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TITLE: Detection of Arthropod-Borne Pathogens By Rapid Panel Assays in Mosquitoes

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13. ABSTRACT (Maximum 200) The overall goal of Phase I of this project was to develop a rapid, one step, rugged immuno-chromatographic dip-stick assay for the identification of malaria and dengue-infected mosquitoes. This assay is based on the existing know-how at Navix by which sensitive test-strip based single analyte or panel assays provide results in less than 15 minutes and the tests are stable at ambient storage conditions. Phase I involved (1) Selection of the most appropriate antibodies for incorporation into the malaria and dengue assay that met the required criteria; (2) Development of prototype malaria assays with the required sensitivity; (3) Development of dengue assays with the required sensitivity and (4) Production of approximately 1000 assays of various malarial sporozoite antigens for laboratory /field evaluation. Tests supplied for evaluation with this report include those for the Plasmodium falciparum, Plasmodium vivax variant 210 and Plasmodium vivax variant 247 sporozoite antigens. The results of this phase have demonstrated that the antibody reagents provided by the contracting agency against various malarial sporozoite antigens can be used effectively to generate highly sensitive and robust assays economically. Since the laboratory results are very encouraging, evaluation of these tests in field condition would provide essential insight in optimization of the performance.				
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FOREWORD

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Where For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Kilth' Lane

6/3/97

PI - Signature

Date

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4. INTRODUCTION:

Purpose and scope of the research effort:

The resurgence of malaria in most endemic areas has stimulated the search for new methods to measure and control transmission. The absence of an effective vaccine, vector resistance to insecticides, and development of drug resistance have contributed to the defeat of control measures.

The causative agent of malaria is a protozoan of the genus *Plasmodium*. The four species of *Plasmodium* that are responsible for disease in humans are *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The life cycle of the four species is generally similar and consists of two discrete phases: sexual and asexual. The asexual stages develop in humans, first in the liver and then in the circulating erythrocytes, the sexual stages develop in the mosquito. In general, infection is initiated by the injection of sporozoites into the bloodstream during a mosquito blood meal. Sporozoites rapidly disappear from the bloodstream because they invade the hepatic cells during one passage through the liver. Within liver cells the sporozoite rapidly differentiates into an intracellular form that undergoes asexual multiplication. One sporozoite can produce up to 20,000 parasites (merozoites) in this process. Clinical disease is initiated by the release from liver cells of parasites which have the capacity to invade reticulocytes and/or erythrocytes. The four human malarias, caused by the above four mentioned species, can be differentiated by the properties of their asexual blood-stage infection and some aspects of parasite morphology. Asexual blood-stage malarial parasites cannot infect mosquitoes. The mosquito-infective forms are sexual forms of the malarial parasite, male and female gametocytes, that are derived from the proliferative asexual cycle. Some blood-stage merozoites enter erythrocytes and instead of developing along the asexual multiplication pathway, they differentiate into sexual forms or gametocytes. The asexual blood-stage parasite is haploid; sexual differentiation does not involve nuclear division. Male and female gametocytes develop into large parasites that almost completely fill the infected erythrocyte. Gametocyte-infected erythrocytes often remain in the circulation for prolonged periods during which the levels of asexual parasites may wane. Mosquitoes that take a blood meal (and that may concomitantly inoculate sporozoites) therefore ingest uninfected erythrocytes, erythrocytes containing asexual parasites, and gametocyte-infected cells. Only the gametocytes survive digestion in the mosquito gut. The host membrane surrounding these sexual stages is ruptured to release a large female gamete and slender, motile male gametes (gametogenesis). The male gametes fertilize the female gametes to produce a diploid zygote. Conversion of intracellular gametocytes to extracellular gametes and fertilization is largely completed within 30 min of blood ingestion. The zygotes remain within the contents of the blood meal for about 24 hr during which they transform into motile ookinets. Mature ookinets cross the mosquito midgut wall and continue development to form an oocyst. These grow and divide to produce many sporozoites which migrate to the mosquito salivary glands from where they enter the vertebrate host during mosquito feeding (1).

Plasmodium falciparum often causes the death of the host; the other malarias rarely cause more than temporary morbidity. The WHO's figures in 1996 World Health Report show that 2.1 millions deaths were caused by Malaria alone in 1995 (2), *Plasmodium falciparum* is widespread throughout the tropics and therefore constitutes an important health threat for millions of people. *Plasmodium vivax* is also widespread, and because of its property of successive relapse from liver and consequent chronicity, constitutes an important cause of morbidity in tropical regions. *Plasmodium ovale* and *P. malariae* are less common, both causing low-grade, chronic diseases, the

latter infection often causing disruption of kidney function through immune-complex deposition. The detailed symptomatology and pathology of the human malarias has been reviewed (3).

The availability of rapid, simple, sensitive and specific diagnostic tools is of prime importance in the control of malaria. Microscopy remains the standard, most cost-effective method. However, it is very labor intensive, requires an effective microscope and skilled technicians. This is not generally accessible in many regions of the tropics. Beyond the clinics where such clinical laboratory based diagnosis is unavailable, the diagnosis of malaria is usually based on symptomatology. The intrinsic inaccuracy of syndrome based diagnosis, however, results in the significant overuse of drugs and may accelerate the development of resistance factors in falciparum malaria.

Besides malaria, dengue and dengue associated hemorrhagic fever occur in epidemic form throughout the tropical areas of world. Dengue virus types 1 through 4 have commonly being tested using serological tests (hemagglutination-inhibition, immunofluorescence and complement fixation) with varying degrees of success. Presently, the only certain method of identification requires the use of standardized reference antiserum in a virus plaque-reduction neutralization assay. Since few field laboratories possess sufficient resources to perform this test with the slowly replicating dengue viruses, new methods are necessary. With the development of monoclonal antibodies against dengue antigens with demonstrated separate specificities for a flavivirus group-common, dengue-complex, sub-complex, and serotype-specific determinants opens the possibility of developing rapid assays to identify the virus strains (4).

The advantages of combining detection and species identification in the same test is obvious. In endemic areas where only one species of *Plasmodium* is present, species-specific determination is unnecessary. More often than not, however, a mixed population of *P. falciparum* and *P. vivax* or *P. malariae* or *P. ovale* occurs in endemic areas, requiring each mosquito sample to be processed multiple times for specific identification. In the present application, we have developed individual as well as panel dip-stick assays whereby specific antigens can be detected on the same strip by visualizing the development of signals in appropriate regions on the strip.

As discussed below, in the Phase 1 of the DOD SBIR grant, we applied our wicking dip-stick detection technology to develop a simple, one-step, field-usable assay capable of detecting arthropod-borne pathogens in mosquitoes.

5. BODY:

5.1 PRICIPLE:

Principle on which the strip performs: The strip contains a combination of unique sets of gold-adsorbed and membrane-immobilized monoclonal and/or polyclonal antibodies to produce a distinctive visual pattern indicating the presence of species specific antigens in the test sample within 15 minutes. In the test procedure, a test sample is allowed to migrate through the absorbent area. If antigen eg A1 is present, labeled antibody-gold binds it, forming a gold-antibody-antigen complex. As the reaction mixture continues to flow along the test membrane, the complex binds to the another anti-A1 antibody immobilized in the test zone producing a red colored band or line. Unbound conjugate binds to the reagents immobilized in the control (C) zone producing a red colored band or line demonstrating proper performance of the test. In the event when instead of antigen A1, the sample has either of antigen A2 to A5 appropriate lines

positioned specifically on the test strip will develop determining the presence of the specific antigen in the test sample.

5.2 Plan and accomplishments of tasks:

In the first quarter, i.e. in the first three monthly progress reports, we described accomplishment of individual analyte assays for Plasmodium falciparum (Pf) and two variants of the Plasmodium vivax, namely Pv247 and Pv210. During this time, we:

1. Prepared and evaluated individual tests for Pf using a combination of antibodies which we had not attempted earlier.
2. Prepared and evaluated panel tests containing Pf, Pv210 and/or Pv247 on the same test strips.
3. Purified 4 anti-dengue virus IgG from ascitic fluid samples received.
4. Prepared the more economical dip-stick format and compared to the lateral formats.
5. Accomplished the development of latex and carbon based reporter system as potential alternative label systems for developing quantitative formats for Phase II.
6. Some preliminary work with antibodies received for Dengue virus types was also reported in that report.

