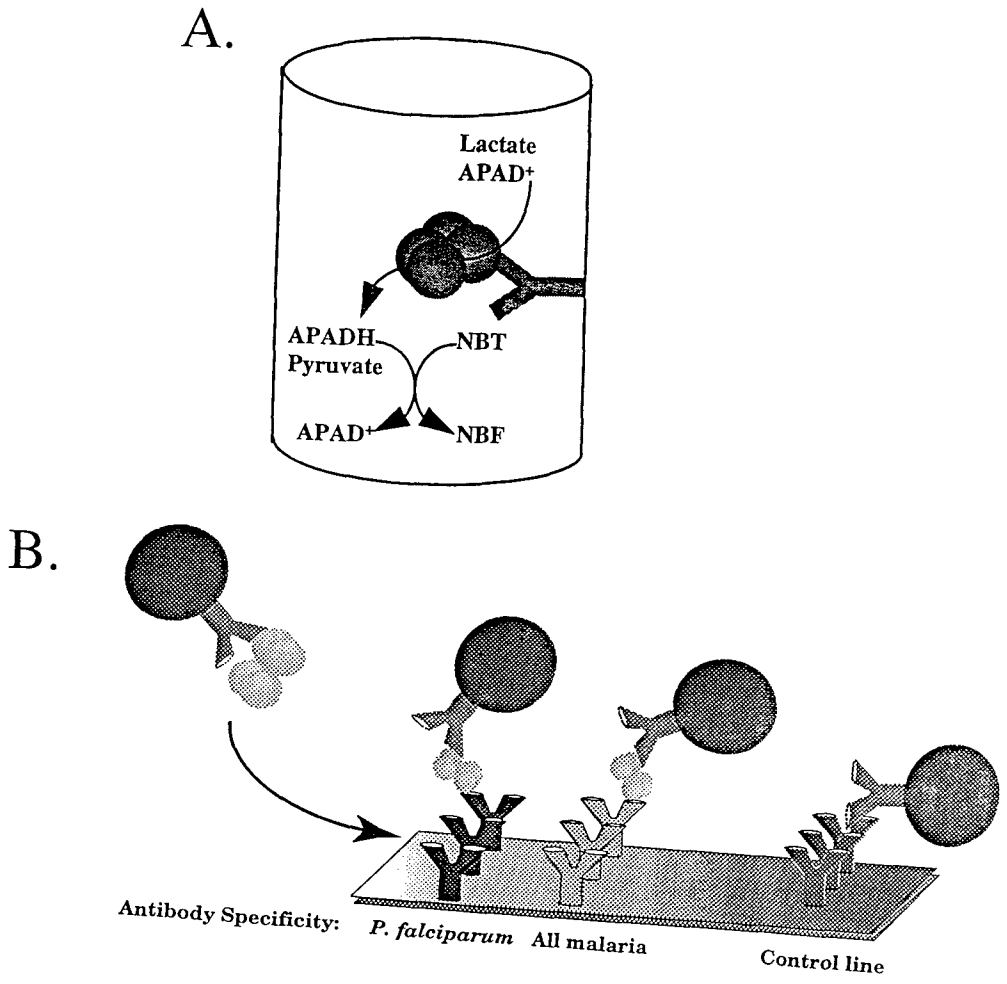


FIGURE 2.

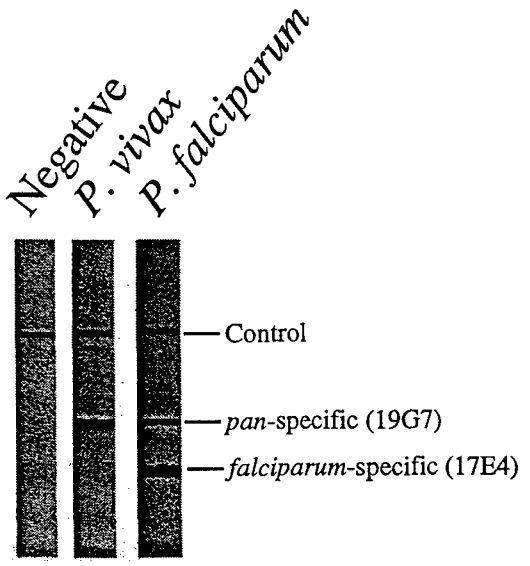
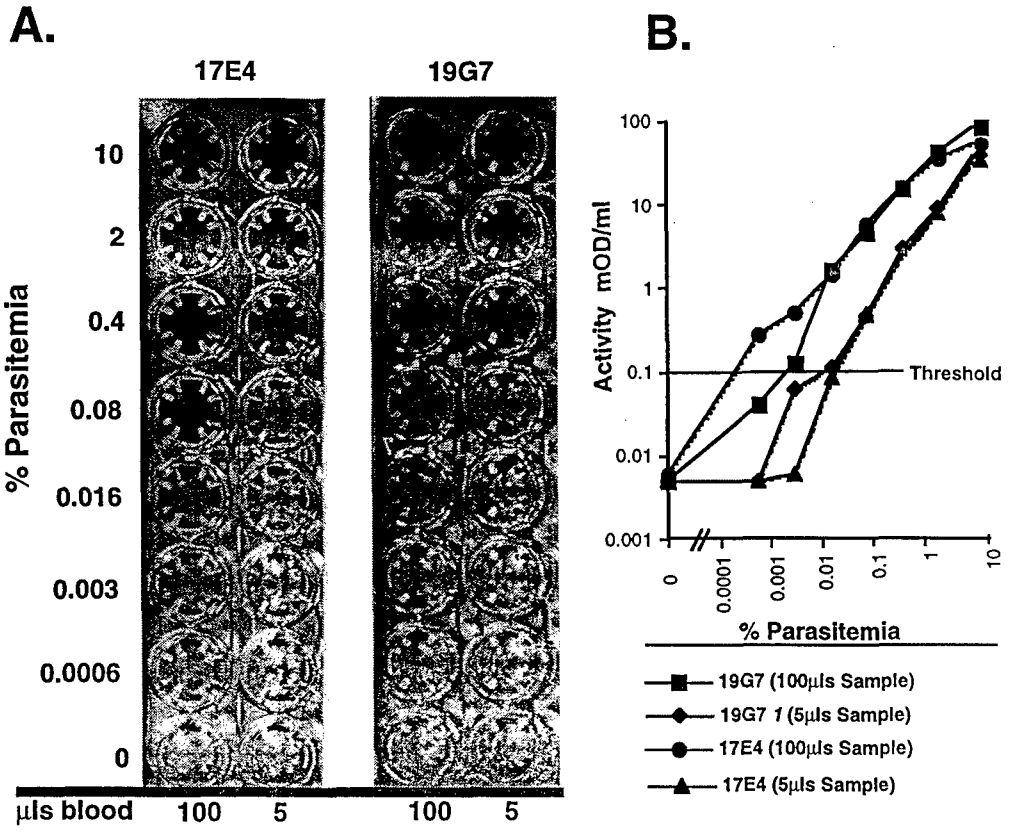


FIGURE 3.



Field Evaluations of the OptiMAL® Assay and ICpLDH Test

Several Studies were performed to evaluate the pLDH in vitro diagnostic test. It was critical for Flow Inc. personnel to be intimately involved in all initial studies. Since the assay was and remains under development, we found it necessary to quickly respond to the technical requirements of the assay both in the laboratory and in the field. It was only after the test format had benefited from this field development period that the test was appropriate to take outside the laboratory and be used by non-Flow personnel.

Several field studies were performed to test the efficacy of the pLDH OptiMAL® test:

Study 1: ~300 samples of whole blood from infected patients under chemotherapy were brought to Flow Inc. by Angela Cook in collaboration with Peter Chiodini at the Hospital for Tropical Disease, London. Both the ICpLDH assay and the OptiMAL® assay were performed on these samples. All tests were conducted by Laura Wentworth with Angela Cook as assistant. All samples were tested in a blinded fashion. The test key was only revealed after Ms. Cook had returned to HTD. The results of these test are shown in Tables I and II and show a very good sensitivity and specificity. Overall we find the OptiMAL® and ICpLDH assay results to be comparable: for samples containing 50 parasites/µl or more the sensitivity for each assay was 96% and 92%, respectively. As a measure of non-specific reactivity we also tested samples that were negative by microscopy. These samples included either persons who had just recovered from malaria (5 total) or persons who had contracted malaria at least 6 months prior to the date of testing (25 total). In these cases we saw no "false positives".

Study 2: Samples were collected from 26 patients in South America (CIDEIM, Cali, Columbia). Since both *P. falciparum* and *P. vivax* are endemic to Columbia, this study provided us with the ability to not only validate the ICpLDH assay and the OptiMAL® assay for the diagnosis of malaria but also allowed us to evaluate how well the assays could distinguish between falciparum malaria and non-falciparum malaria (e.g. *P. vivax*). The range of parasitemias tested were 42 - 130,000 parasites/µl (0.001-2.6% parasitemia) for 10 *P. falciparum* samples and 200-39,500 (0.004-0.8% parasitemia) for 12 . 8 negative samples were also included in this study. The patient samples were prepared in one of two ways: samples were either stored at -20°C until evaluation or were absorbed to sheets of Whatman 3M paper, dried and stored at room temperature. The frozen samples were thawed and tested using the ICpLDH assay (Table III) or the OptiMAL® assay (Table IV). To assay the dried samples, a 0.5 cm^2 area of the paper was soaked in 300 uls of PBS for 20 min. 200 µls of this solution was used in the ICpLDH test with the *pan*-specific 19G7 antibody (Table V). In all cases, we could use the ICpLDH assay and the OptiMAL® assay was able to identify samples from patients infected with either *P. falciparum* or *P. vivax*. Furthermore, both assays were able to distinguish samples of *P. falciparum* (which reacted with both 17E4 and 19G7 antibodies in either assay) from samples of *P. vivax* which reacted only with the 19G7 antibody in either assay. These data show that both the ICpLDH assay and the OptiMAL® assays can be used as sensitive tests for the diagnosis of malaria that are capable of identifying and distinguishing *P. vivax* infections and *P. falciparum* infections.

Study 3: ~370 samples were analyzed during May, June, July at HTD. The study was conducted by Liz Gabbet (an Honors student at the University of Aberdeen) under the direction of Angela

Cook and Peter Chiodini. No Flow personnel were present for this study. While this study was conducted with reagents from our earlier formulations and test formats, the performance characteristics of the test were quite favorable. These results are presented in Table VI.

Study 4: In July, Dr. Makler visited Dr. Pierre Drhuile at the Pasteur Institute and examined ~200 samples collected from patients in the Senegal. This study was performed blinded and only after the study was completed did Dr. Drhuile provide the key. The results of this study are presented in Table VII. These results confirm the sensitivity and specificity of the pLDH assay. In this study the calculated sensitivity was somewhat better than that with samples from HTD. This difference is most likely related to the differences between how each group quantitates parasitemia.

Study 5: In July, Dr. Makler also visited Dr. Jaques LeBras at the Hopital Bichat in Paris. This study examined pLDH levels in serial blood samples taken from patients undergoing chemotherapy. Jaques was also able to compare the presence of pLDH with the presence of HRP II in these samples. We found that HRPII persisted in patients long after parasitemia had been cleared. In contrast, pLDH levels closely followed peripheral parasitemia (see next section). This study combined with the above studies performed at the HTD address one of the main goals of this grant: "Relate the levels of pLDH whole blood to the percent parasitemia and the severity of disease". It appears from both studies that pLDH is an accurate indicator of viable parasites.

Study 6: In November, Angela Cook visited the MRC in the Gambia to evaluate the Opti**MAL**® test in ~400 patients. A copy of the protocol is included in Attachment 3. Our preliminary findings are that the pLDH test was ~95% sensitive and 95% specific. A full and intensive microscopic examination has now been performed by field microscopists in the Gambia, by Flow microscopist Junita Reis, and by Angela Cook and Tony Moody of the Hospital for Tropical Disease. These results are presented in Table VIII and show that Opti**MAL**® performs quite well. In fact, there is as much variability among the microscopists used in this study than there is in comparison to Opti**MAL**®.

