

Detection of Dengue Infected Mosquitoes by Direct Fluorescent Antibody Microscopy

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OBJECTIVE : To detect the presence of dengue virus in human serum using the mosquito inoculation technique.

BACKGROUND : This is a continuation of work previously reported (1). For the identification of dengue antigens in mosquitoes a direct fluorescent antibody (DFA) technique was added to the plaque isolation technique. This report reviews the experience with both techniques for the identification of dengue infections in patients seen at the Bangkok Children's Hospital (BCH) in 1975.

DESCRIPTION : To the previously described standard plaque assay in LLC-MK₂ tissue culture (TC) and the mosquito amplification technique, inoculation to *Aedes aegypti* followed by the standard plaque assay (MI/TC), was added an immunofluorescent technique similar to that used at the Pacific Research Section of the National Institute of Allergy and Infectious Disease, Hawaii. (2).

Anti-dengue fluorescent antibody conjugate was made from immune high-titered human sera. The globulin in immune sera was precipitated using 50% ammonium sulfate. The precipitate which contained gamma globulin and traces of albumin was dissolved in 0.01 M phosphate buffer pH 7.5 (P.B.) to a final volume approximately equal to that of the original serum sample, and then dialyzed against PB overnight.

After determining the total amount of protein present, the globulin solution was diluted with PB to a final concentration of 10 mg protein/ml and chilled in an ice bath. A freshly made carbonate-bicarbonate buffer (0.5 M, pH 9.0), was added to the chilled globulin in an amount equal to 10% by volume of diluted globulin. To the chilled globulin 0.0125 mg FITC/mg protein was added slowly with constant stirring. The solution was stirred overnight in the cold (12-18 hrs.), then dialyzed against PB to remove any unbound fluorescein dye. Merthiolate 1:10,000 was added as a preservative and the conjugate was frozen in aliquots.

The heads of infected mosquitoes were squashed under a coverslip (16 heads per slide) and stained with fluorescein conjugated antibody by a direct method. In early experiments each individual mosquito body was ground and tested by micro plaque assay. Later, pooled mosquito bodies were tested by a standard plaque assay.

Control mosquitoes consisted of non-infected mosquitoes, and mosquitoes infected with D 1, 2, 3 and 4 reference suckling mouse brain strains.

Each reading was made by two observers: — S.V. and W.H.B. or S.V. and A.N.

PROGRESS : A comparison of the results of virus isolation using the standard plaque isolation technique with and without mosquito amplification is shown in Table 1. Using the mosquito isolation step five strains of dengue were identified beyond those found by plaque isolation alone.

Only one strain was isolated using the plaque isolation technique without mosquito amplification. The five additional strains isolated using the mosquito amplification step came from sera with titers of 1:2560, 1:640, 1:40 and 1:20.

For the DFA technique, test sensitivity and specificity was determined after arraying the results in a "decision matrix" and calculating the "conditional probability" of true positive results (sensitivity) and true negative results (specificity) (3). Each decision matrix was built by comparing DFA test results to some "either-or" characteristic of the test materials, e.g., successful isolation of virus from individual mosquitoes or from individual patients. Separate determinations were made for each observer, S.V., W.H.B. and A.N. Each matrix was constructed as follows :

	Infection +	Infection -
DFA+	a	c
DFA-	b	d

$$\text{True positive (TP)} = \frac{a}{a + b}$$

$$\text{True negative (TN)} = \frac{d}{c + d}$$

Each ratio gives the conditional probability.

DFA ability to detect dengue in control mosquitoes was shown in the following matrices.

S.V.

Dengue 1

	TC +	TC -
DFA +	0	5
DFA -	1	7

$$\text{TP} = 0.0$$

$$\text{TN} = 0.58$$

W.H.B.