In Month 4 we:

1. Purified anti-dengue virus IgG from some more ascitic fluid samples.
2. Prepared dip-stick assays using the purified antibodies to Dengue and evaluated these with inactivated Dengue virus types 1 to 4.
3. Further evaluated the materials (antibodies mainly and antigen to some extent) provided for the Dengue assay by ELISA.
4. Made more progress in the development of latex and carbon based reporter system as potential alternative label systems for developing quantitative formats for Phase II. Prepared preliminary prototypes for a possible quantitative assay by generating multi-line- signals as concentration of analytes increase.

In Month 5 we:

1. Prepared more dip-stick assays, repeating the assay conditions standardized for Pf and Pv tests and using the purified antibodies to Dengue evaluated the tests with inactivated Dengue virus types 1 to 4.
2. Further evaluated the materials (antibodies mainly and the inactivated viral antigen to some extent) provided for the Dengue assay by ELISA.
3. Continued development of latex based reporter system as potential alternative label systems for developing quantitative formats for Phase II. Prepared preliminary prototypes for a possible quantitative assay by generating quantifiable signals as concentration of analytes increase.

In Month 6 we:

1. Manufactured over 1000 assays for Pf, Pv210 and Pv247 and evaluated these tests.
2. We have started preparing manufacturing documents for the above tests.
3. Preparation of kit insert and packaging.

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5.3 MATERIALS:

Antibodies and positive control in quantities enough to initiate the project were provided by the contracting agency. This included:

53.1 For Malaria tests:

Mab Pf2A10 (474)
Mab Pf1B2.2 (93-3-5)
Positive Control Pf-R32tet32 (104189)
Mab Pv-210 (NSV#3)
Positive Control Pv-210 (105232)
Mab Pv-247 (1G12.1)
Positive control Pv-247

5.3.2 For Dengue tests:

The following antibodies were received:

Polyclonal (1mL each):

1. Dengue 1 Polyclonal mouse hyperimmune ascitic fluid
2. Dengue 2 Polyclonal mouse hyperimmune ascitic fluid
3. Dengue 3 Polyclonal mouse hyperimmune ascitic fluid
4. Dengue 4 Polyclonal mouse hyperimmune ascitic fluid

Other ascitic fluid (1 mL each):

- | | | |
|------------------|--------------|------------|
| 1. J3-11B9-3 | MAF 7-22-86 | REF-552-25 |
| 2. J2-802-6 | MAF 2-14-84 | REF-429-15 |
| 3. D3-1A2-6 | MAF 6-27-84 | REF-429-26 |
| 4. D1-3H1-5-10 | MAF 5-29-87 | REF-552-39 |
| 5. D6-16C7-4 | MAF 9-26-86 | REF-552-27 |
| 6. D1-4G2-4-15 | MAF 8-31-87 | REF-552-47 |
| 7. D6-14A4-8 | MAF 8-19-86 | REF-552-27 |
| 8. D2-15F3-1-15 | MAF 5-2-87 | REF-429-20 |
| 9. D6-9E1-3 | MAF 03-05-90 | REF-725-13 |
| 10. D1-4E6-4 | MAF 11-25-86 | REF-552-27 |
| 11. D6-16C7-4 | MAF 9-26-86 | REF-552-27 |
| 12. D2-7E11-1 | MAF 12-4-91 | REF-725-42 |
| 13. D6-9E1-5 | MAF 8-15-86 | REF-552-27 |
| 14. D7-3F6-9 | MAF 5-4-84 | REF-429-20 |
| 15. D1-4E5-4 | MAF 8-31-87 | REF-552-47 |
| 16. D1-2C6-1 | MAF 12-8-86 | REF-552-29 |
| 17. D2-5C11-6-19 | MAF 1-20-87 | REF-552-31 |
| 18. D6-16C7-4 | MAF 2-17-84 | REF-429-15 |
| 19. D1-1B10-3 | MAF 1-16-87 | REF-552-31 |
| 20. D3-2H2-9-15 | MAF 9-9-86 | REF-552-27 |
| 21. D6-17H7-2 | MAF 9-8-86 | REF-552-27 |
| 22. D7-3E9-8 | MAF 03-01-90 | REF-725-13 |

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23. D6-802-1	MAF 7-9-84	REF-429-26
24. D1-4E5-4	MAF 12-10-91	REF-725-44
25. D2-13E7-9-10	MAF 6-20-84	REF-429-26
26. J2-2F1-12	MAF 8-15-86	REF-552-25
27. D6-4F12-11	MAF 9-8-86	REF-552-27
28. D8-1G6-2	MAF 01-09-90	REF-725-11
29. D1-3G11-9	MAF 11-7-86	REF-552-29
30. D8-6D2-7	MAF	REF-552-39
31. D3-4G3-2-13	MAF 2-2-87	REF-552-31
32. D8-2B4-2	MAF	REF-552-39
33. D1-4E5-6-5	MAF 10-24-86	REF-552-29
34. D2-1F1-3	MAF 2-15-90	REF-725-13
35. D3-4B10-3-11	MAF 1-16-87	REF-552-31
36. D7-6B6-10	MAF 10-29-86	REF-552-29
37. D3-5B2-12	MAF 2-2-87	REF-552-31
38. D8-3D9-5	MAF	REF-552-39
39. D2-13B6-6	MAF 1-16-87	REF-552-31
40. D6-1B7-5	MAF 9-10-93	REF-778-17
41. D1-2G4-4-7	MAF 11-5-86	REF-552-29
42. D7-5F10-7	MAF 10-29-86	REF-552-29
43. D1-3G11-9	MAF 1-10-85	REF-429-36
44. D2-13E7-9-10	MAF 8-27-86	REF-552-27
45. D2-8B9-9	MAF 1-16-87	REF-552-31
46. D6-14E9-7	MAF 12-08-93	REF-778-24
47. D6-15H5-8	MAF 12-01-93	REF-778-24
48. D6-4F12-11	MAF-2-15-84	REF-429-15
49. D3-5C9-1	MAF 8-27-86	REF-552-27
50. D6-9E1-3	MAF 3-18-85	REF-429-42
51. D4-2F3-10	MAF 2-14-84	REF-429-15
52. D6-17H7-2	MAF 6-25-84	REF-429-26
53. D2-15F3-1-15	MAF 5-2-84	REF-429-20
54. D1-3H5-1-21	MAF 1-09-90	REF-725-10
55. D3-1H10-6-7	MAF 8-25-87	REF-552-47

Only the last three, namely

1. D2-15F3-1-15 MAF 5-2-84 REF-429-20
 2. D1-3H5-1-21 MAF 1-09-90 REF-725-10
 3. D3-1H10-6-7 MAF 8-25-87 REF-552-47
- which were referenced in Henchal et al: Rapid Identification of Dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay, in Am. J. Trop. Med. Hyg. 32(1), 1983, pp 164-169, were used this time for IgG purification as shown below.

5.4 Purification of antibody:

5.4.1 For Plasmodium sporozoite assays (Pf, Pv210 and Pv247):

Since the antibodies provided were purified, no further treatment was necessary.

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5.4.2 For Dengue assays (all four variants):

In order to adsorb antibodies to colloidal gold and/or immobilize on membrane for the Dengue assays, the ascites and polyclonal mouse hyperimmune fluid were used as start-up materials to recover purified IgG.