Study 7: In November, Robert Piper and Miguel Quintana conducted a field study in Los Cevita, Honduras, outside the town Tacoa. The majority of the population was a symptomatic with a large proportion of infected individuals having *P. vivax*. A copy of the study protocol is included as Attachment 4. The results have not been fully analyzed yet, however, initial comparisons are very good. Out of 370 patient samples tested, only 3 samples gave an answer with the pLDH assay that could not be confirmed by one of two microscopists used in the study (with the exception of *P. falciparum* samples: see below). All of these samples were scored positive by microscopy but negative by pLDH, however, these samples contained very few parasites and are likely to be below the threshold of detection for the pLDH assay. We also found several samples (7) that gave a clear positive test result for *P. falciparum* on the pLDH assay but all were scored as negative by microscopy. These samples were later confirmed with *P. falciparum* by the HRP II assay and PCR indicating that in these asymptomatic patients, the pLDH assay was able to detect sequestered parasites. Further data analysis is required to complete this study.

Table I. Performance of ICpLDH assay on *P. falciparum* samples from HTD.

% Parasitemia	Parasites/µl	Total	ICpLDH Positive	ICpLDH Negative	Sensitivity	Specificity
>0.03	>1500	31	31	0	100%	-
0.01-0.03	500-1500	11	10	1	91%	-
0.001-0.01	50-500	8	5	3	62%	-
<=0.0001	<=5	17	7	10	41%	-
Negative	0	10	0	10	0%	100%

Table II. Performance of Opti**MAL**® assay on *P. falciparum* samples from HTD.

% Parasitemia	Parasites/µl	Total	ICpLDH Positive	ICpLDH Negative	Sensitivity	Specificity
>0.03	>1500	40	40	0	100%	-
0.01-0.03	500-1500	18	17	1	94%	-
0.001-0.01	50-500	11	9	2	81%	-
<=0.0001	<=5	22	13	9	60%	-
Negative	0	30	0	30	0%	100%

Table III. Performance of the ICpLDH assay on *P. falciparum* and *P. vivax* Samples.

Parasite Species	Parasites/µl Range	Total	ICpLDH Positive 17E4 (Visually)	ICpLDH Positive 19G7 (Visually)	Sensitivity	Correct Speciation	Specificity
P. falciparum	42-129,000	10	10	10	100%	100% falciparum	-
P. vivax	200-39,500	11	0	11	100%	100% non-falciparum	
Negative	50-500	8	0	0	0%		100%

Table IV. Performance of Opti**MAL**® assay on *P. falciparum* and *P. vivax* Samples.

Parasite Species	Parasites/µl Range	Total	Two Reaction bands (17E4 and 19G7)	One reaction band only (19G7)	Sensitivity	Correct Speciation	Specificity
P. falciparum	42-129,000	10	10	0	100%	100% *falciparum*	-
P. vivax	200-39,500	12	0	12	100%	100% non-*falciparum*	
Negative	50-500	8	0	0	0%		100%

Table V. Performance of the ICpLDH assay on dried blood samples of P. *falciparum* and *P. vivax*

Parasite Species	Parasites/µl Range	Total	ICpLDH Spectrophotometry	ICpLDH (Visual)	Sensitivity	Correct Speciation	Specificity
P. falciparum	42-129,000	10	10	10	100%	100% *falciparum*	-
P. vivax	200-39,500	13	13	13	100%	100% non-*falciparum*	
Negative	50-500	8	1	0	0%		100% (90%)

Table VI. Performance of Opti**MAL**® assay on *P. falciparum* samples from HTD-July 1996.

% Parasitemia	Parasites/µl	Total	OptiMAL Positive	OptiMAL Negative	Sensitivity	Specificity
>1	50,000	21	21	0	100%	-
0.1-0.9	5,000	54	54	0	100%	-
0.01-0.09	500	32	32	0	100%	-
0.001-0.009	50	26	12	14	46%	-
<=0.0001	5	16	6	10	50%	-
Negative*	0	20	2	18	10	90

* These negatives were from patients that were undergoing malaria therapy. Although negative by microscopy on the day the sample was taken, each patient was positive for parasites on the previous day.

Table VII. Performance of Opti**MAL**® assay on *P. falciparum* samples from Pasteur Institute.

% Parasitemia	Parasites/µl	Total	ICpLDH Positive	ICpLDH Negative	Sensitivity	Specificity
>1	50,000	-	-	-	-	-
0.1-0.9	5,000	3	3	0	100%	-
0.01-0.09	500	21	21	0	100%	-
0.001-0.009	50	23	21	2	91%	-
0.0001-0.0009	5	28	15	13	53%	-
<0.0009	<5	12	3	9	25	-

Table VIII. Performance of Opti**MAL**® assay in the Gambia

GOLD STANDARD	sensitivity/ specificity MRC	sensitivity/ specificity HTD	sensitivity/ specificity Junita	sensitivity/ specificity OptiMAL®
MRC	----------	98/94	92/94	96/93
HTD	90/99	----------	87/97	92/96
Junita	91/95	95/92	----------	85/94
OptiMAL®	87/98	94/95	91/92	----------

These data compile results of a field study in the Gambia. Note that 3 separate microscopists were used. Opti**MAL**® was found to perform as good or better as any microscopist. This study not only substantiates the Opti**MAL**® assay as a good performer in the field but these data also underscore the need for a standardized assay in the diagnosis of malaria given the variability amongst highly trained microscopists.

Therapeutic Monitoring and Detection of Drug-Resistance

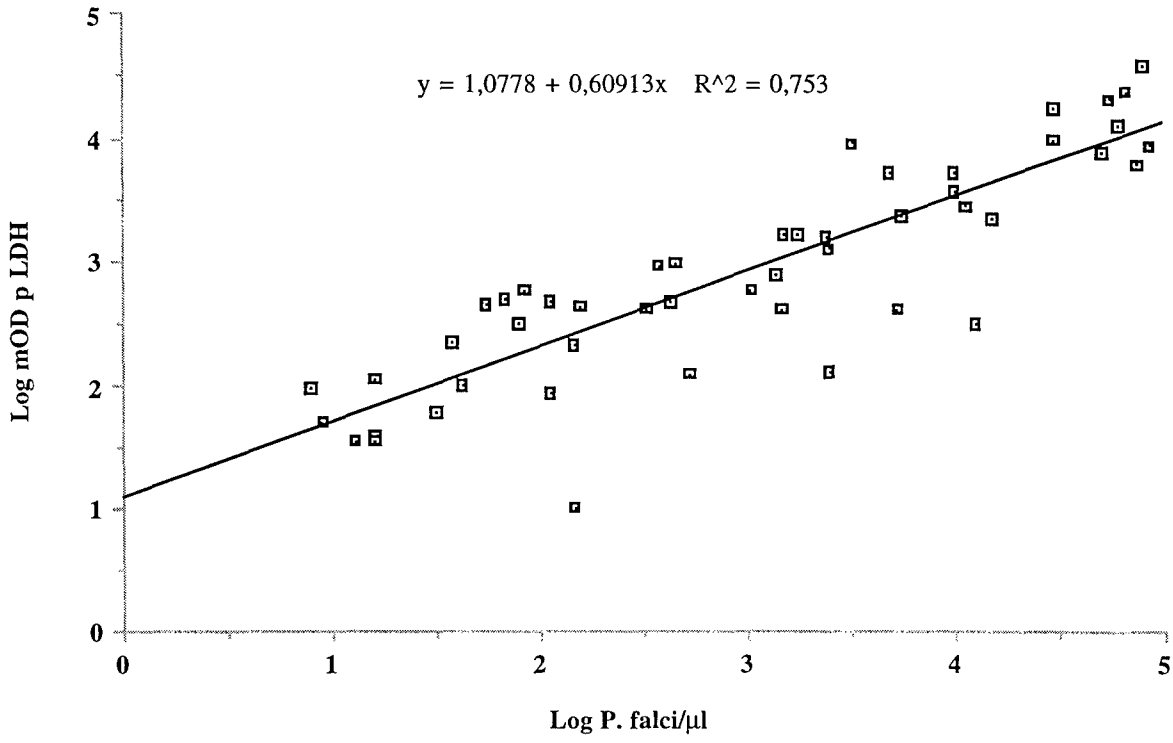
All of our previous experiments indicated that pLDH levels follow the level of parasitemia. This is not only the basis of the i*n vitro* culture and sensitivity test developed with the MalStat™ reagent but might also serve as the basis by which the ICpLDH assay and the Opti**MAL**® assay could be used to monitor the success of drug therapy and thus detect drug-resistant infections. To test this we examined pLDH levels by the ICpLDH assay in samples taken daily from several patients undergoing therapy. This was done in two separate field studies. The first was performed with the Hospital for Tropical Disease and was comprised of the same patients as reported in Table I and II. The second study was performed with Jaques LeBras at the Hopital Bichat Claude Bernard (HBCB), Paris, France. All patients from HTD were admitted and started on intraveneous quinine/tetracyline. Blood smears were examined daily. For studies at HBCB, 16 malaria cases from Africa were followed by daily or 4X daily blood smears. Among all 29 patients from HTD and 16 patients from HBCB followed with this method, we found that pLDH levels qualitatively matched the peripheral parasitemias. Importantly, pLDH levels were gone on the day each patient was found to be free of parasites by microscopic examination. The absence of false positives even with samples from patients that had sustained recent infection should prove to be a useful aspect of these pLDH-based assays since this feature allows for the monitoring of therapy. To further test this feature, we also examined whether the Opti**MAL**® assay could be used to follow therapy. We obtained 5 samples from different patients who had undergone chemotherapy for *P. falciparum* infection. These samples were obtained on the day the corresponding blood smear was declared negative; in all 5 cases the blood films from the previous day had been positive for parasites. Consistent with the results of the ICpLDH assay, we found that Opti**MAL**® assay was negative for all 5 of these negative samples thus making it possible to monitor therapy using the Opti**MAL**® rapid dipstick assay (Table II).

In general, we also found that parasite levels correlated with peripheral parasitemia when comparing among different patients. These data from studies at HBCB show the potential of using pLDH levels as an absolute and quantitative measure of parasite density (Figure 4). We have found significant variation on this point, however, and caution that additional studies are required before this calibration can be made.

Examples of the data following patients from HTD is shown in Figure 5. These data are supplemented in Appendix 1. Patient data from HBCB is shown in Figure 5, 6, and 7. Patient #4 (Fig. 7) from HBCB was interesting in that they came to the hospital with a short course of fever but a malaria diagnosis could not be made by either microscopy, Opti**MAL**®, ICpLDH, *Para*Site, or the QBC-test™. A positive diagnosis was made 7 days later upon a return visit by the patient.

This ability to monitor infections as they occur is also evident in the study by HTD presented in Table VI (Appendix 1) where one patient(patient 7: samples 7a-7w) were taken from a patient over time who recrudesced.

Figure 4.



Correlation of parasitemia and Parasite Lactate Dehydrogenase (pLDH) activity measured by ICpLDH for the 49 positive values obtained in the follow-up of 16 treated patients at HBCB.

Figure 5.