Dengue 1

	TC +	TC -
DFA +	0	5
DFA -	1	7

$$\text{TP} = 0.0$$

$$\text{TN} = 0.58$$

Dengue 2

	TC +	TC -
DFA +	10	3
DFA -	2	2

$$\text{TP} = 0.83$$

$$\text{TN} = 0.40$$

Dengue 2

	TC +	TC -
DFA +	4	2
DFA -	6	3

$$\text{TP} = 0.40$$

$$\text{TN} = 0.60$$

Dengue 3

	TC +	TC -
DFA +	0	4
DFA -	0	12

TP = unknown

TN = 0.75

Dengue 3

	TC +	TC -
DFA +	0	0
DFA -	0	14

TP = unknown

TN = 1.00

Dengue 4

	TC +	TC -
DFA +	6	4
DFA -	1	6

TP = 0.14

TN = 0.60

Dengue 4

	TC +	TC -
DFA +	0	0
DFA -	6	8

TP = 0.0

TN = 1.0

All negative controls were arbitrarily considered to be DFA negative by both observers and none yielded a virus isolate.

The plaque isolation technique did not appear to be capable of isolating virus from all mosquitoes inoculated with suckling mouse brain strains. This suggests that either the tissue culture or the mosquito was not very sensitive to mouse adapted dengue strains.

The results of DFA for mosquitoes inoculated with plasma of DHF patients are summarized in Table 2. There was a correlation between the DFA readings of the pair observers. Separate determination of matrices were made for each observer, S.V., W.H.B. and A.N. as follow :-

S.V.

	TC +	TC -
DFA +	7	28
DFA -	3	62

n = 100

TP = 0.7

TN = 0.69

and

W.H.B.

	TC +	TC -
DFA +	9	8
DFA -	1	82

n = 100

TP = 0.9

TN = 0.9

	S.V.	
	TC +	TC -
DFA +	7	33
DFA -	0	40

n = 80
 TP = 1.0
 TN = 0.55

and

	A.N.	
	TC +	TC -
DFA +	4	34
DFA -	3	39

n = 80
 TP = 0.6
 TN = 0.56

DISCUSSION AND SUMMARY : As has been found previously; the mosquito amplification step gave a greater number of virus isolations than the standard plaque assay alone. The DFA test as performed in this laboratory is difficult to interpret. Although it appears to be relatively sensitive in some cases it showed a very poor specificity. In the absence of a second test capable of detecting dengue antigens *per se*; it is difficult to determine the usefulness of the DFA test.

REFERENCES :

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Table 1. Isolation of Dengue Viruses from Patients' Plasma by Standard and Mosquito Amplification Techniques

HI Titer	Number	Isolation	
		Standard TC	MI/TC
≤20	23	9 (39)*	10 (43)
40	4	0 (0)	1 (25)
80	5	2 (40)	1 (20)
160	19	1 (5)	1 (5)
320	20	2 (10)	2 (10)
640	27	0 (0)	2 (7)
1280	39	0 (0)	0 (0)
2560	32	0 (0)	1 (3)
5120	17	0 (0)	0 (0)
10240	7	0 (0)	0 (0)
Total	193	14 (7.2)	18 (9.3)

*Percent of number tested at each titer.

Table 2. Detection of Dengue Antigen in Mosquitoes
by Direct Fluorescent Antibody
(Performance of Two Observers, S.V. and W.H.B. or S.V. and A.N.)

HI D2 Titer	Observers		Observers		Isolation + FA + by both observers	Isolation + FA + by one observer only
	S.V.	W.H.B.	S.V.	A.N.		
<20	11/20	6/20	9/15	12/15	8	2
20	0/2	0/2	2/3	2/3	1	—
40	1/3	1/3	1/3	1/3	—	—
80	3/5	1/5	—	—	—	1
160	3/9	1/9	2/5	2/5	—	—
320	3/9	2/9	3/9	3/9	—	2
640	6/13	2/13	7/10	6/10	1	—
1280	2/17	3/17	5/13	4/13	—	—
2560	5/16	1/16	5/9	5/9	—	—
5120	1/6	0/6	4/8	4/8	—	—
>10240	—	—	2/5	2/5	—	—
Total	35/100	17/100	40/80	41/80	—	—