Example: Purification of MAb D2-15F3-1-15 MAF 5-2-84 REF-429-20

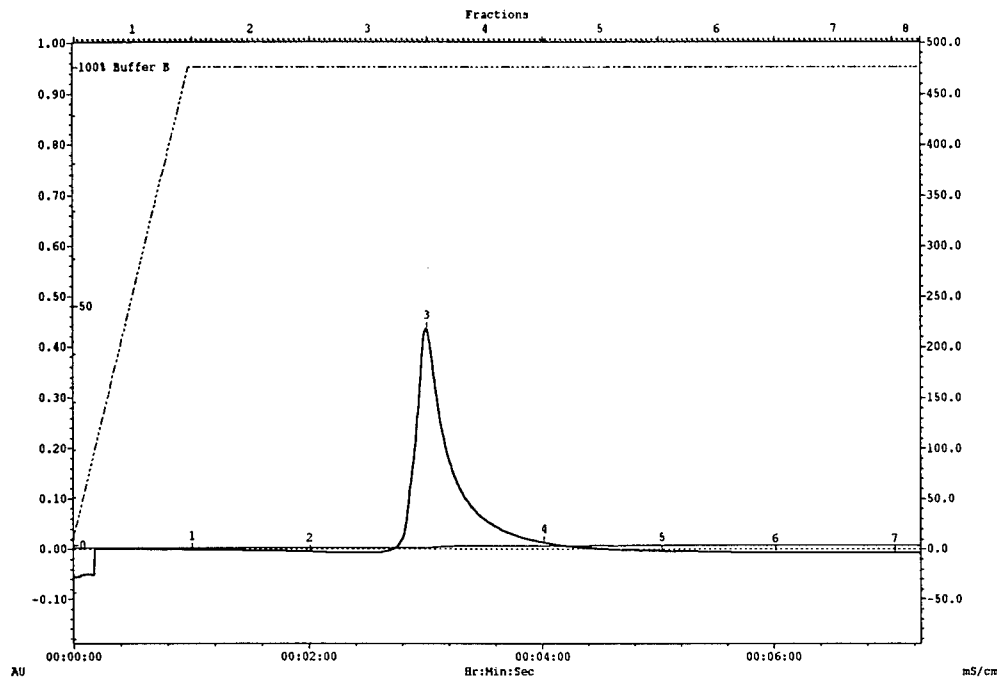


Figure 1: Material was affinity purified on a 1mL Protein G Sepharose 4 Fast Flow column (Pharmacia) equilibrated with binding buffer (0.1M glycine, pH 9.0) and eluted with 0.1M glycine, pH 3.0 at a flow rate of 1mL/min and stored in 0.1M Glycine, 0.1M Tris at pH 7.5. IgG is around fraction 23.

The IgG content in these samples is low and the following amount of IgG was recovered:

				IgG purified
1.	D2-15F3-1-15	MAF 5-2-84	REF-429-20 (Received 0.85mL)	0.35 mg
2.	D1-3H5-1-21	MAF 1-09-90	REF-725-10 (Received 1mL)	0.22 mg
3.	D3-1H10-6-7	MAF 8-25-87	REF-552-47 (Received 1mL)	0.7 mg
4.	Dengue-1 Polyclonal mouse hyperimmune fluid	(Received 1mL)		2.0 mg

5.5 Preparation of Gold colloid:

Mono-disperse colloidal gold suspensions were prepared using the reductive process on chloroauric acid (HAuCl₄) to create 15 to 35 nm particles. For the antibodies that are adsorbed on these particles, and for sensitivity and other issues, we have selected gold colloid which has a absorption peak at 520nm which corresponds to about 20-25nm particles. Since the reduction

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process (we used sodium citrate) determines these sizes, reproducible preparations can be obtained by carefully monitoring reagent source (supplier, quality), concentration, temperatures and times of the reaction.

5.6 Preparation of Gold-Antibody complexes:

During the this entire phase we have defined the conditions we need to achieve optimal gold-antibody complexes. The phenomenon of "adsorption" is relatively a simple process, however, the factors responsible for creating stable gold-antibody conjugates are complex. Therefore, the optimization of this include many experiments which study individual parameters. Following steps describe the sub-tasks necessary to create each different kind of assay:

Selection of optimal coupling pH which is dependent on the antibody pI (isoelectric point).

Selection of buffer type and concentration to be used

Determination of minimum amount of antibody required to stabilize the colloidal gold being used.

Time for reaction.

Method for further stabilizing with extraneous proteins/polymers

Method for separating (centrifugation - time and speed)

Fixing composition of resuspension buffer, pH, concentration and stability verification.

5.7 Preparation of test strips:

5.7.1 Individual Tests (to test one antigen type in one test strip)

Tests prepared for evaluation used the following antibodies:

Conjugate name	Antibody used for conjugation and printing
59711-Pf 2A10- KPL/1	Pf 2A10-725-49; Lot:TF097: KPL vial, 1 mg/ml
59712-Pf 2A10- HAZ/1	Pf 2A10 (HAZ); Lot:22:123:14A; 2 mg/ml
59713-Pv 247/1	Pv 247; Lot:1G12 # 498; 7 mg/ml
59714-Pv 210/2	Pv 210 (HAZ); Lot:9-292-219C;7.4 mg/ml
59715-Pf 1B2.2/1	Pf 1B2.2; Lot:93-3 (3-5); 8 mg/ml

These tests are provided with the report for evaluation.

No.	Tests carried	Antibody on Gold:	Capture Antibody: (on membrane)
1.	<i>P. falciparum</i>	Mab Pf2A10	Mab Pf2A10
2.	<i>P. falciparum</i>	Mab Pf1B2.2	Mab Pf2A10
It was interesting to find how the Pf assay performs when the Pf2A10 is present on the gold as well as on the membrane and similarly in the case of Pf1B2.2.			
3	<i>P. vivax</i> 210	Mab Pv210	Mab Pv210
4	<i>P. vivax</i> 247	Mab Pv247	Mab Pv247

5.7.1.1 Plasmodium falciparum DipStick assay: Pf2A10*gold

These tests were made with Pf2A10 monoclonal antibody adsorbed to gold. The capture antibody in this case was also Pf2A10. The sensitivity limit of the Plasmodium falciparum sporozoite assay between 1 and 0.1 ng/mL.

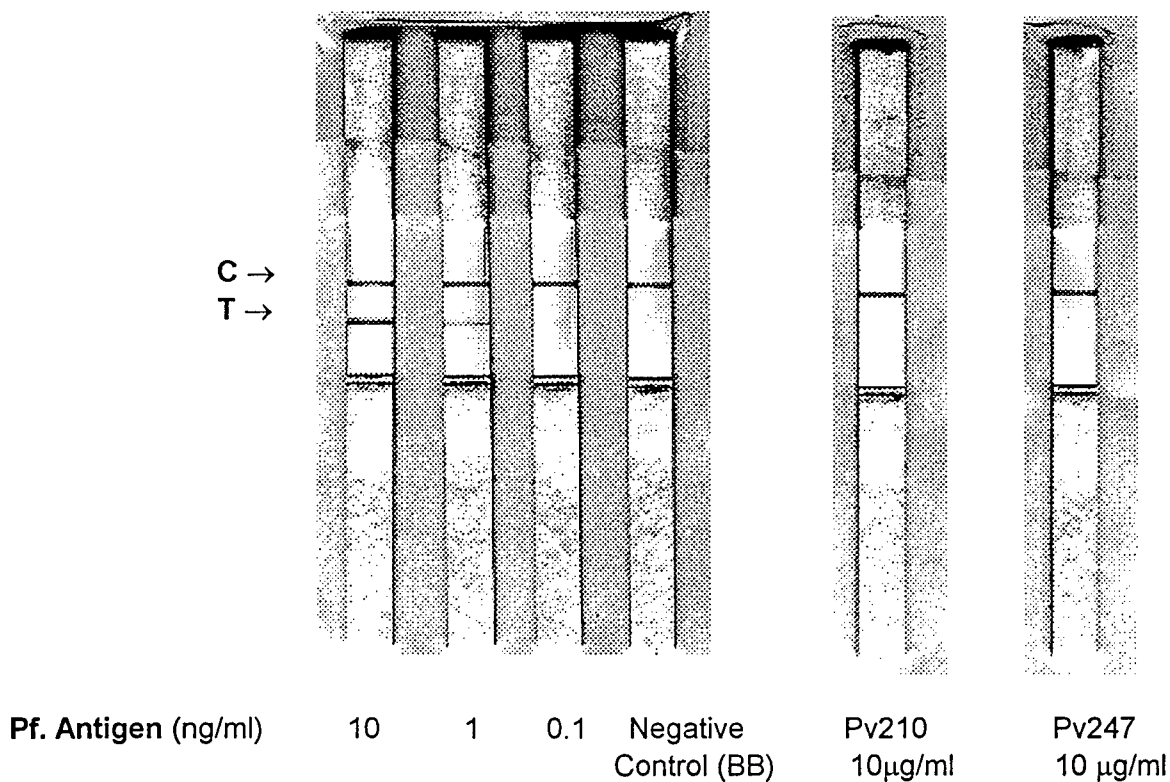


Figure 2: The three strips on left were tested with positive control (Plasmodium falciparum, received 5/97). The test line (T) results are clear in concentrations as low as 1 ng/mL. The fourth strip on the