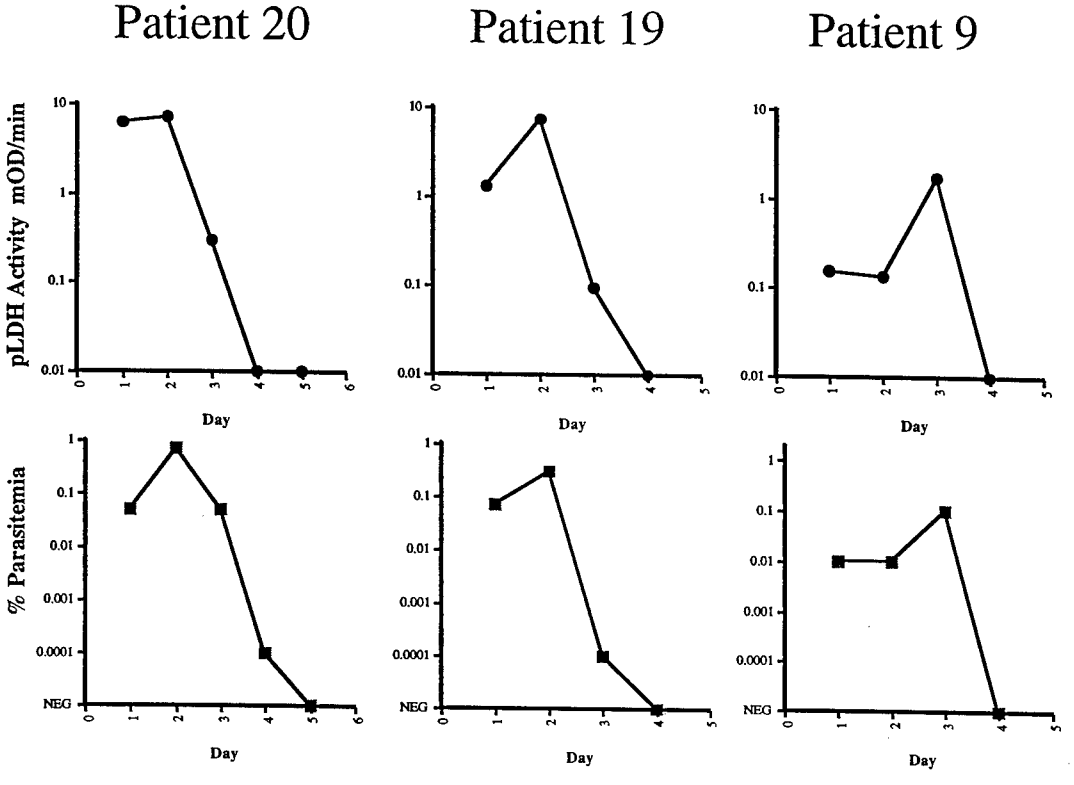
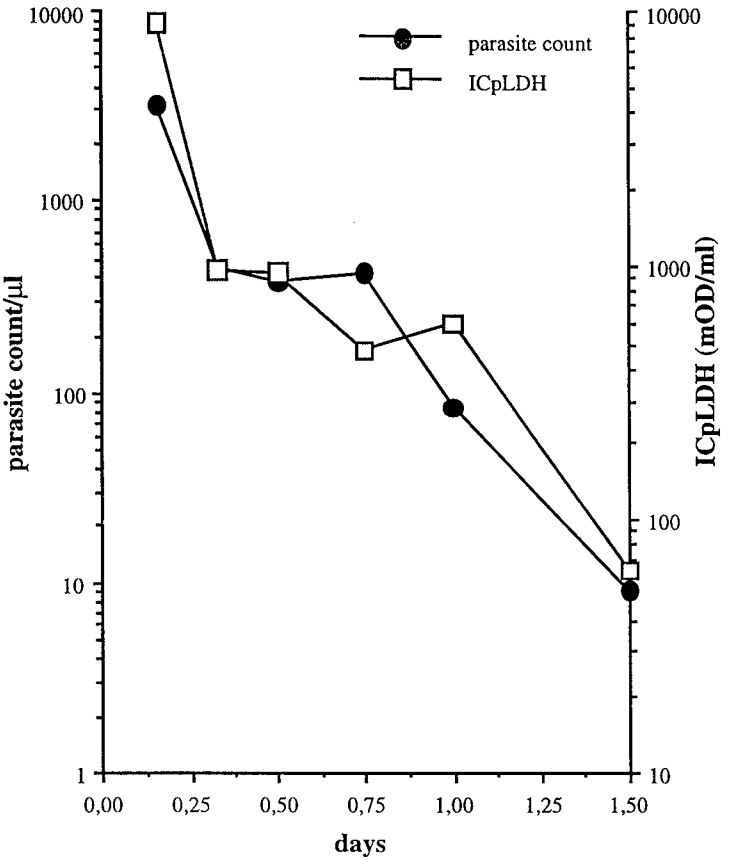
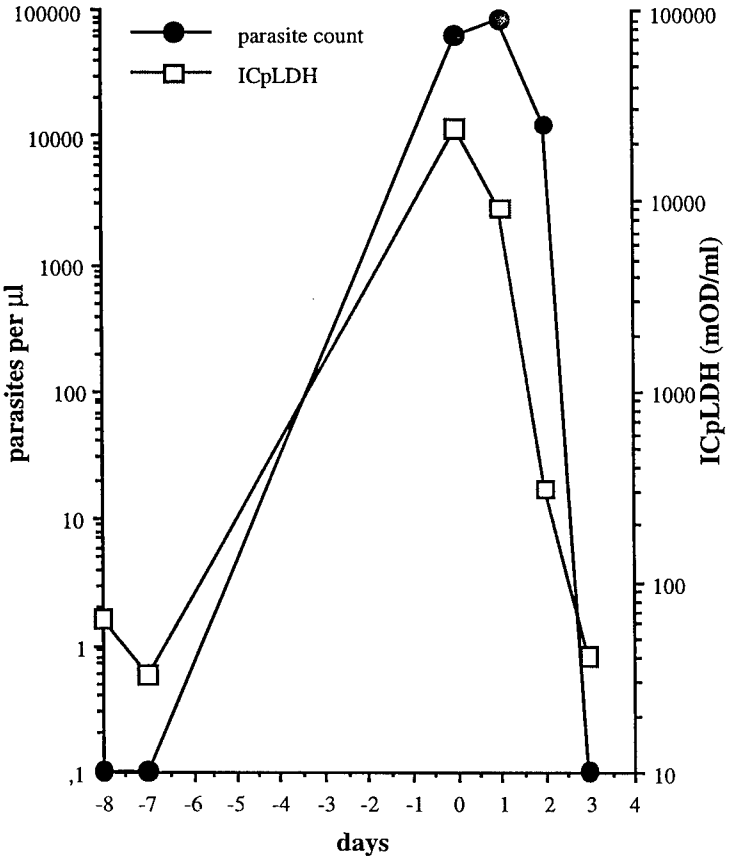

Patient 20
Patient 19
Patient 9
pLDH Activity mOD/min
% Parasitemia
Day
NEG

Figure 6.



Change in parasitic densities and intra-erythrocytic parasitic lactate dehydrogenase activities within 3 days of treatment of patient # 3 at HBCB.

Figure 7.



Patient # 4 presented with a short course of fever on the day he returned from Africa to France and the following day but we were unable to confirm malaria with **any** of the tests (included the QBC-test©) until 7 days later.

Note on human subjects experimental protocols
All field studies were strictly operated under protocols approved by the Flow Inc. human ethics board and remain in strict compliance with federal guidelines. As such at no time was patient testing performed for any purpose specific to the activities of Flow Inc. or on behalf of Flow Inc. Patient blood samples were tested only once they had been collected by non-Flow personnel for routine clinical reasons other than on behalf of Flow Inc. projects. As such, the clinicians in charge also kept the identity of all patients confidential from Flow personnel. A partial collection of protocols is included for inspection in Appendix 2 and 3.

Commercial Activities
Flow Incorporated has under taken significant activities to effectively commercialize the pLDH assays developed under this SBIR grant. For marketing reasons we have elected to focus on the commercialization of the Opti**MAL**® "dip-stick" assay format. Toward that end we have completed the kit design and are currently manufacturing and selling this kit. Kit manufacture under cGMP standards will be forth coming and Flow Inc. is actively pursuing the necessary business arrangements to effect this. We have also prepared promotional literature as well as an informative internet site to promote the sale of Opti**MAL**®. So far the response has been favorable and overwhelming.

CONCLUSIONS:

Under this SBIR grant period we have accomplished the following goals:

1) Design and format a simple, rapid, and sensitive "wet" (ELISA-like) method for assaying pLDH activity.
2) Promote this basic design to detect and even possibly differentiate the 4 species of malarial parasites (*P. falciparum, P. vivax, P. malariae,* and *P. ovalae*)
3) Subject the new test format to a rigorous "test of principle" to demonstrate that pLDH is an accurate indicator of malarial infection.
4) Subject the new test format to a rigorous "test of principle" to demonstrate whether pLDH can be used to monitor therapy.
5) Format a test kit that can be used to measure pLDH activity in the clinic that has practical and commercial application.
6) Produce a prototype dry "dip-stick" format that can measure pLDH levels in "far forward" conditions that utilizes little to no equipment.
7) Finished small scale manufacture of a field ready kit based on the prototype "dip-stick format.
8) Extensively field tested the manufactured malaria test kit and have found it to perform well for diagnosing malaria, differentiating malaria species, and assist in following the success anti-malarial chemotherapy.

LIST OF PUBLICATIONS

Part of this work has been presented in Poster form at:
The British Society of Parasitology, October, 1995
Woods Hole Meeting for Parasitology, October, 1995
This work has also been presented as a seminar at:
The American Society of Tropical Medicine and Hygiene, Baltimore, MD December, 1996

The British Society of Parasitology, October, 1995
Woods Hole Meeting for Parasitology, October, 1995
A pLDH Enzyme Capture Diagnostic Assay for Drug-Resistant Malaria

Abstract
The diagnosis of *Plasmodium sp.* has traditionally been performed by microscope. We have developed a procedure that perr to diagnosis the presence of the malaria parasite by the detection of a unique parasite enzyme . This parasite lactate dehydrogenase (pLDH) is able to utilize an analog of NAD, 3 acetyl pyridine adenine dinucleotide (APAD), to convert lact pyruvate . The human LDH does not readily use this analog. This fact allows the pLDH to be specifically measured with t use of this analog. The pLDH diagnostic enzyme assay is easy to perform and quantitative. The percent parasitemia has be standardized with the use of recombinantly expressed pLDH. The sensitivity of the original pLDH enzyme assay is howev limited to 0.01% parasiitemia, thus the assay has limited value in the developed world where sensitivities of 0.001% are req for diagnosis. Consequently, using the recombinant expressed pLDH we have produced monoclonal antibodies in mice to t pLDH. These monoclonal antibodies are able to capture and concentrate the active enzyme and can be used to move the ac away from red cell lysates. Initial studies show that the use of the monoclonal antibodies with the pLDH assay reagents w increase the sensitivity and specificity of the pLDH diagnostic assay to a level required in the developed world. This study reformat the current pLDH test based on these new reagents and evaluate whether the measurement of pLDH activity is a g diagnostic indicator of malaria. The pLDH enzyme capture assay will be formatted into a test using whole blood, red bloo cells, or serum/plasma. This technology may also be used in the future to test blood products for malaria and to measure drug-sensitivity of parasites isolated from individual patients.

The American Society of Tropical Medicine and Hygiene, Baltimore, MD December, 1996
Opti**MAL**(Immunochromatographic Assay for Diagnosis and Therapeutic Monitoring of Malaria.

Angela Hunt-Cooke*, Anthony Moody*, Elizabeth Gabbett*, Peter Chiodini*, David VanderJagt***, Frederique Marquet#, Sandrine Houze#, Jacques Le Bras#, Pierre Druilhe^,Marcel Hommel+, Martha Cecilia Acosta **, Ayoade Odoula++, Lim Chae Seung", Jennifer Kimmerlein>>, Junita Ries>>, Jean Williams>>, Laura Wentworth:: , Robert Piper::, Michael T. Makler::>>.

*Hospital for Tropical Diseases, UK,***U of New Mexico, #Hopital Bichat-Claude Bernard, France, ^Institute Pasteur, France, +Liverpool School of Tropical Medicine, UK, **CIDEIM, Columbia,++ U of Ibadan, Nigeria, "Republic of Korea Army, >>Veterans Affairs Medical Center, USA, ::Flow Inc. Or. USA

We describe an immunochromatographic assay , designated the Opti**MAL** assay for the diagnosis of malaria. The Opti**MAL** (assay is easy to perform, rapid to complete, sensitive, quantitative and able to monitor anti-malarial therapy. The Opti**MAL** (assay is directed to Plasmodium lactate deydrogenase(pLDH), a conserved enzyme (antigen) involved in the glycolytic pathway of the *Plasmodium* parasite. Monoclonal antibodies to several epitopes on this enzyme have been developed which permit differentiation of *P. falciparum* from *P.vivax, malariae* and *ovale.* The pLDH is expressed in all blood stages. The Opti**MAL** dip sticks contains both a *pan*-specific and *falciparum*-specific monoclonal antibody. The Opti**MAL** assay was used to examine 312 cases of malaria. The assay detected 277 *falciparum* -specific specimens and 35 specimens that only reacted with the *pan*-specific antibody, indicating the presence of either *vivax, malariae,* or *ovalae.* These results exactly confirmed results obtained from thin and thick microscopy. The sensitivity of the Opti**MAL** assay was recently evaluated in several European Institutes and from samples obtained from laboratories around the world. The data is to be reviewed. The assay is 100% sensitive to 100 paraistes/µl , 91% sensitive to 50-100 parasites/µl, and , and 20-60% sensitive to 1-50 parasites/µl . Thespecificity of the assay to 100 parasites/µl is 100% (>400n). Since pLDH is a product of viable parasites the Opti**MAL** assay is able to monitor anti-malarial therapy. In all cases of effective anti-malarial therapy tested to date, the fall of pLDH coincides exactly with the decline in % parasitemia. After 5-6 days there is no pLDH signal if there is no parasitemia detected by microscopy. In a single case of recrudescene of the parasite, pLDH was noted. This ability to monitor thereapy is not the case with either the Parasight F test or the ICT malaria assays. Both these tests detect HRP2. This antigen is known to persists for up to 3 weeks after anti-malarial therapy is completed and after the patient is free of clinical symptoms.

LIST OF PERSONNEL

Flow Inc.

Robert Piper, Principle Investigator
Michael Makler, Medical Director
Laura Wentworth, Research Technician
Jean Williams, Research Technician
Junita Rees, Research Technician
Nia Bryant, Molecular Biology Consultant

Hospital for Tropical Disease

Peter Chiodini
Angela Cook
Liz Gabbett

Hopital Bichat Claude Bernard

Jaques LeBras

Comparison of ICpLDH Assay and OptiMAL® Assay on P. falciparum Samples: Correlation with Parasitemia during Drug Treatment.

This study investigated the following questions:

1) How does the Opti**MAL**® Dipstick compare with the ICpLDH assay
2) What levels of parasitemia can be measured using the ICpLDH assay and the Opti**MAL**® Dipstick
3) Can pLDH levels be used to follow drug therapy

Methods:

Frozen samples collected in 1993 at the Hospital for Tropical Disease were shipped to Flow for evaluation of ICpLDH assay. Samples were collected as part ofthe regular activities of HTD. Samples were stored for up to 5 months at -20°C prior to shipping at 4°C. Sequencial samples were available on some patients. The pLDH activity (mOD/min) of some of these sample series are plotted below the corresponding graph of % parasitemia determined by microscopy. Both Activity and Parasitemia are plotted as a function of days during antimalarial therapy.
Opti**MAL**® assays were run for 10 min with PIP buffer.
ICpLDH assays were performed with both the *P. falciparum* specific antibody (17E4) and the *pan*-specific antibody (19G7).

Results:

pLDH was found to correlate quite well with the presence of malaria. Both the ICpLDH assay and the Opti**MAL**® Dipstick assays were able to pick up most samples even at low parasitemias. OptiMAL® was able to detect samples of 0.01% parasitemia (or 200-500 parasites/µl) very consistently. Opti**MAL**® did not detect some of the samples with lower levels of parasites, however, all of these low parasitemia samples came from patients that had and were currently undergoing drug therapy.

ICpLDH Assay

% Parasitemia	Total	ICpLDH Positive	ICpLDH Negative	Sensitivity	Specificity
>0.03	32	32	0	100%	
0.01	16	16	0	100%	
0.001	19	15	4	78%	
<=0.0001	21	15	6	71%	
negative	30	0	34		100%

OptiMAL® **Dipstick Assay**

% Parasitemia	Total	Opti**MAL**® Positive	Opti**MAL**® Negative	Sensitivity	Specificity
>0.03	40	40	0	100%	
0.01	18	17	1	94%	
0.001	20	20	12	72%	
<=0.0001	22	22	15	68%	
negative	30	0	30		100%

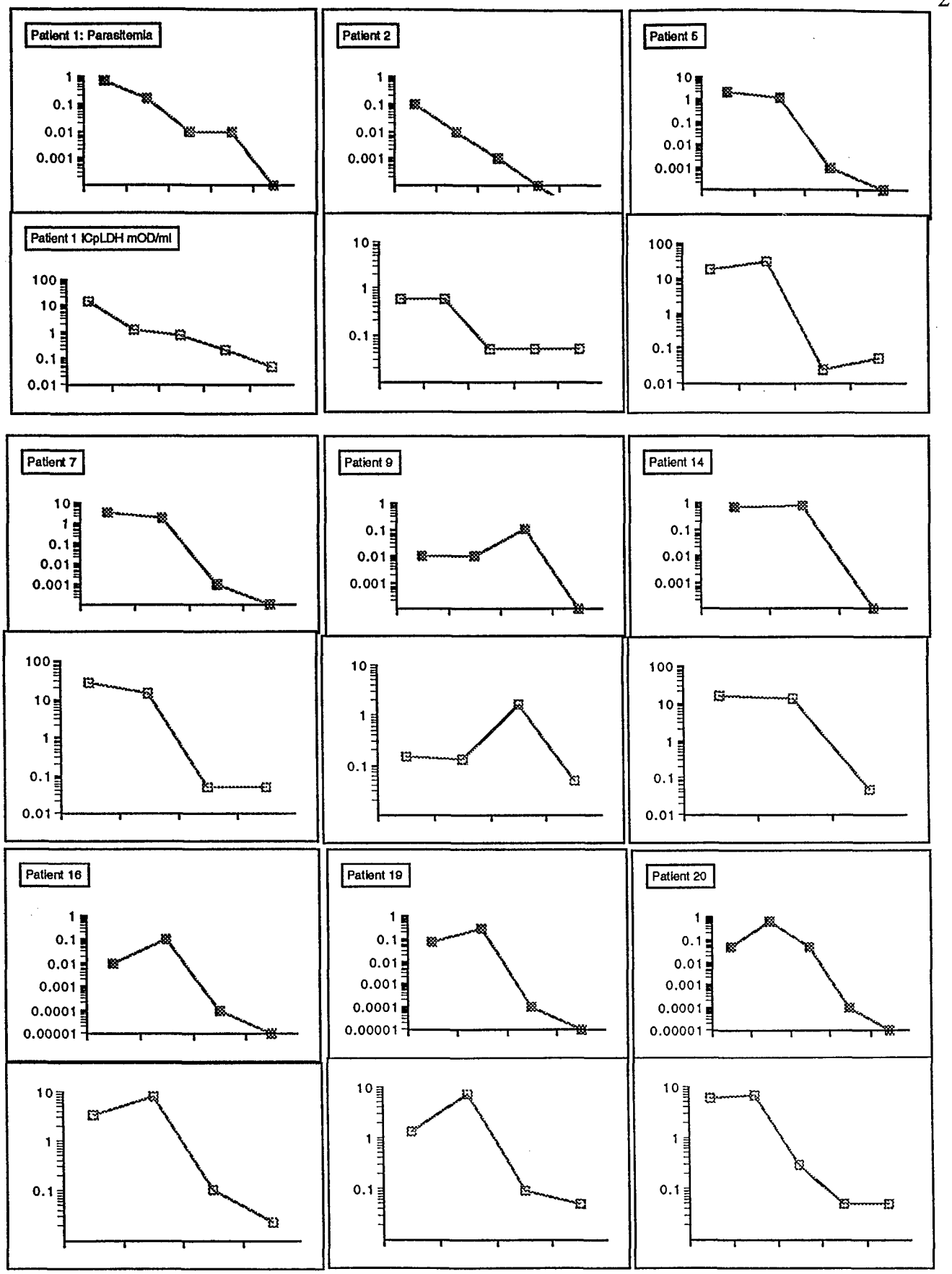
1996 samples from Hospital of Tropical Disease
Performed at FLOW Inc. by Angela Cook and Laura Wentworth
Samples collected and stored at -20°C
whole blood EDTA venous samples

OptiMAL dipstick assay and ICpLDH assay
JUNE, 1996

NEG* means possible faint line but was called negative.

Patient KEY	Parasitemia	OptiMAL 17E4	Kinetic 650 ICpLDH antibody/19G7 antibody	Comments
1a	0.7	POS	13.95/13.97	
1b	0.15	POS	1.250/1.020	
1c	0.01	POS	0.728/0.717	
1d	0.01	POS	0.193/0.114	
1e	0.0001	NEG*	0.000/0.000	Extremely low parasitemia at end of drug therapy
2a	0.1	POS	0.597/0.433	
2b	0.01	POS	0.608/0.833	
2c	0.001	POS	0.000/0.133	
2d	0.0001	POS	0.000/0.084	
2e	GAMS	POS	0.000/0.107	
3a	0.3	POS	0.229/2.002	
3b	0.05	POS	0.323/2.083	
3c	0.001	POS	0.224/0.364	
4a	0.2	POS		
4b	0.3	POS		
4c	0.01	POS		
4d	0.0001	NEG*		Extremely low parasitemia at end of drug therapy
5a	2	POS	18.17/28.95	
5b	1.3	POS	31.21/20.39	
5c	0.001	POS	0.024/0.016	
5d	0.0001	?		
6a	0.01	POS	3.043/2.600	
6b	0.01	POS	0.000/0.016	
6c	0.01	NEG*	0.000/0.080	Low parasitemia at end of drug therapy
6d	GAMS	POS	0.038/0.076	Positive by OptiMAL but did not attain threshold cuttoff by ICpLDH
7a	3.6	POS	26.71/60.60	
7b	1.8	POS	14.35/31.07	
7c	0.001	POS		
8a	0.0001	NEG*	0.000/0.000	
8b	NEG	NEG	0.034/0.000	
9a	0.01	POS	0.000/0.152	
9b	0.01	POS	0.134/0.299	
9c	0.1	POS	1.689/0.965	
9d	0.0001	POS	0.000/0.000	Extremely low parasitemia at end of drug therapy
10a	3.2	POS	19.77/59.80	
10b	4	POS	17.31/51.80	
10c	0.001	POS	7.695/4.676	
10d	GAMS	POS	10.60/7.146	
11a	0.001	POS	0.071/0.057	Positive by OptiMAL but did not attain threshold cuttoff by ICpLDH
11b	0.001	POS	0.000/0.000	
11c	NEG	NEG*	0.000/0.016	
12a	0.2	POS		
12b	0.01	POS	0.446/0.170	
12c	GAMS	POS	0.269/0.129	
13a	0.4	POS	17.31/15.72	
13b	0.2	POS	1.285/0.795	
14a	0.7	POS	16.09/15.04	
14b	0.8	POS	13.81/15.39	
14c	0.0001	NEG	0.000/0.000	Extremely low parasitemia at end of drug therapy
15A	0.01	POS		
15b	0.001	POS	0.146/0.051	
15c	NEG	NEG*	0.000/0.005	
16a	0.01	POS	3.521/3.109	
16b	0.1	POS	8.215/1.580	
16c	0.0001	POS	0.105/0.225	
16d	GAMS	POS	0.024/0.175	
17a	1.5	POS	7.303/8.393	
17b	0.5	POS	0.158/0.139	