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left is a negative control test where the test strip was evaluated with only the dilution buffer BB. All tests developed a control line (C). Assays were carried out as described in the insert. 0.2 mL sample was used for all assays described and 50 μ L of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Conclusion: As seen in the figure above, when Pf assay was checked at 1 ng/mL Pf antigen concentration, the signal development was very clear. In this test Pv210 and Pv247 antigens at 10 μ g/ml did not give any nonspecific signal.

5.7.1.2 Plasmodium falciparum DipStick assay: Pf1B2.2*gold

These tests were made with Pf1B2.2 monoclonal antibody adsorbed to gold. The capture antibody in this case was also Pf2A10. The sensitivity limit of this Plasmodium falciparum sporozoite assay is again between 1 and 0.1 ng/mL.

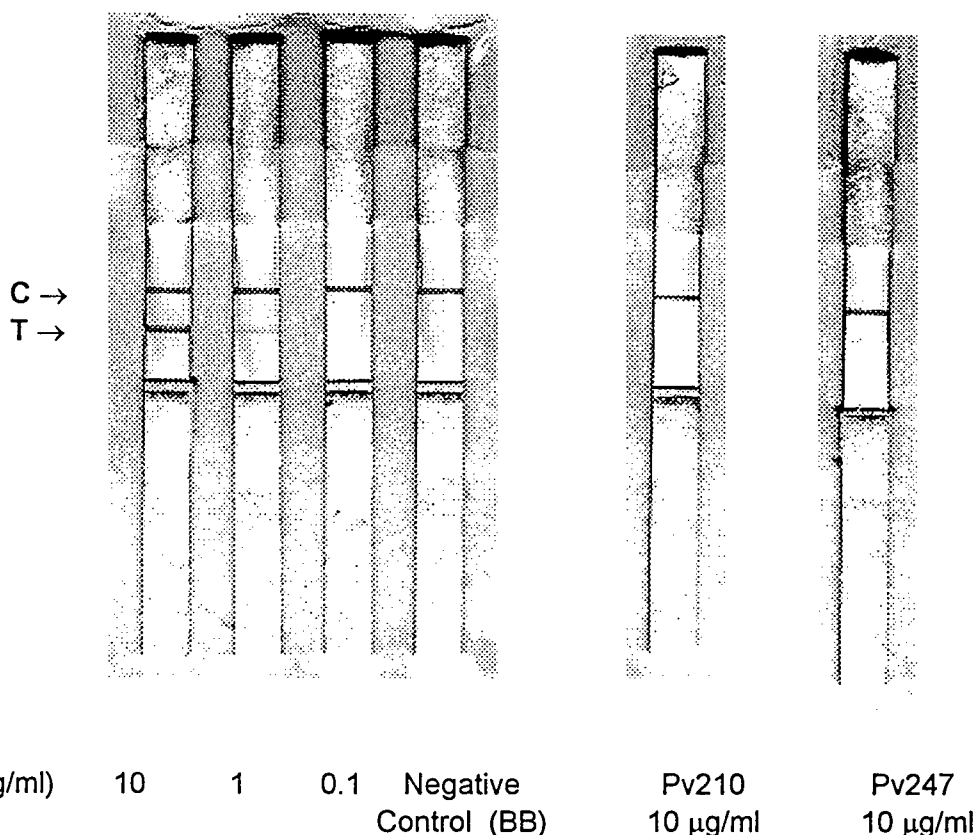


Figure 3: The three strips on left were again tested with positive control (Plasmodium falciparum, received 5/97). The test line (T) results are clear in concentrations as low as 1 ng/mL. All tests developed a control line (C). Assays were carried out as described in the insert. 0.2 mL sample was used for all assays described and 50 μ L of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Conclusion: As seen in the figure above, when the Pf assay was checked at 1 ng/mL Pf antigen concentration, the signal development is clear. However, this signal appears slightly less intense (about 15% on the densitometer). Again, in this test Pv210 and Pv247 antigens at 10 µg/ml did not give any nonspecific signal.

5.7.1.3 Plasmodium vivax 210 DipStick assay: Pv210*gold

These tests were made with Pv210 monoclonal antibody adsorbed to gold. The capture antibody in this case was also Pv210. The sensitivity limit of this Plasmodium vivax sporozoite assay is close to 0.1 ng/mL.

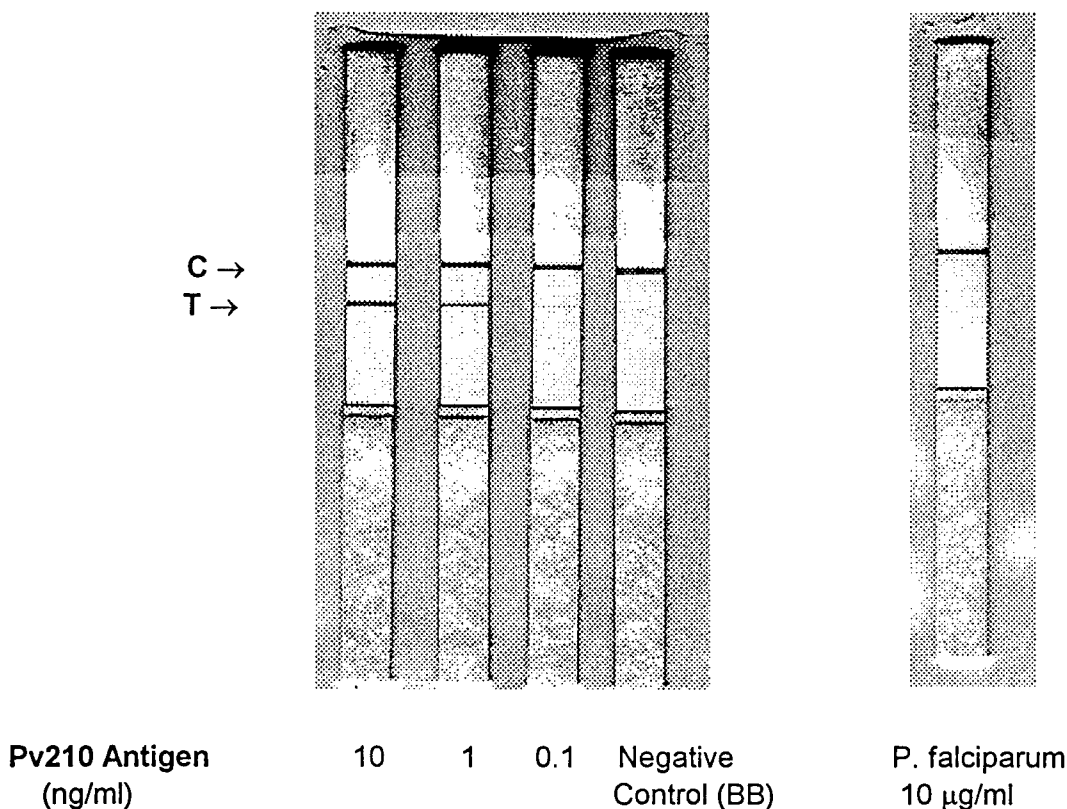


Figure 4: The three strips on left were again tested with positive control (Plasmodium vivax 210, received 5/97). The test line (T) results are clear in concentrations as low as 0.1 ng/mL. All tests developed a control line (C). Assays were carried out as described in the insert. 0.2 mL sample was used for all assays described and 50 µL of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Conclusion: The Pv210 assay is sensitive at 0.1 ng/mL Pv210 antigen concentration and the signal development is clear. Again, in this test the Pf antigen at 10 µg/ml did not give any nonspecific signal.