Patient KEY	OptiMAL® Parasitemia	Kinetic 650 ICpLDH 17E4 antibody/19G7 antibody	Comments
17c	NEG	NEG*	0.098/0.049	
18a	1.6	POS		
18b	1	POS		
18c	0.3	POS		
18d	0.01	NEG*		Low parasitemia at end of drug therapy
19a	0.07	POS	1.323/1.192	
19b	0.3	POS	7.448/5.252	
19c	0.0001	POS	0.095/0.159	
19d	GAMS	NEG*	0.000/0.000	
20a	0.05	POS	6.111/5.043	
20b	0.7	POS	7.115/7.099	
20c	0.05	POS	0.294/0.055	
20d	0.0001	NEG	0.000/0.017	Extremely low parasitemia at end of drug therapy
20e	GAMS	NEG	0.00/0.0000	Extremely low parasitemia at end of drug therapy
21a	0.0001	POS		
21b	GAMS	NEG		
22a	0.65	POS	17.47/19.70	
22b	12	POS	15.89/19.07	
22c	12	POS	11.65/7.762	
22d	0.0001	POS	1.186/0.735	
23a	0.01	NEG	0.082/0.000	Low parasitemia during drug therapy. Did not get initial sample
23b	0.0001	NEG	0.000/0.014	Extremely low parasitemia at end of drug therapy
24a	0.05	POS	0.751/0.472	
24b	NEG	NEG*	0.107/0.105	
25a	0.4	POS	5.097/7.307	
25b	0.5	POS	14.52/12.14	
26a	0.02	POS		
26b	GAMS	POS		
27a	1	POS		
27b	0.01	POS		
28a	0.05	POS	1.133/0.867	
28b	0.001	NEG	0.000/0.000	Low parasitemia at end of drug therapy
29a	0.001	NEG		Low parasitemia at end of drug therapy
29b	0.001	POS		
TN1	NEG	NEG		
TN2	NEG	POS	0.000/0.000	
TN3	NEG	NEG		
TN4	NEG	NEG	0.000/0.014	
TN5	NEG	NEG		
TN6	NEG	NEG		
TN7	NEG	NEG		
TN8	NEG	NEG		
TN9	NEG	NEG		
TN10	NEG	NEG		
TN11	NEG	NEG		
TN12	NEG	NEG		
TN13	NEG	NEG		
TN14	NEG	NEG		
TN15	NEG	NEG		
TN16	NEG	NEG		
TN17	NEG	NEG	0.000/0.000	
TN18	NEG	NEG		
TN19	NEG	NEG		
TN20	NEG	NEG		
TN21	NEG	NEG		
TN22	NEG	NEG	0.000/0:000	
TN23	NEG	NEG		
TN24	NEG	NEG		
TN25	NEG	NEG	0.021/0.013	


Patient 1: Parasitemia
Patient 2
Patient 5
Patient 1 lCpLDH mOD/ml
Patient 7
Patient 9
Patient 14
Patient 16
Patient 19
Patient 20

Comparison of ICpLDH Assay and OptiMAL® Assay on *P. falciparum* and *P. vivax* Samples: Cali Colubia, 1996

This study investigated the following questions:
1) What levels of parasitemia can be measured using the ICpLDH assay and the Opti**MAL**® Dipstick
2) How does the Opti**MAL**® Dipstick and the ICpLDH assay perform with *P. vivax* and *P. falciapurm*
3) Can the ICpLDH assay and the Opti**MAL**® Dipstick correctly speciate *P. vivax* from *P. falciparum*
4) What are the levels of pLDH in whole blood, plasma and red cell fractions from *P. falciparum* and *P. vivax* infected patients
5) Can blood samples be dried before assaying making it possible to test field samples in central facility.

Methods:
Samples were stored for up to 3 months at -20°C prior to shipping at 4°C. Red cell fractions were separated from Plasma fractions by allowing samples to settle. Quantitiation of samples by microscopy was performed on think smears. Dried blood samples were prepared by spotting 50 µls of whole blood onto filter paper and allowing paper to dry at room temperature. Dried samples were eluted by soaking filter paper in 200 µls of water.

Opti**MAL**® assays were run for 10 min with FB4 buffer.
ICpLDH assays were performed with the *pan*-specific antibody (19G7).

Results:
Both the ICpLDH assay and the Opti**MAL**® Dipstick assays were able to pick up all of the p. falciparum and P. vivax samples even at low parasitemias. This was observed for samples of whole blood as well as for red cell fractions. We also found 100% concordance with samples that hed been dried onto filter paper. Opti**MAL**® was also able to correctly speciate all P. vivax from P. falciparum in whole blood samples and red blood cell fractions. We also found enzyme activity in plasma fractions of both *P. falciparum* and *P. vivax* samples.

Below are results of the Opti**MAL**® assay's ability to differentiate *P. falciparum* malaria from *P. vivax*

WHOLE BLOOD SAMPLES

	Total	Two reaction lines *falciparum*-specific line	One reaction line *pan*-specific line only
P. falciparum	10	10	0
P. vivax	12	0	12

RED CELL FRACTIONS

	Total	Two reaction lines *falciparum*-specific line	One reaction line *pan*-specific line only
P. falciparum	10	10	0
P. vivax	13	0	13

Samples of P. falciparum and P. vivax from Columbia
Several sample types
WHOLE BLOOD, RED CELL FRACTION, PLASMA FRACTION, and samples dried onto filter paper
All samples stored at -20°C and shipped at 4°C
Analysis performed at FLOW Inc.
Threshold for ICpLDH Assay using K650 read is >0.100 mOD/ml

Whole BLOOD			ICpLDH		DipStick		KEY
SAMPLE #	CALI KEY	#PARASITE	K650	VISUAL	OptiMAL	%	Parasitemia
GE-1	P.vivax	39500	0.822	1 1/2+	POS-PV		0.800
GE-13	P.vivax	15000	6.215	4 +	POS-PV		0.304
C3+	P.vivax	11592	64.6	4 +	POS-PV		0.235
GE-14	P.vivax	5225	0.711	1 1/2+	POS-PV		0.106
GE-17	P.vivax	3500	4.74	4 +	POS-PV		0.071
GE-25	P.vivax	2808	N/S	N/S	POS-PV		0.057
GE-22	P.vivax	2469	9.034	4 +	POS-PV		0.050
GE-16	P.vivax	1283	8.422	4 +	POS-PV		0.026
GE-11	P.vivax	783	7.379	4 +	POS-PV		0.016
GE-6	P.vivax	741	6.036	3 +	POS-PV		0.015
GE-10	P.vivax	290	5.428	4 +			0.006
GE-12	P.vivax	203	6.218	3 +	POS-PV		0.004
C1+	P.fal	129937	61.2	4 +	POS-PF		2.631
C2+	P.fal	14467	4.36	3 +	POS-PF		0.293
GE-5	P.fal	10000	3.155	3 +	POS-PF		0.203
GE-20	P.fal	4180	7.448	3 1/2+	POS-PF		0.085
GE-18	P.fal	2475	1.766	2 +	POS-PF		0.050
GE-26	P.fal	2103	1.933	3 +	POS-PF		0.043
GE-3	P.fal	1000	5.544	4 +	POS-PF		0.020
GE-24	P.fal	890	0.569	1/2 +	POS-PF		0.018
GE-23	P.fal	230	2.513	2 +	POS-PF		0.005
GE-7	P.fal	42	0.289	1/2 +	?		0.001
C3-	NEG	NEG	-0.012	NEG	NEG		
C2-	NEG	NEG	0.037	NEG	NEG		
C1-	NEG	NEG	-0.006	NEG	NEG		
GE-21	NEG	NEG	0.04	NEG	NEG		
GE-19	NEG	NEG	0.026	NEG	NEG		
GE-15	NEG	NEG	0.033	NEG	NEG		
GE-8	NEG	NEG	0.028	NEG	NEG		
GE-4	NEG	NEG	0	NEG	NEG		

Red Blood Cell Fraction			ICpLDH				KEY
Patient	CALI KEY	#PARASITE	K650	VISUAL	OptiMAL	%	Parasitemia
GE-1	P.vivax	39500	1.094	2+	POS-PV		0.800
GE-13	P.vivax	15000	5.188	4+	POS-PV		0.304
C3+	P.vivax	11592	19.53	4+	POS-PV		0.235
GE-14	P.vivax	5225	10.12	4+	POS-PV		0.106
GE-17	P.vivax	3500	9.426	4+	POS-PF		0.071
GE-25	P.vivax	2808	4.918D	4+	POS-PV		0.057
GE-22	P.vivax	2469	10.85	4+			0.050
GE-16	P.vivax	1283	11.75	4+	POS-PV		0.026
GE-11	P.vivax	783	6.379	4+	POS-PV		0.016
GE-6	P.vivax	741	5.786	3+	POS-PV		0.015
GE-2	P.vivax	320	5.186	4+	POS-PV		0.006
GE-10	P.vivax	290	5.998	4+	POS-PV		0.006
GE-12	P.vivax	203	6.251	4+	POS-PV		0.004
C1+	P.fal	129937	69	4+	POS-PF		2.631
C2+	P.fal	14467	1.277	2+	POS-PF		0.293
GE-5	P.fal	10000	2.655	3+	POS-PF		0.203
GE-20	P.fal	4180	6.565	3 1/2+	POS-PF		0.085
GE-18	P.fal	2475	1.367	2+	POS-PF		0.050
GE-26	P.fal	2103	1.428	2+	POS-PF		0.043
GE-3	P.fal	1000	7.248	4+	POS-PF		0.020
GE-24	P.fal	890	0.535	1/2+	POS-PF		0.018
GE-23	P.fal	230	2.054	2+	POS-PF		0.005
GE-7	P.fal	42	0.609	1/2+	POS-PF		0.001
C3-	NEG	NEG	0.009	NEG	NEG		
C2-	NEG	NEG	0.028	NEG	NEG		
C1-	NEG	NEG	0.034	NEG	NEG		
GE-21	NEG	NEG	0.051	NEG	-/+ PF		
GE-19	NEG	NEG	0.051	NEG	-/+ PF		
GE-15	NEG	NEG	0.016	NEG	NEG		
GE-8	NEG	NEG	0.026	NEG	NEG		
GE-4	NEG	NEG	-0.014	NEG	NEG		

PLASMA Patient	CALI KEY	#PARASITE	ICpLDH K650	VISUAL	%	KEY Parasitemia
GE-1	P.vivax	39500	0.01	NEG		0.800
GE-13	P.vivax	15000	0.153	NEG		0.304
C3+	P.vivax	11592	0.905	2+		0.235
GE-14	P.vivax	5225	-0.004	NEG		0.106
GE-17	P.vivax	3500	0.698	1+		0.071
GE-25	P.vivax	2808	2.173	3+		0.057
GE-22	P.vivax	2469	2.477	3+		0.050
GE-16	P.vivax	1283	1.673	2 1/2+		0.026
GE-11	P.vivax	783	0.127	NEG		0.016
GE-6	P.vivax	741	0.314	NEG		0.015
GE-2	P.vivax	320	0.035	NEG		0.006
GE-10	P.vivax	290	0.339	1/2+		0.006
GE-12	P.vivax	203	0.257	*+/-		0.004
C1+	P.fal	129937	0.04	NEG		2.631
C2+	P.fal	14467	-0.008	NEG		0.293
GE-5	P.fal	10000	0.14	*+/-		0.203
GE-20	P.fal	4180	0.114	*+/-		0.085
GE-18	P.fal	2475	0.034	NEG		0.050
GE-26	P.fal	2103	0.172	NEG		0.043
GE-3	P.fal	1000	0.227	1/2+		0.020
GE-24	P.fal	890	0.074	NEG		0.018
GE-23	P.fal	230	0.072	NEG		0.005
GE-7	P.fal	42	0.021	NEG		0.001
C3-	NEG	NEG	0.003	NEG		
C2-	NEG	NEG	0.051	*+/-		
C1-	NEG	NEG	0.009	NEG		
GE-21	NEG	NEG	0.036	NEG		
GE-19	NEG	NEG	0.029	NEG		
GE-15	NEG	NEG	0.038	NEG		
GE-8	NEG	NEG	-0.01	NEG		
GE-4	NEG	NEG	0.017	NEG		