5.7.1.3 Plasmodium vivax 247 DipStick assay: Pv247*gold

These tests were made with Pv47 monoclonal antibody adsorbed to gold. The capture antibody in this case was also Pv247. The sensitivity limit of this Plasmodium vivax sporozoite assay is between 10 and 1 ng/mL.

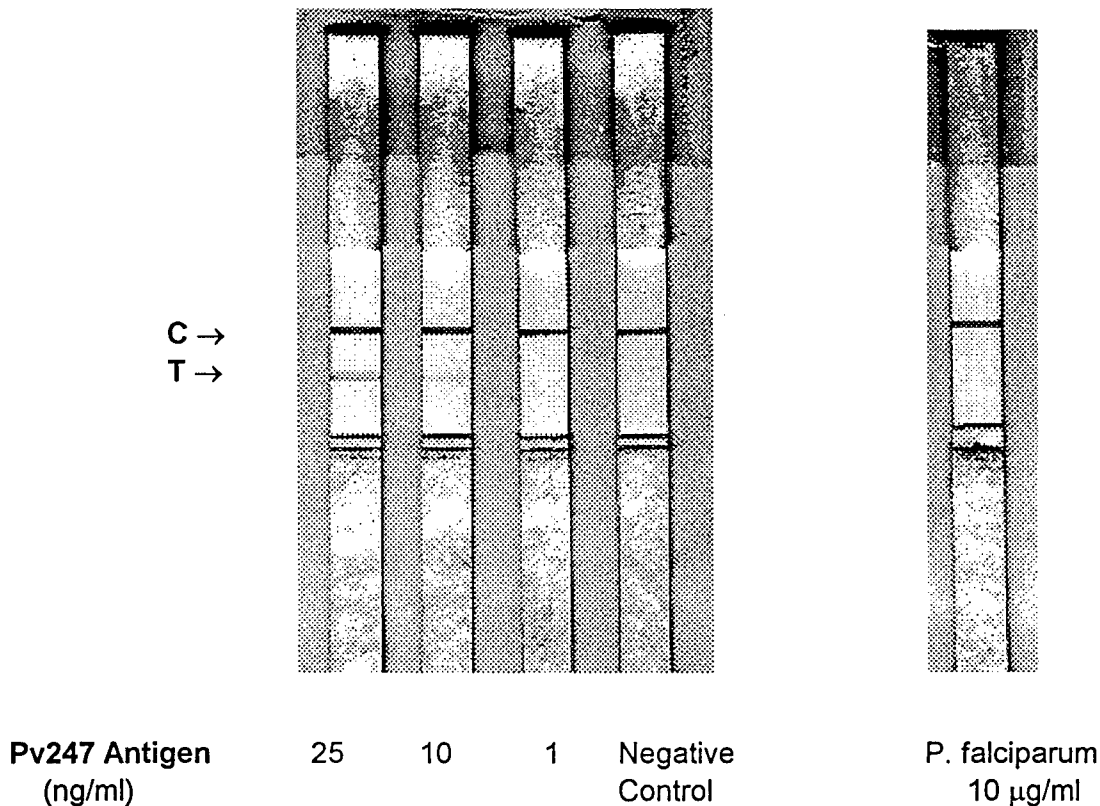


Figure 5: The three strips on left were again tested with positive control (Plasmodium vivax 247, received 5/97). The test line (T) results are clear in concentrations as low as 10 ng/mL. All tests developed a control line (C). Assays were carried out as described in the insert. 0.2 mL sample was used for all assays described and 50 µL of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Conclusion: As seen in the figure above, when the Pv247 assay was checked at 10 ng/mL Pv247 antigen concentration, the signal development is clear. At a concentration of 1 ng/mL, the signal is very weak. Again, in this test the Pf antigen at 10 µg/mL did not give any nonspecific signal.

5.7.2 PANEL TESTS (TWO OR MORE ANTIGEN TESTED ON SINGLE TEST STRIP)

Combined Pv210 and Pf strip assay: Panel assays were developed whereby specific antigens such as both Pv210 and Pf can be detected on the same strip. Three lines were printed including the control line and the gold conjugates against each antigens were mixed in the conjugate pad.

Printed lines: control
Mab Pv 210
Mab Pf 2A10

Gold conjugates - mixed:
Mab Pv 210*gold
Mab Pf 2A10*gold

Assay for Pv 210 antigen dilution in the combined Pv210 and Pf test:

These tests were made with Pf2A10 and Pv210 monoclonal antibody adsorbed to gold. The capture antibodies in this case were Pv210 (proximal to the control line) and Pf2A10 (distal to the control line). The sensitivity limit of this combined *Plasmodium falciparum* - *Plasmodium vivax* 210 sporozoite assay is as low as 0.25 ng/mL Pv210.

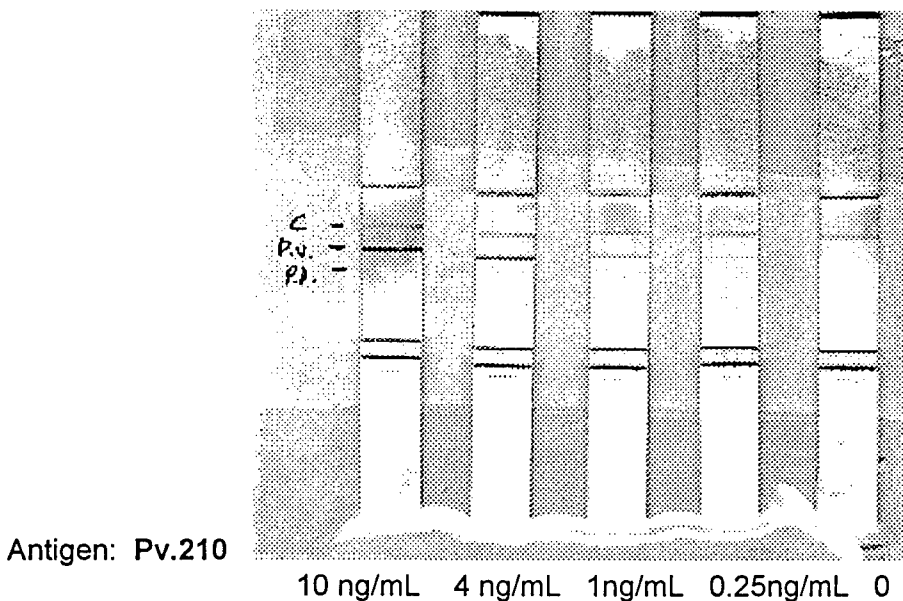


Figure 6: The four strips on left were tested with positive control (*Plasmodium vivax* 210). The test line (T) results are clear in concentrations as low as 0.25 ng/mL. All tests developed a control line (C). Assays were carried out as described in the insert. 0.25 mL sample was used for all assays described and 50 μ L of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Conclusion: When Pv210 antigen is tested on the Pf+Pv combo test strip, only the specific Pv signal is seen. No non-specific Pf signal developed in these assays.

Assay for Pf antigen dilution in the combined Pv210 and Pf test:

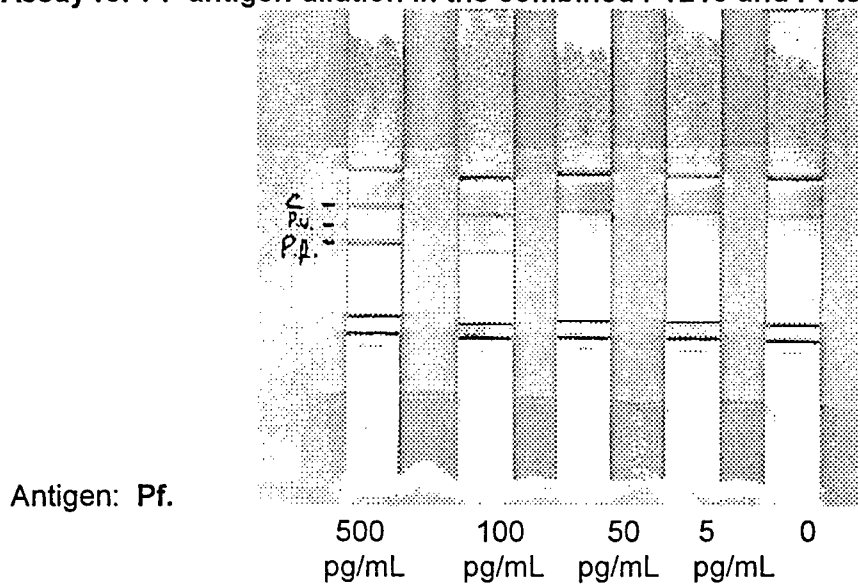


Figure 6: The four strips on left were tested with positive control (Plasmodium falciparum). The test line (T) results are clear in concentrations as low as 100 pg/mL. All tests developed a control line (C). Assays were carried out as described in the insert. 0.25 mL sample was used for all assays described and 50 μ L of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Conclusion: Similarly, when Pf antigen is tested on the Pf+Pv combo test strip, only the specific Pf signal is seen.

Assay for Pv210 and Pf antigens' dilution in the combined Pv210 and Pf strip tests:

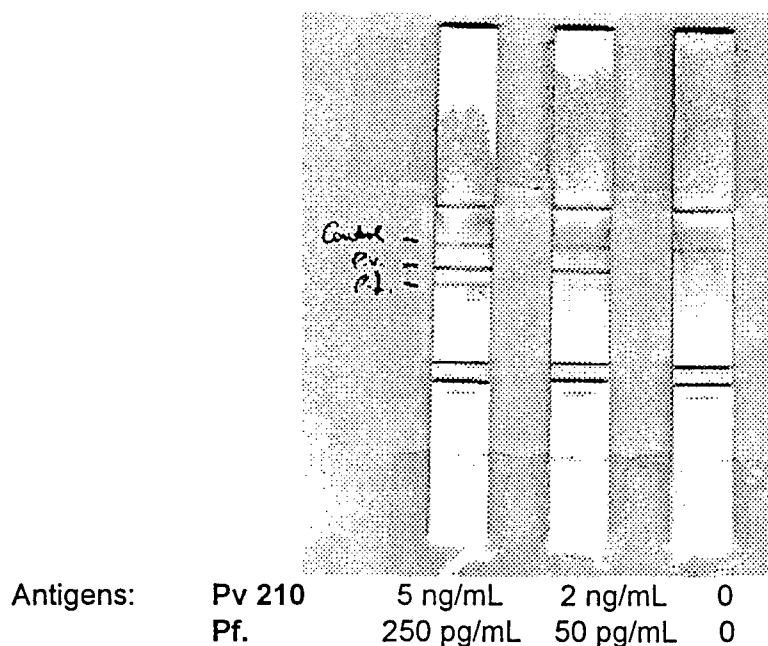


Figure 6: The two strips on left were tested with positive control (Plasmodium falciparum as well as

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Pv210 mixture). The test line results are comparable to those achieved in individual assays. All tests developed a control line. Assays were carried out as described in the insert. 0.25 mL sample was used for all assays described and 50 μ L of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Conclusion: The combination test of these two antigens was successful. Sensitivity is comparable (same) for both analytes in the combined assay as is in the individual assays.

5.7.3 DENGUE TESTS

Many different combinations had to be tried in order to characterize the given material. The line of experiments followed this order:

1. Dengue assays prepared in which the printed capture antibodies were selected (based on literature) original monoclonal ascitic fluid samples (55 types of which were provided by WRAIR). These unpurified samples were diluted 1:5 and printed on the membrane.
2. Dengue assays prepared in which the printed capture antibodies were purified polyclonal mouse hyperimmune ascitic fluid presented in elution buffers.

Preparation of Dengue Assays using unpurified monoclonal ascitic fluids diluted 5X as capture antibody (printed on membrane) :

In tests prepared with capture antibody: 2F3 MAF printed membrane [this is anti- Dengue 1/ anti- Dengue 3 according the Henchal et al, 1982, Am J. Trop. Med. Hyg., 31(4), pp 830-836] and conjugate antibody: Gold conjugated anti-Dengue 3 Pcl HMAF (purified) various Dengue types 1 to 4 were examined.

Dengue 3 assay:

With 10X dilution of Dengue 3 gives a weak signal whereas with 100X dilution of the same and with negative control (dilution buffer only) gave no signal.

In another set of strips, diluted Dengue 2 and diluted Dengue 4 gave signals in combination of respective anti-Dengue 2 and anti-Dengue 4 Pcl HMAF (purified) present on the gold with 2F3 HMAF printed antibodies. However, according to Henchal et al, 1982, the printed antibody 2F3 HMAF is supposed to recognize only Dengue 1 and Dengue 3.

Dengue 2 and 3 assay using:

2H2 MAF printed membrane: [anti- Dengue 1/ anti- Dengue 2/anti- Dengue 3/ anti- Dengue 4 according the Henchal et al, 1982, Am J. Trop. Med. Hyg., 31(4), pp 830-836].

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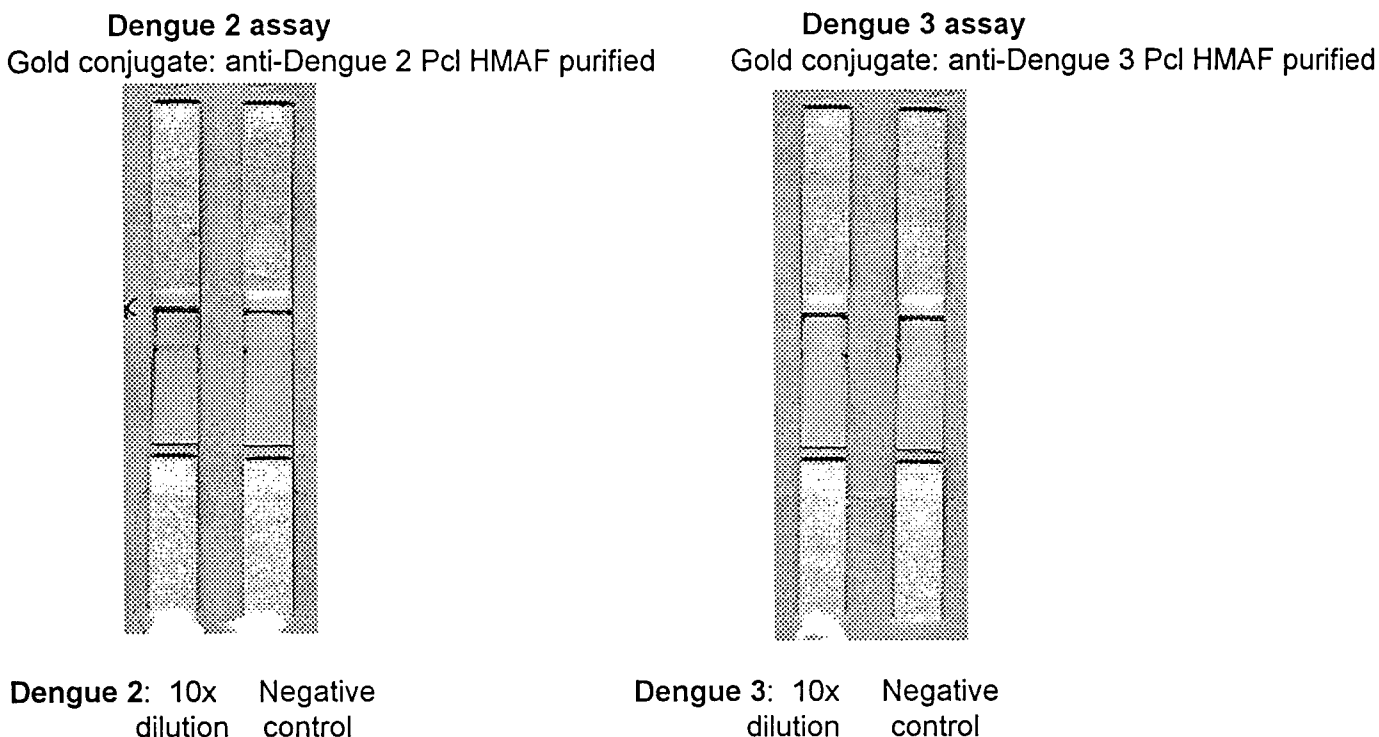