CONTROLS	AB K650	AB VIS
CRBC RW	0.045	NEG
	0.036	NEG
PLDH	27	4+
	33.36	4+
P.FAL OK	30.71	4+
	23.24	4+

Dried Filter Paper samples			ICpLDH			KEY
Patient	CALI KEY	#PARASITE	K650	VISUAL	%	Parasitemia
GE-1	P.vivax	39500	0.255	1/2+		0.800
GE-13	P.vivax	15000	9.478	3 1/2+		0.304
C3+	P.vivax	11592	7.06	4+		0.235
GE-14	P.vivax	5225	0.732	1+		0.106
GE-17	P.vivax	3500	4.106	3+		0.071
GE-25	P.vivax	2808	14.3	4+		0.057
GE-22	P.vivax	2469	8.126	3 1/2+		0.050
GE-16	P.vivax	1283	5.11	3+		0.026
GE-11	P.vivax	783	2.393	2+		0.016
GE-6	P.vivax	741	1.312	2+		0.015
GE-2	P.vivax	320	0.939	1+		0.006
GE-10	P.vivax	290	1.656	2+		0.006
GE-12	P.vivax	203	0.757	1+		0.004
C1+	P.fal	129937	18.64	4+		2.631
C2+	P.fal	14467	1.09	2+		0.293
GE-5	P.fal	10000	1.216	2+		0.203
GE-20	P.fal	4180	4.476	3+		0.085
GE-18	P.fal	2475	0.497	1+		0.050
GE-26	P.fal	2103	1.623	2+		0.043
GE-3	P.fal	1000	6.272	3+		0.020
GE-24	P.fal	890	0.163	1/2+		0.018
GE-23	P.fal	230	0.606	1+		0.005
GE-7	P.fal	42	0.109	1/2+		0.001
C3-	NEG	NEG	0	NEG		
C2-	NEG	NEG	0.046	NEG		
C1-	NEG	NEG	0.165	NEG		
GE-21	NEG	NEG	0	NEG		
GE-19	NEG	NEG	0	NEG		
GE-15	NEG	NEG	0	NEG		
GE-8	NEG	NEG	0	NEG		
GE-4	NEG	NEG	0.002	NEG		

Comparison of OptiMAL® Assay on P. falciparum Samples:
Correlation with Parasitemia during Drug Treatment at HTD July 1996

This study investigated the following questions:
1) What levels of parasitemia can be measured using the lCpLDH assay and the OptiMAL® Dipstick
2) How does the test perform in non-Flow personnel hands
3) How will can OptiMAL® track successful drug treatment

Methods:
Samples were collected at HTD according to standard procedure. During the 3 month course of this study, samples were collected and run with the OptiMAL® assay. Sequential samples were available for many patients. Patients that were smear positive for parasites were admitted and followed until they were delcared smear negative. These smear negative samples are termed "terminal negatives" OptiMAL® assays were run for 10 min with FB4 buffer.

Results:

All Plasmodium falciparum positive blood samples
sample size 177

%Parasitemia	total tested	OptiMAL® positive	OptiMAL® negative	%positive
>/=1%	21	21	0	100
0.1-0.9	54	54	0	100
0.01-0.09	32	32	0	100
0.001-0.009	26	12	14	46.15
0.0001-0.0009	12	6	6	50
0.00001-0.00009	4	0	4	0
gametocytes only	8	1	7	12.5
terminal negatives	20	2	18	10

July, 1996
Liz Gabbett, Angela Cook Study
Samples collected and evaluated at HTD by non-FLOW persons
All P. falciparum samples--some from patients undergoing drug therapy

Different patients are denoted by different numbers (1,2,3..)
Samples taken from sequential days from the same patient are denoted (1a, 1b, 1c...)
Disparate samples are in bold

Patient	GIEMSA %parasitemia	OptiMAL® Result P. falciparum-POS	
1a l	0.1	pos	
2a l	0.01	pos	
3a l	0.01	pos	
4a l	**0.001**	**NEG**	**low parasitemia**
4b	0.01	pos	
4c	**0.001**	**NEG**	**low parasitemia at the end of drug therapy**
5a l	0.01sc	pos	
6a l	0.6	pos	
7a l	4	pos	
7b	4	pos	
7c	4.5sc	pos	
7d	0.8	pos	
7e	0.005	pos	
7f	**0.0001**	**NEG**	**extremely low parasitemia**
7g	**gametocytes only**	**NEG**	**extremely low parasitemia**
7h	NEG	NEG	
7i	1.5	pos	
7j	1	pos	
7k	0.15	pos	
7l	0.7	pos	
7m	0.001	pos	
7n	0.05	pos	
7o	**gametocytes only**	**NEG**	**extremely low parasitemia**
7p	0.01/g	pos	
7q	**gametocytes only**	**NEG**	**extremely low parasitemia**
7r	**0.001**	**NEG**	
7s	**gametocytes only**	**NEG**	**low parasitemia**
7t	NEG	NEG	
7u	NEG	NEG	
7v	**gametocytes only**	**NEG**	**extremely low parasitemia**
7w	NEG	NEG	
8a l	0.05	pos	
9a l	0.00001	NEG	
9b	0.00001	NEG	
9c	gametocytes only	NEG	
10a l	**0.001**	**NEG**	**low parasitemia**
10b	**0.001**	**NEG**	**low parasitemia**
11a l	**0.001**	**NEG**	**low parasitemia**
11b	**0.001**	**NEG**	**low parasitemia**
12a l	0.01	pos	
13a l	**0.001**	**NEG**	**low parasitemia**
13b	1	pos	
13c	NEG	NEG	
14a l	0.8	pos	

Patient	GIEMSA %parasitemia	OptiMAL® Result P. falciparum-POS	
15a I	0.05	pos	
16a I	0.6	pos	
17a I	6	pos	
18a I	0.1	pos	
19a I	0.8	pos	
20a I	**0.001**	**NEG**	**low parasitemia**
21a I	0.001	pos	
22a I	0.3	pos	
22b	0.3	pos	
22c	0.001	pos	
22d	NEG	NEG	
24a I	0.01	pos	
25a I	**0.00001**	**NEG**	**extremely low parasitemia**
26a I	0.1	pos	
27a I	0.1	pos	
28a I	0.005	pos	
29a I	NEG	NEG	
29b	**0.005**	**NEG**	
29c	**0.0001**	**NEG**	**extremely low parasitemia**
29d	**0.0001**	**NEG**	**extremely low parasitemia**
29e	NEG	NEG	
29f	NEG	NEG	
30a I	0.01	pos	
31a I	0.6	pos	
31b	0.2	pos	
31c	0.0001	pos	
31d	0.0001	pos	
31e	**0.00001**	**NEG**	**extremely low parasitemia**
32a I	1	pos	
32b	0.7	pos	
32c	0.1	pos	
32d	0.005	pos	
32e	**0.001**	**NEG**	**low parasitemia**
33a I	0.07+psch	pos	
33b	1	pos	
33c	0.05	pos	
33d	**0.0001**	**NEG**	**extremely low parasitemia**
33e	NEG	NEG	
34a I	0.05+psch	pos	
34b	0.01+psch	pos	
34c	0.1	pos	
34d	0.01	pos	
34e	0.001	pos	
34f	**NEG**	**pos**	**false postive; however from patient that tested posti**
34g	NEG	NEG	
35a I	0.005	pos	
36a I	6	pos	
37a I	0.03	pos	
38a I	**gametocytes only**	**NEG**	**extremely low parasitemia**
39a I	0.05	pos	
40a I	0.01	pos	
41a I	0.0001	pos	
43a I	0.4	pos	
44a I	0.6	pos	

Patient	GIEMSA %parasitemia	OptiMAL® Result P. falciparum-POS	
44b	0.8	pos	
44c	**0.0059**	**NEG**	**low parasitemia**
44d	NEG	NEG	
44e	**gametocytes only**	**NEG**	**extremely low parasitemia**
45a I	0.3	pos	
46a I	0.4	pos	
47a I	2.5	pos	
47b	0.8	pos	
47c	0.2	pos	
47d	0.1	pos	
47e	0.001	pos	
47f	NEG	NEG	
48a I	0.5	pos	
49a I	0.01	pos	
50a I	0.1	pos	
51a I	1.5	pos	
53a I	0.05	pos	
55a I	**0.0001**	**NEG**	**extremely low parasitemia**
56a I	0.2	pos	
56b	0.1	pos	
56c	0.1	pos	
56d	**0.001**	**NEG**	**low parasitemia**
56e	NEG	NEG	
57a I	0.8	pos	
57b	0.4	pos	
57c	0.001	pos	
57d	**0.0001**	**NEG**	**extremely low parasitemia**
57e	NEG	NEG	
58a I	1.2	pos	
59a I	1	pos	
60a I	0.3	pos	
61a I	0.005	pos	
62a I	1.2	pos	
63a I	0.1	pos	
64a I	0.1	pos	
65a I	0.1	pos	
65b	0.005	pos	
65c	0.0001	pos	
65d	NEG	NEG	
66a I	0.1	pos	
66b	0.06	pos	
67a I	0.01	pos	
68a I	5.5	pos	
69a I	0.2	pos	
70a I	0.6	pos	
71a	0.0001	pos	
72a I	0.2	pos	
73a I	10	pos	
74a I	g	pos	
75a I	0.01	pos	
76a I	0.01	pos	
77a I	0.2	pos	
78a	0.01	pos	
79a I	0.2	pos	

Patient	GIEMSA %parasitemia	OptiMAL® Result P. falciparum-POS	
80a I	0.01	pos	
81a I	0.1	pos	
82a I	**0.001**	**NEG**	**low parasitemia**
83a I	0.5	pos	
83b	0.8	pos	
83c	0.2	pos	
83d	0.05	pos	
83e	NEG	pos	
84a I	0.8	pos	
84b	0.8	pos	
84c	0.01	pos	
84d	NEG	NEG	
85a I	0.01	pos	
85b	0.1	pos	
86a	0.5	pos	
86b	1.5	pos	
86c	0.01	pos	
86d	0.0001	pos	
87a I	0.2	pos	
88a I	2	pos	
89a I	0.08	pos	
90a I	0.2	pos	
91a I	1.6	pos	
92a I	0.1	pos	
93a I	1	pos	

Evaluation of OptiMAL at the Pasteur Institute with Samples from Senegal

This study investigated the following questions:
1) What levels of parasitemia can be measured using the Opti**MAL**® Dipstick
2) What are the levels of pLDH in whole blood, plasma and red cell fractions from *P. falciparum*

Methods:
Samples were stored for up to 3 months at -20°C prior to analysis. Analysis was performed in a double blinded fashion at the Pasteur institute with Institute and Flow personnel. Opti**MAL**® assays were run for 10 min with FB4 buffer.

Results:

Table 1- Whole Blood (Pasteur)

parasitemia

	total tested	positive	negative	%positive
>/=1%	-	-	-	-
0.1-0.9	3	3	0	100
0.01-0.09	21	21	0	100
0.001-0.009	23	21	2	91
0.0001-0.0009	28	15	13	53
0.00001-0.00009	12	3	9	25
total 87				

Study at Pasteur Institute, 1996
Samples of P. falciparum from Senegal
Samples counted and stored frozen until OptiMAL strips run

Whole Blood Samples

Patient	Thick Smear per white cell	% Parasitemia	OptiMAL®
4230	f80,m0,025	0.08	fal
4363	f56%	0.056	fal
4256	f52%	0.052	fal
4465	f26%,gf1	0.026	fal
4220	f24,gf5	0.024	fal
4305	gf 21 gameto	0.02	fal
4318	f15%,gf10	0.015	fal
4295	f15%,m0,094	0.015	fal
4385	f13%	0.013	fal
4325	f 12%	0.012	fal
4380	f11%	0.011	fal
4384	f10%,m0,03	0.01	fal
4215	f9%	0.009	fal
4304	f9%,gfi	0.009	fal
4217	f8%,m0,09	0.008	fal
4301	f8%	0.008	fal
4407	f8%	0.008	fal
4402	f8%	0.008	fal
4387	f8%	0.008	viv?
4371	f8% gf1	0.008	fal
4443	f7%	0.007	viv/fal?
4423	f7%	0.007	fal/mix
4335	f4%,gfé,m0,03	0.004	fal
4345	f4%	0.004	fal
4369	f4%	0.004	fal
4322	f3%,gf,m0,03	0.003	fal
4449	f3%	0.003	fal
4439	f3%	0.003	neg
4440	f3%	0.003	fal
4425	f3%	0.003	fal
4404	f3%gf1	0.003	fal
4409	gf3%	0.003	fal
4310	f3%,gf1,m0.06	0.003	fal
4365	f3% gf1	0.003	fal
4376	f3%	0.003	fal
4426	f2.1%	0.0021	fal
4433	m2.1%	0.0021	fal
4311	f2%,m11%	0.002	fal
4389	f2%,gf2	0.002	fal
4233	f2%	0.002	fal

Appendix 2: Protocol for Field Study: The Gambia

Title

Comparison Of A pLDH-Based Antigen Detection Assay With Microscopy For The Detection Of Malarial Parasites In Human Blood Samples.