Figure 7: The strips on left in each group were tested with positive control (Dengue 2 and Dengue 3 respectively). The test line (T) results are clear in 10X diluted heat-inactivated Dengue virus positive control. All tests developed a control line (C). Assays were carried out as described in the insert. 0.25 mL sample was used for all assays described and 50 μ L of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Result: Dengue 2 assay gives clear but weak signal. Dengue 3 assay is weak.

Conclusion: As seen in the figure above, identification of dengue using the present format is possible. However the sensitivity of the test can be improved when material is available.. At present with the availability of these materials being low, only the basic feasibility experiments were carried out. An additional problem with the dengue assay is that the whole (live) virus antigen that was used for the assays here was inactivated by heat denaturation in order to inactivate the virus. It may be that this step is affecting the epitope to an extent that the antibodies do not recognize the epitope. Regarding this formaldehyde inactivated virus could be used.

5.7.4 Development of tests with latex instead of gold

It is possible to develop the various sporozoite antigen tests using colored (dyed) latex particles.

5.7.4.1 Quantitative Plasmodium vivax 210 Strip Assay using latex conjugate:

Since the detection of "red" colored gold signal is a limiting aspect for densitometric based readers, we are concurrently developing latex and carbon particle based assays. In our experience with

cardiac marker assays (e.g. cardiac troponin I), we have applied dark blue and black latex and carbon particles which have provided very quantifiable signals. Here we have attempted to apply the blue latex conjugated anti Pv210 antibodies to develop Pv assays which were evaluated for this purpose:

5.7.4.2 Preparation of Latex conjugates:

Particle type: Carboxy modified died PS latex, 10% suspension

IgG: 1mg/ml solution in phosphate buffer

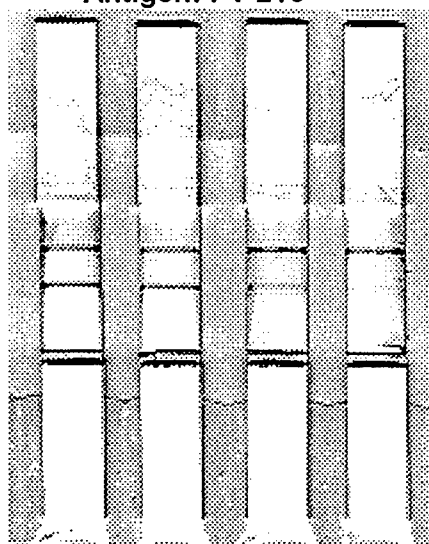
Method of coupling: one step EDAC mediated covalent coupling

The following table describes the characteristics of the latex -antibody complex prepared and used for the test strips in this report.

Conju-gate name	Ab used for conjugation	Ab amount used for conjugation	Particle used for conjugation	Method of coupling	Final suspension volume	Final suspension conc. for particle number /ml	Preparation of conjugate pad
B1860CB-PV210	Mab Pv 210 NSV #3	122 µg	Carboxy modified died PS latex, 0.18 micron, dark blue, Bangs Lab. Inc.	One step EDAC mediated covalent coupling	8 ml	3.5 x 10 ¹¹	Without further dilution dried at 41°C

Mab Pv210 (printing with 1mg/ml Ab) - Mab Pv210*latex

Antigen: Pv-210



1

2

3

4

Test strip #4 is negative control (run with BB buffer only).
Positive control used in all these tests is Pv-210 (cl #) 105232.

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Figure 8: The three strips on left were tested with positive control (Plasmodium vivax 210). The test line (T) results are clear in concentrations lower than 1ng/mL. All tests developed a control line (C). Assays were carried out as described in the insert. 0.2 mL sample was used for all assays described and 50 µL of 10% Tween-20 (Developer solution) was utilized. All assays were read 15 minutes from start.

Results:

Test strip	Concentration of test antigen	Amount of test antigen	Result at 20 minutes (appearance of test line)
1.	80 ng/ml	200 µl	3+
2.	10 ng/ml	200 µl	3+
3.	4 ng/ml	200 µl	2+
4.	0	200 µl	no signal

CONCLUSION: The sensitivity for Pv210 antigen using latex conjugate is comparable to the sensitivity using gold conjugate. Optimization of latex conjugate is under development. Using gold conjugate we got 3+ result for 4 ng/ml antigen and 2+ result for 1 ng/ml antigen.

5.7.5 Plastic Cassette for lateral-flow formats:

During this phase we have also developed a prototype for the lateral immunoassay approach. We designed the plastic cassette containing an innovative filter assembly. The filter assembly consists of a plastic hollow casket and a plastic ring that clips on to the casket. Between the casket and the ring we plan to place specific filter material which will filter out debris present in mosquito/parts extracts. The filter assembly is removable and use is optional when the test solution is relatively free from debris.

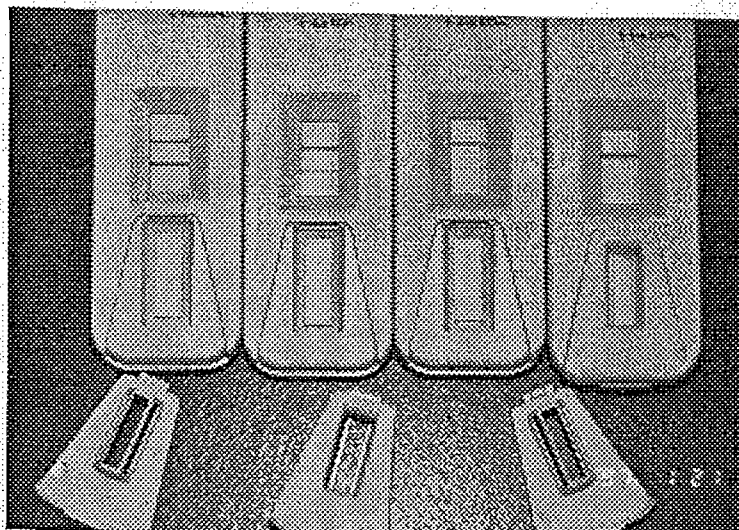


Figure 9: Lateral-flow assays contained in plastic cassettes were made and evaluated for Pf and other assays. The sensitivity of this test is comparable to that for the dip-stick assay described before.

6. CONCLUSION

SUMMARY OF OBJECTIVES ACCOMPLISHED IN PHASE I

The overall goal of Phase I of this project was to develop a rapid, one step, rugged immuno-chromatographic (dip-stick technology) assay for the identification of malaria and dengue-infected mosquitoes.

The results of this phase have demonstrated that the antibody reagents provided by the contracting agency against various malarial sporozoite antigens can be used effectively to generate highly sensitive and robust assays economically. Since the laboratory results are very encouraging, evaluation of these tests in field condition would provide essential insight in optimization of the performance.

This work has set the stage for Phase II, whose objective is to develop a larger number of such tests with appropriate optimization to suit the nature of the sample, and have optimum sensitivity and specificity.

Specific accomplishments of this phase are:

- 1: Selection and incorporation of the most appropriate antibodies into the malaria and dengue assay. From the given set of antibodies, pairs have been identified that can function in a sandwich assay.
- 2: Development and successful laboratory evaluation of prototype assays for single and sporozoite panel. These include Plasmodium falciparum, Plasmodium vivax 210 and 247 variants.
- 3: Development of a prototype dengue assay. From the available material, it was possible to demonstrate the feasibility of dengue assay. Antibodies to the specific types of Dengue namely Type 1 to 4 were applied and the tests evaluated with inactivated dengue virus types. Preliminary sandwich pair identification has been accomplished and when more material is made available, a panel of antibody pairs in sandwich format capable of detecting 10^4 plaque-forming units of dengue virus in a pool of 25 mosquitoes can be used to develop the dengue assay.
4. Production of about 1000 assays in several different formats (individual PF, Pv210 or Pv247) to allow for initial laboratory/ field validation of the assay. These dip-stick format can be manufactured at relatively low cost.
5. An alternative lateral-flow format has also been developed. This format has a unique filter which can remove cellular debris or particulate matter and allow the immunochromatography process in a cleaner background

For the detection of various sporozoite antigens, the following types of dipsticks have been made for evaluation and supplied with this report:

1. Plasmodium falciparum 2A10 (6 kits each with 25 tests each)

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2. Plasmodium falciparum 2A10 500 tests (these are in 5 boxes each containing 100 tests) This is the same test as #1.
3. Plasmodium falciparum 1B2.2 (1 kit with 25 tests)
4. Plasmodium vivax 210 (4 kits each with 25 tests)
5. Plasmodium vivax 247 (2 kits each with 25 tests)

Further work:

This work has set the stage for Phase II, whose objective is to develop a larger number of such tests with appropriate optimization to suit the nature of the sample, and have optimum sensitivity and specificity.

Once the test combinations are selected, they will be prepared under GMP environment, individually packed in foil pouches with desiccant or in air-tight canisters with desiccant as supplied for the evaluation. The provided insert in the kit provides the direction of use.

Additionally a low cost miniature system capable of quantifying and recording results by inserting the strip into the machine can have worthwhile applications.

7. REFERENCES:

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2. Day, M., Scourge of infections kills Third World's Young, In: New Scientist, 150, No 2031, p6, 1996.
3. Miller, L.H., Transmissible Disease and Blood Transfusion (Eds T. J. Greewait and G. A. Jamieson) Grune and Stratton, N.Y., 1975.
4. Henchal, E.A., McCown, J.M., Seguin, M.C., Gentry, M.K., Brandt, W.E. Am.J.Trop.Med.Hyg. 32(1), pp.164-169, 1983.
5. Hermanson, G.T. In: Preparation of Colloidal-Gold-Labeled Proteins, in Bioconjugate Techniques, Academic Press, N.Y. 1996.

8. APPENDICES

8.1 INSERT (description, contents and procedure) OF KITS SUPPLIED WITH THIS REPORT FOR EVALUATION: Attached at the end

8.3 LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
Pf	Plasmodium falciparum
Pv210	Plasmodium vivax 210
Pv247	Plasmodium vivax 247
BB	Blocking buffer: 100mM phosphate buffer with 1% BSA and 0.5% Gelatin
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
MAS	Medical Analysis Systems, Inc.
RIA	Radioimmune Assay
SBIR	Small Business Innovation Research

9 BIBLIOGRAPHY

PERSONNEL involved in this research and development (SBIR)

• NAME • PROJECT RESPONSIBILITY	• EDUCATIONAL BACKGROUND • POSITIONS HELD • EMPLOYERS/WORK YEARS	• RELATED EXPERIENCE
• Kirti Dave Project manager	• Ph.D. in Biochemistry • Texas A& M University, 5 years Research Scientist • SA Scientific, 2 years, Project Manager • Navix, 2 years, Project Manager	• product development • immunoassay development • conjugate production • solid phase materials
• Eva Sinter Immunologist	• MS in Biochemistry • L. Eotvos University, Budapest, 2 years, Research Scientist • Navix Hungary, 2 years, Immunologist • Navix, 3 years, Immunologist	• immunoassay development • antibody production • clinical & stability studies • data management
• Janos Botyanszki Protein chemist	• Ph. D. in Biochemistry @ E. L. University, Hungary • Research Group for Peptide Chemistry, Hungarian Academy of Sciences, Research Fellow, 10 years • NAVIX Hungary, 1 year • Navix, 2 year, Protein Chemist	• immunoassay development • protein purification • latex conjugation • protein immobilization
• Lisa Short Junior Scientist	• B.S. in Genetics @ UC Davis • Development of liquid stable protein controls • Navix, 1 year	• Immunoassays • clinical & stability studies • programmed printing

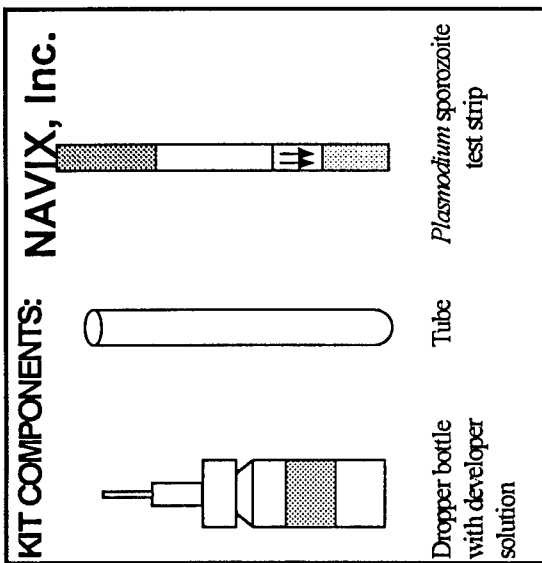


NAVIX, Inc.

Plasmodium Sporozoite Antigen Tests

Store at room temperature

FOR INVESTIGATIONAL USE ONLY



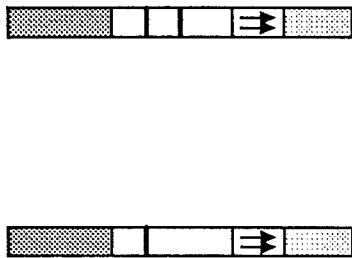
READING RESULT:

Positive

Interpretation of results:

- The test should be interpreted positive only if both lines appear on the strip.
 - The test is negative when only one line (control line) develops on the strip.
 - Discard strip if no line develops.
- The test results are not valid if control line does not develop.

Negative



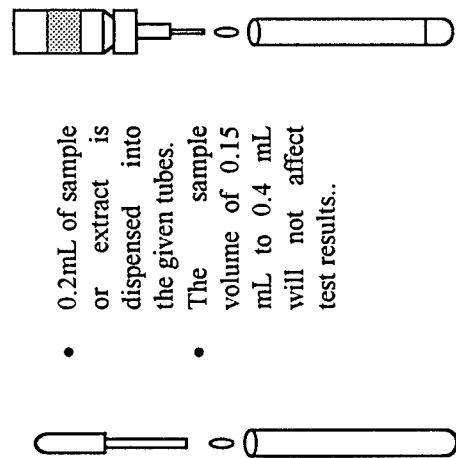
PERFORMING THE TEST:

Put strip into tube

Add developer

on

Add sample:



- Holding the labeled end, place the Dipstick in the sample tube as shown in the figure.
- Wait 15 minutes before reading results.

- Add 2 drops or 50µL of given Developer solution to the sample in the tube

- 0.2mL of sample or extract is dispensed into the given tubes.
- The sample volume of 0.15 mL to 0.4 mL will not affect test results..

524 Flynn Road, Camarillo, CA 93012

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

REPLY TO
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MCMR-RMI-S (70-1y)

4 Dec 02

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FOR THE COMMANDER:

Encl


PHYLIS M. RINEHART
Deputy Chief of Staff for
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