Abstract

Many clinical settings require alternative techniques to diagnose malaria than the traditional inspection of Giemsa stained blood films. Measurement of an abundant malarial enzyme, *Plasmodium* Lactate Dehydrogenase (pLDH), provides a viable alternative. We intend to evaluate a new immunochromatographic test that can detect the presence of pLDH in whole blood samples for its effectiveness as a diagnostic test for malaria. The immunochromatographic test will be compared to the conventional method of microscopic analysis of Giemsa stained thin smears.

Review of Human Ethics Board

Flow Inc.

Robert Piper, Ph.D., Flow Inc. (Chair)
Lisa Hess, MD.., Dept. Ob/Gyn, University of Indianapolis
Andre Makler, Ph.D., Flow Inc.
David Sewell, MD.., Dept. Microbiology, Veterans Administration Hospital, Portland Oregon.

Approved 9/1/96

Investigators

Flow Inc.
SW Corbett, Portland, Oregon, 97201, USA

Robert Piper, Ph.D., Scientific Director.
Michael T. Makler, Medical Director.
Laura Wentworth, Technical Advisor.
Jean Williams, Research Technician

Medical Research Council, Malaria Programme
Fajara, PO Box 273, Banjui, The Gambia, West Africa

Margaret Pinder, Head.
Tom Doherty, Clinical Scientist.

Hospital for Tropical Diseases
St. Pancras, London, UK

Peter Chiodini, M.D., Consultant Parasitologist
Angela Hunt Cook, Senior Scientific Officer

Scientific Background

Microscopic examination of blood smears is the most widely used method of determining malaria infection in humans. The procedure does not require sophisticated equipment and individuals can be trained relatively quickly on preparing blood smears and staining slides. However, microscopic examination is labor intensive and individuals that examine slides need to be experienced to differentiate parasites from artifact. Also, microscopic examination of smears is not always definitive with low level parasitemias and underscores the variability among different clinics that utilize microscopy for diagnosis of malaria.

These limitations justify the development and implementation of simple to use dipstick antigen-capture assays that have been recently developed. One such test has been developed that detects P*lasmodium falciparum* histidine-rich protein 2 in peripheral blood (PfHRP-2). The assay can be done quickly and easily, but the test can only detect *P. falciparum* infections and the sensitivity decreases at lower levels of parasitemia. Another problem with the assay is that the circulating antigen is detectable even several days after viable parasites have been eliminated from the peripheral blood stream (Beadle et al. 1994). This makes it difficult for health providers to **accurately** assess the effectiveness of drug therapy.

Makler et al, (1993) have shown that *Plasmodium* infections can be accurately detected by the unique ability for the parasites lactate dehydrogenase (pLDH) to utilize the 3-acety pyridine adenine dinucleotide (APAD) as a cofactor. A dipstick based on these finding has been developed by Flow Inc. and is ready to be field tested. Similar to the currently available dip-stick tests that detect HRP-2, the test made by Flow Inc. is based on the detection of pLDH.

The Flow Inc. pLDH assay stick (OptiMAL™ assay) detects the malarial parasites by detecting the presence of the pLDH antigen in lysed whole blood. The Flow Inc. pLDH assay stick detects the presence of parasites in a 10 µl of fresh, frozen or dried whole blood samples (finger stick or venopuncture) collected in EDTA/ACD/heparin. The pLDH first binds to a labeled antibody particle. This complex then migrates up the test strip where it is captured by an immobilized second antibody. At the reaction site a visual antibody-antigen -antibody complex is formed.

The current configuration of theOptiMAL™ assay potentially offers the following advantages over currently available rapid tests based on the detection of HRP-2:

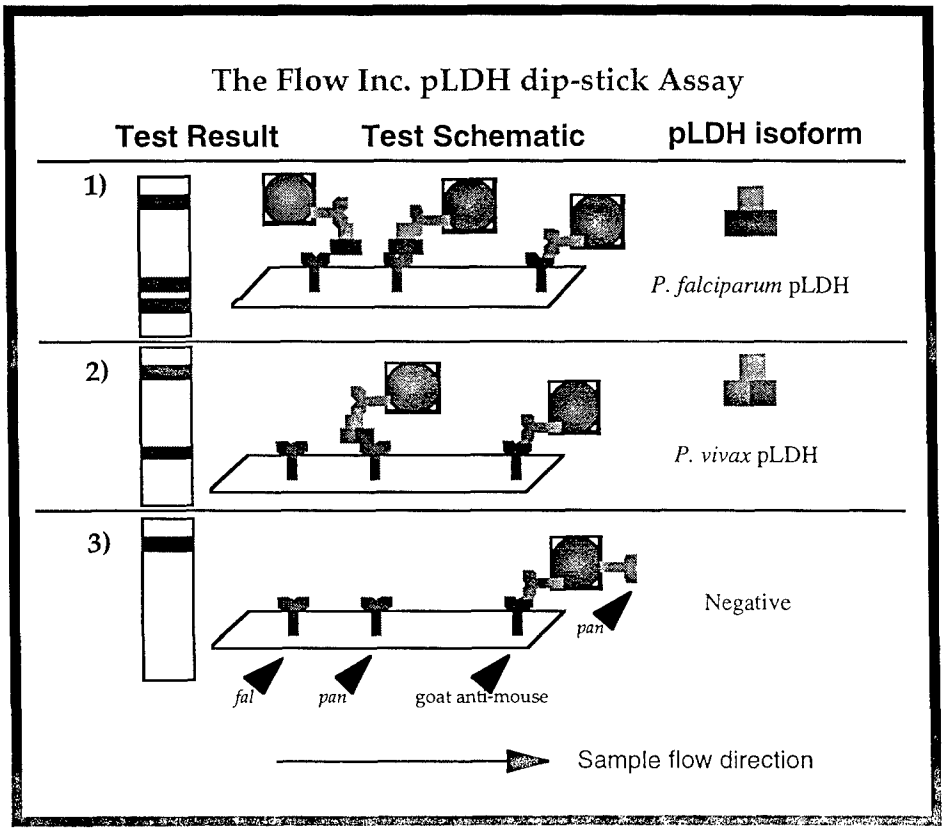
1) **The pLDH-based** test recognizes all major forms of human malaria
2) Samples infected with *P. vivax* are clearly and easily distinguished from those infected with *P. falciparum.*
3) The test follows the course of infection since preliminary data shows that a profound drop in circulating pLDH activity occurs immediately after parasites are cleared from peripheral blood.

The current format of the OptiMAL™ assay that includes the ability to detect and speciate human malarial pathogens is shown below:

The OptiMAL™ assay is designed to diagnose all forms of malaria and also differentiate between *P. falciparum* and the other three species of malaria. This differentiation is clinically relevant since the salient feature of malaria diagnosis is determined whether a malarial infection is positive or negative for *P. falciparum*. In the Flow Inc. pLDH-based dip-stick assay, there are two diagnostic zone react containing different antibodies. A monospecific antibody is present in the bottom reaction zone which recognizes only *P.falciparum*. A second pan-specific antibody is present immediately above this zone, this monoclonal antibody recognizes the pLDH isoform of *P. vivax*, *P. ovalae*, and *P. malariae*. A third reaction zone is present at the top of the immunochromatographic test strip where an antibody which captures the excess colloid conjugate and serves as a positive control for the assay. The colloid conjugate is coupled with a third monoclonal antibody that is pan specific.

The Interpretation of the assay test strip is as follows:

1. POSITIVE - *P. falciparum*: One control band plus **two** test bands.
2. POSITIVE - *P. vivax*: One control band plus **one** band.
3. NEGATIVE - One control band at the top of the test strip.



Procedures

a) Study Area: The study site will be the MRC facility in Fajara, The Gambia. This site has been used for a variety of similar studies and is well suited for the experimental protocols proposed herein.

b) Patient Population
Geographic area of residence of patient population: Persons visiting the MRC facility for routing blood evaluation represent a geographical radius of $\leq$50 miles.
General: Samples from persons undergoing routine diagnosis for malaria at the MRC will be entered into this study. No samples will be collected from persons for the express purposes of this study
Age of patient population: Only persons over the age of 8 shall be entered into the study protocol unless approval by the human ethics committee is granted.

c) Patient Identity
Patient Identity will be kept confidential by all parties to the extent that is reasonably possible. To enhance the objective nature of the study, patient samples, slides, test strips, etc. will be assigned a number that can be correlated with a patient history.
Patient histories will be made available after encryption and correlation to the number scheme used to code the blood/test samples.

d) Clinical History of Patients
Previous studies have shown that the recent clinical history of the patient may impact on the interpretation of the test results. Therefore, the following parameters will be ascertained and documented in a patient catalog described above that makes the patients identity available only to the attending physician:
1) Estimated time of onset of recent sickness
2) Documentation of any anti malarial chemotherapy, self administered or otherwise.
3) Estimate of previous malarial infections
4) Age, sex, pregnancy status
5) CBC, blood lactate and glucose levels if available

e) Patient Consent.
Volunteer consent forms will be signed by all volunteers over the age of 18 and by the legal guardians for minors under the age of 18.
Copy of Form in Appendix

f) Impact of study on established clinical operations
All patients with slide positive malaria as determined by standard operating procedures of the clinic will be treated according to accepted and standard guidelines for the management of malaria. Information regarding the result of the pLDH-based dip-stick assay or of the blood film inspected by Flow Inc. personnel will NOT be made available to the attending physician and will not be used to guide treatment.

g) Blood Collections: Finger stick blood samples will be collected from 500 - 600 individuals during the month of November, 1996 on site at the MRC, The Gambia. Approximately 300-400 µl of addition blood will be drawn from the same stick using micro-pipette-capillary blood tubes that contains an anti-coagulating agent (potassium EDTA). This blood will be divided for evaluation under the following prescription:

1) 1 Thin smear labeled with the patients name (for physician use) and with a numeric code (for the purposes of this protocol
2) 1 Thick smear labeled with the patients name (for physician use) and with a numeric code (for the purposes of this protocol)
3) 1 additional Thin/Thick smear to remain unstained for later inspection at the discretion of Flow personnel
4) 10 µls for use in the pLDH-based dip-stick assay.

Any remaining blood will be labeled with the numeric code and stored at -20°C.

h) Thin and Thick Film Microscopy: Two sets of Thin and Thick films will be prepared for all individuals participating in the study. One set will be Giemsa stained and examined by an experienced MRC microscopist that will designated prior to the initiation of this study. It is understood that inspection of the slides will be performed as the normal standard at the MRC facility. All stained blood films will be examined within 3 working days of collection. It is understood that samples from individuals that have symptoms consistent with a malaria infection may be given priority. More detailed inspection of blood films by multiple microscopists may be employed to verify the study parameters. The other slide will be catalogued and returned to Flow Inc. for analysis.

The normal standard for microscopic analysis at the MRC facility is defined by:
(example 1)
One hundred (100) oil immersion fields (100x) from thin films will be examined from each slide before declaring it negative. The number of red cells per field will be estimated every 10th field. Parasite densities on the positive slides will be recorded as the number of parasites per 100 fields examined as a function of he number of red cells counted multiplied by 10 (percent parasitemia). If a CBC is available, the number of red cells/µl will be incorporated into the parasite density estimation. In this case parasite densities will be calculated as the number of parasites/µl whole blood.

(example 2)
Fifty (50) oil immersion fields (100x) from thick films will be examined from each slide before declaring it negative. The number of white cells per field will be estimated in every field.

Parasite densities on the positive slides will be recorded as the number of parasites over the number of white cells. If a CBC is available, the number of white cells/µl blood will be incorporated into the parasite density estimation. In this case parasite densities will be calculated as the number of parasites/µl whole blood.

i) Dipstick Assay: Whole blood from each donor will be used to with the OptiMAL™ assay and reagents provided by Flow Inc. of Portland, OR. Flow Inc. personnel or personnel trained by Flow Inc. will be present to advise and if necessary perform the assays and record the results. Each dipstick will be labeled with the patient identification number and used in the corresponding blood sample on the day of collection according to instruction. The results of the dipstick test will be recorded immediately after the test is complete and the dipstick will be preserved and mounted in a laboratory notebook.

Assay method:

2 drops of reagent A (30 µls of colloid/buffer solution) are added to a test tube or a configured well plate. Four drops of reagent B (80µl of clearing solution) are added to the second test tube or well. 10 µls of blood are then placed into the first test tube with gentle mixing. The OptiMAL™ assay test strip is then placed into the test tube and the sample is allowed to wick up the test strip. After 8 minutes the OptiMAL™ assay test strip is moved to the second test well containing the clearing buffer for an additional 2 minutes. Interpretation of the assay result should be performed immediately after completion of the clearing step; approximately 10 minutes after the test is initiated.

i) Treatment: Individuals that have positive blood smears as determined by MRC personnel using the established standard procedure will be treated by local government health workers in accordance with standard local protocols.

j) Outcomes: Of the 500-600 individuals tested it is expected that between 100-300 individuals will be infected with malaria as diagnosed using thin or thick film microscopy. Most of these individuals are anticipated to be infected with *P. falciparum.* The evaluation parameters of the OptiMAL™ assay dip-stick test will be to determine:

1) specificity of detection of malaria
   Fraction of False positives
2) specificity of distinguishing falciparum and non-falciparum infections
   Fraction of *P. falciparum* containing samples that test positive but do not react with *falciparum*-specific antibody line.
3) overall sensitivity of assays
   Fraction of all true positives identified
4) sensitivity threshold
   Fraction of true positives identified within the range of % parasitemias of ≥1%; 1.0-0.1%, 0.1-0.01%, 0.01-0.001%, and
   0.001- 0.0001%.

k) Records

Besides the secondary unstained Thin/Thick blood smear, Flow Inc. will have access to all recorded data including:
Complete patient histories encrypted with the above described test code
The completed and mounted dip-sticks
Copies of microscopy notes

Bibliography

Beadle C., G.W.Long, W.R. Weiss, P.D. McElroy, S.M. Maret, A.J. Oloo, S.L. Hoffman(1994). Diagnosis of Malaria by detection of *Plasmodium falciparum* HRP-2 antigen with a rapid dipstick antigen-capture assay. Lancet. 343: 564-68

Makler M.T., J. Ries, J. Williams, J. Bancroft, R. Piper, B.l Gibbins, and D. Hinrichs (1993). Parasite lactate dehyrogenase as an assay for *Plasmodiurm falciparum* drug sensitivity. Am. J. Trop. Med. Hyg. 48(6): 739-741.

(Appendix) **This form will be translated into language of donor, as required**

Medical Research Council Laboratories

Fajara, Banjul, The Gambia

Flow Incorporatec

CONSENT FORM

Title: Comparison Of A pLDH-Based Antigen Detection Assay With Microscopy For The Detection Of Malarial Parasites In Human Blood Samples.

The location of the field study : ***Fajara, Banjul, The Gambia.***

The Investigators are:

Tom Doherty
Angela Hunt Cook

Purpose

The purpose of this study is to analyze a new diagnostic test for malaria.

Permission

I understand that I was selected, or my child was selected as a volunteer for this study because he, she or I may have malaria.

I hereby authorize a qualified phlebotomist designated by the **MRC** to withdraw ~0.4 mls (a few drops) from a finger prick for the purpose of medical research related to the diagnosis of malaria. No more than 10 mls will be removed. I understand that I will be informed of any change in the nature of the study or the procedures, as described above, as they may occur. My local physician or health care provider will answer any questions that I have.

I understand that the procedure described above involves the following possible risks and discomfort: the possibility of bruising, infection, fainting, pain or discomfort. I understand that all normal precautions will be taken to minimize these risks. I understand the purpose of these tests is to gain information about the potential of a new diagnostic test for malaria, and they are not intended as a direct benefit to me.

I consent to the use of the results of the studies performed with this sample of blood for publication for scientific purposes, excluding my identity.

I understand that a committee exists which has reviewed, and continues to review this study from a scientific and ethical standpoint. I further understand that I am free to withdraw my voluntary consent and discontinue my volunteer participation at any time without prejudice.

I understand that there is no compensation available for your participation in this research study; however, you understand that this not a waiver or release of my legal rights.

_____ _____

DONOR'S Signature DATE

_____

Donor Name (printed)

Attachment 3: Protocol for Field Study: Honduras

Title

Comparison Of A pLDH-Based Antigen Detection Assay With Microscopy For The Detection Of Malarial Parasites In Human Blood Samples.

Abstract

Many clincal settings require alternative techniques to diagnose malaria than the traditional inspection of Giemsa stained blood films. Measurement of an abundant malarial enzyme, *Plasmodium* Lactate Dehydrogenase (pLDH), provides a viable alternative. We intend to evaluate a new immunochromatographic test that can detect the presence of pLDH in whole blood samples for its effectiveness as a diagnostic test for malaria. The immunochromatographic test will be compared to the conventional method of microscopic analysis of Giemsa stained thin smears.

Review of Human Ethics Board

Flow Inc.

Robert Piper, Ph.D., Flow Inc. (Chair)
Lisa Hess, M.D., Dept. Ob/Gyn, University of Indianapolis
Andre Makler, Ph.D., Flow Inc.
David Souell, M.D., Dept. Microbiology, Veterans Administration Hospital, Portland Oregon.

Approved 9/1/96

Investigators

Flow Inc.

SW Corbett, Portland, Oregon, 97201, USA
Robert Piper, Ph.D., Scientific Director.
Michael T. Makler, Medical Director.
Laura Wentworth, Technical Advisor.
Jean Williams, Research Technician

Walter Reed Army Institute of Research

Cpt. Miguel Quintana Principle Investigator
Cpt. Lisa Bollan, Nurse
LTC Samueal Martin Senior Officer

Scientifc Background

Microscopic examination of blood smears is the most widely used method of determining malaria infection in humans. The procedure does not require sophisticated equipment and individuals can be trained relatively quickly on preparing blood smears and staining slides. However, microscopic examination is labor intensive and individuals that examine slides need to be experienced to differentiate parasites from artifact. The Flow Inc. pLDH assay stick detect the malarial parasites by detecting the presence of the pLDH antigen in lysed whole blood. The Flow Inc. pLDH assay stick detects the presence of parasites in a 10 µl of fresh, frozen or dried whole blood samples (finger stick or venopuncture) collected in EDTA/ACD/heparin. The pLDH first binds to a labeled antibody particle. This complex then

migrates up the test strip where it is captured by an immobilized second antibody. At the reaction site a visual antibody-antigen -antibody complex is formed.

Personnel from Walter Reed Army Institute of Research (WRAIR) are currently conducting a malaria survey in northern Honduras, under the authorization of the Honduran Ministry of Health (HMOH). One objective of the study is to estimate the prevalence of malarial infection among individuals residing in the region. Preliminary results indicated that 23 percent of the inhabitants in some communities in the study area are infected with either *P.vivax* or *P. falciparum* using microscopic examination of blood smears.
In November, the WRAIR investigators will return to Honduras to continue monitoring malaria in the human population. As part of the study the Flow optiMAL™ dip stick could be evaluated under field conditions and compared to microscopy and polymerase chain reaction (PCR) analysis. This protocol outlines the procedures to be used in such a study.

Procedures

Study Area: The study site will include several communities located in the Department of Colon, Honduras. All of the communities can be easily reached by a two-wheel-drive vehicle and are within 30 minutes of HMOH laboratories. Thc communities in the study site range in size from 20 - 500 homes that are clustered closely together. *Plasmodium vivax*, the most common form of malaria in the area accounts for 70 percent of the cases treated by the HMOH, while the remaining cases have been diagnosed as *P. falciparum* (DVEP, Honduras 1992).

Blood Collections: Finger stick blood samples will be collected from 450 - 500 individuals during the month of November. Blood for the thick and thin smears will be taken directly from finger sticks. Approximately 300 ul of addition blood will be drawn from the same stick using microvette-capillary blood tubes that contains an anti-coagulating agent (potassium EDTA) and placed on ice packs. A portion of this blood sample will be utilized for the optiMAL™ dipsticks (10ul) and PCR assays. The remaining blood will be frozen and stored at WRAIR. Microvette-capillary tubes will be used to ensure that sufficient blood is collected from each volunteer to carry out the three assays. Blood will be obtained during home visits by HMOH personnel under the supervision of the Captain Miguel Quintana. **All individuals in the house hold over the age of 6 months will be asked to volunteer for the study**, even if they do not have any symptoms of malaria. It is expected that approximately 120 homes will be visited during the study. Volunteer consent forms will be signed by all volunteers over the age of 18 and by the legal guardians for minors under the age of 18.

Thin and Thick Film Microcopy: A set of Thin and Thick films will be prepared for all individuals participating in the study during the home visits. Prepared slides will then be transported to the appropriate HMOH Area Of fice where the **thick films** will be Giemsa stained and examined by an experienced HMOI I microscopist. Afterwards the thin and stained thick film will be transported back to Flow Inc. and read in a double blinded fashion by an expert microscopist.

In Honduras, one hundred oil immersion fields (100x) ***thick or thin*** will be examined from each slide before declaring it negative. Parasite densities on the positive slides will be recorded as the number of parasites per 1 00 fields examined. All ***stained*** thin and thick films will be examined within 5 working days of collection. Slides from individuals that have symptoms consistent with a malaria infection will be given priority, with their blood samples transported to the HMOH laboratory immediately after collection. Individuals with positive blood slides will be identified and treated within 24 hours of sampling by HMOH personnel. All slides will be archived at the WRAIR (Room 1085) after examination by a HMOH microscopist and the Flow Inc microscopist. Only after both microscopists have reported their findings will the entire results be released to all parties. Slides for reexamination will be randomly selected, without knowledge of initial results.

Dipstick Assay: Whole blood from each donor will be used with the optiMAL™ dipstick and reagents furnished by Flow Inc of Portland, Or. A Flow staffer will be on hand to advise and to, if necessary, perform the assays and record the results. Each dipstick will be labeled with the volunteer identification number and transported to the closest HMOH hospital laboratory for processing. The laboratories are located in Tocoa and Trjuillo, Colon. A representative from Flow Inc. will process the samples according to protocols developed .

PCR: Blood samples for PCR analysis will be collected on PCR template preparation dipsticks, manufactured by Schleiher & Schuell Inc. (ISOCODE™ STIX). One booklet with four dipsticks will be used per individual. The triangle end of the four dipsticks will be saturated with blood, 12-14 ul per dipstick. The dipsticks will be labeled with the donor number and allowed to dry. Isolation of DNA template will be conducted at WRAIR according to directions outlined by Schleiher & Schuell. Primers for PCR amplification will include those used in previous studies for plasmodium species identification at WRAIR (Li et al. 1996). Two reactions will be carried out to determine malaria parasites species. The first reaction will use primers to amplify a small subunit ribosomal RNA gene that is specific for the plasmodium genus. The second reaction will utilize probes to amplify DNA from the first reaction that are species-specific. Electrophoresis using agarose gels with ethidium bromide will be used to separate, visualize, and measure the size of the amplified products.

Treatment: Individuals that have positive blood smears will be treated by local government health workers in accordance with protocols set forth by the division of Vector Borne Disease, HMOH

Outcomes: Of the 450-500 individuals tested it is expected that between 70-110 individuals will be infected with malaria as diagnosed using thin and thick film microcopy. No less than 20 of these individuals are anticipated to be infected with *P. falciparum* with the remaining cases being infected with *P. vivax.* Descriptive statistics will be used initially to evaluate the results of the three methods. The Department of Biometrics, WRAIR will establish additional statistical tests once the descriptive statistical analysis is completed.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Dec 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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