

Comparison of the Mosquito Inoculation Technique
with Cell Culture Techniques for Isolating
Dengue Viruses from Dengue Patients

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OBJECTIVES : To compare *Toxorhynchites splendens* to LLC-Mk2 cells for isolating dengue viruses from plasma and cellular components of the blood of dengue patients

BACKGROUND : Dengue virus isolation rates from dengue patients have been shown to vary according to the assay technique and the type of specimens assayed for virus. Recently, the isolation rate reported for the mosquito inoculation assay¹⁻³ has been substantially higher than the rates obtained by cell culture techniques⁴⁻⁹. In preliminary studies, the rate of isolation of dengue viruses from peripheral blood leukocytes was over three times greater than that from plasma of dengue patients. This difference, however, may have been due to the use of a different assay technique for the two types of specimens. The present report describes the results of investigations designed to evaluate and compare the mosquito inoculation assay to cell culture for isolating dengue viruses, and to identify the component(s) of the blood of dengue patients that yielded the maximum number of virus isolations.

METHODS: Plasma and platelet and leukocyte fractions were obtained from blood of dengue patients in the Children's Hospital during 1978^{10, 11}. *Toxorhynchites splendens* were from a laboratory colony maintained as described previously¹². LLC-Mk2 cell cultures were propagated with medium 199, with 15% calf serum, in 1 oz glass bottles

Figure 1 shows the scheme used to assay fractions of the blood of dengue patients for virus. Blood fractions were assayed for virus by the mosquito inoculation technique employing *Tx. splendens*¹³, and in LLC-Mk2 cells by the direct and delayed plaque assay¹⁴ and the infectious center assay¹⁰. Eight or more *Tx. splendens* were inoculated intra-thoracically with each of the undiluted

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cellular specimens and a 1:5 dilution of plasma, 0.85 ul per mosquito. After 14 days incubation at 32°C, mosquitoes were stored at -70°C. Head squashes of individual mosquitoes were examined for virus by the direct fluorescent antibody technique¹³ and corresponding thorax-abdomens were tested for virus by the direct and delayed plaque assay. The amount of inoculum was 0.3 ml per cell culture. Viruses were identified by the plaque reduction neutralization test using monospecific dengue virus types 1, 2, 3, and 4 antisera prepared in rhesus monkeys. Cell culture assays were performed at 35°C.

RESULTS : Isolation rates for dengue viruses from plasma and cellular components of the blood of dengue patients, by the mosquito inoculation and by the direct and delayed plaque assay, are shown in Table 1. An analysis of the variation in rates obtained by the mosquito inoculation and the cell culture assays for the different types of blood fractions is presented in Table 2. A total of 17 virus isolations were obtained from the same plasma fractions by both the mosquito inoculation and the cell culture assays. An additional seven plasma fractions yielded virus isolations by the mosquito inoculation technique, while one additional virus isolation was obtained by the cell culture technique.

In contrast, the total number of isolations obtained by the mosquito inoculation and the cell culture assays from each of the cellular fractions were comparable. However, there was considerable variation in the two techniques for detecting virus in the same cellular fractions. Forty-six percent of the virus isolations from mononuclear cell fractions, 43% of the isolations from polymorphonuclear cells, and 33% of the isolations from platelet fractions were not detected by both techniques. Virus isolations obtained from blood fractions only by cell culture assay, were by the delayed plaque assay technique. Thus, the mosquito inoculation technique was more effective than the direct plaque assay, but comparable to the delayed plaque assay for obtaining dengue viruses from cellular fractions of the blood of dengue patients.

The variation in the virus isolation rates obtained by the mosquito inoculation and the cell culture assays for the different blood fractions was analysed according to the HI antibody and the neutralizing antibody titers of the patients. Isolations of dengue viruses only from plasma by the mosquito inoculation technique appeared to be related to the antibody titers, especially HI antibody (Table 3). The geometric mean antibody titer for patients from whom virus isolations were obtained from the same plasma fractions by both techniques was 1:33 while the titer was 1:352 for patients from whom viruses were isolated only by the mosquito inoculation technique. A similar relationship was noted between HI antibody titers and the isolation of dengue viruses from patients. In contrast, the variation in the pattern of virus isolation from platelets and polymorphonuclear fractions by the two techniques was not related to antibody titers of the patients (Tables 4 and 5). Isolation of dengue viruses from mononuclear cell fractions appeared to be related to antibody titer of the patient, but the pattern in regard to the techniques was the opposite of that observed for plasma fractions (Table 6). Four virus isolations

were obtained only by the mosquito inoculation technique from mononuclear cell fractions of patients with an HI antibody less than 10 (in most cases), while 8 isolations were obtained only by the delayed plaque assay, from patients primarily with high fixed antibody titers.

Sixty-nine adherent and nonadherent cell fractions were tested for virus by the infectious center assay in LLC-Mk2 cells. The rate of isolation of dengue viruses by the above technique as compared to the mosquito inoculation and the direct and delayed plaque assay is presented in Table 7. Isolation rates for adherent and nonadherent cell fractions were higher than those observed for other fractions assayed by either the mosquito inoculation or the direct and delayed plaque assay. However, the rates were similar to those obtained for mononuclear cell fraction by each of the other techniques. Overall isolation rates for the mononuclear cell fractions assayed by both the mosquito inoculation and the direct and delayed plaque assay were 36%. The isolation rates for the adherent and nonadherent fractions tested by the infectious center assay was 35%. This similarity in the number of virus isolations is not surprising since the adherent and nonadherent cell originated from the mononuclear cell fractions. The only difference in the techniques was the volume of inoculum for the infectious center assay which was approximately twice that used for either of the other techniques. The larger volume of inoculum may have been responsible for the higher isolation rates associated with the infectious center assay as compared to each of the other techniques.

Table 8 shows the F.A. assay results for head smears of *Tx. splendens* in comparison to the results of direct and delayed plaque assays of corresponding thorax-abdomen suspensions. An exceptionally high correlation was observed between F.A. virus positive mosquito heads and the recovery of virus from corresponding thorax-abdomen suspensions by the direct and delayed plaque assay. Isolation of dengue viruses from thorax-abdomen suspensions with no visible fluorescence in corresponding mosquito heads, however, was observed more frequently. Overall, for the different blood fractions, virus was detected only in thorax-abdomen suspensions for approximately one of every 10 virus isolations. In each instance, the isolate was dengue virus type-2.

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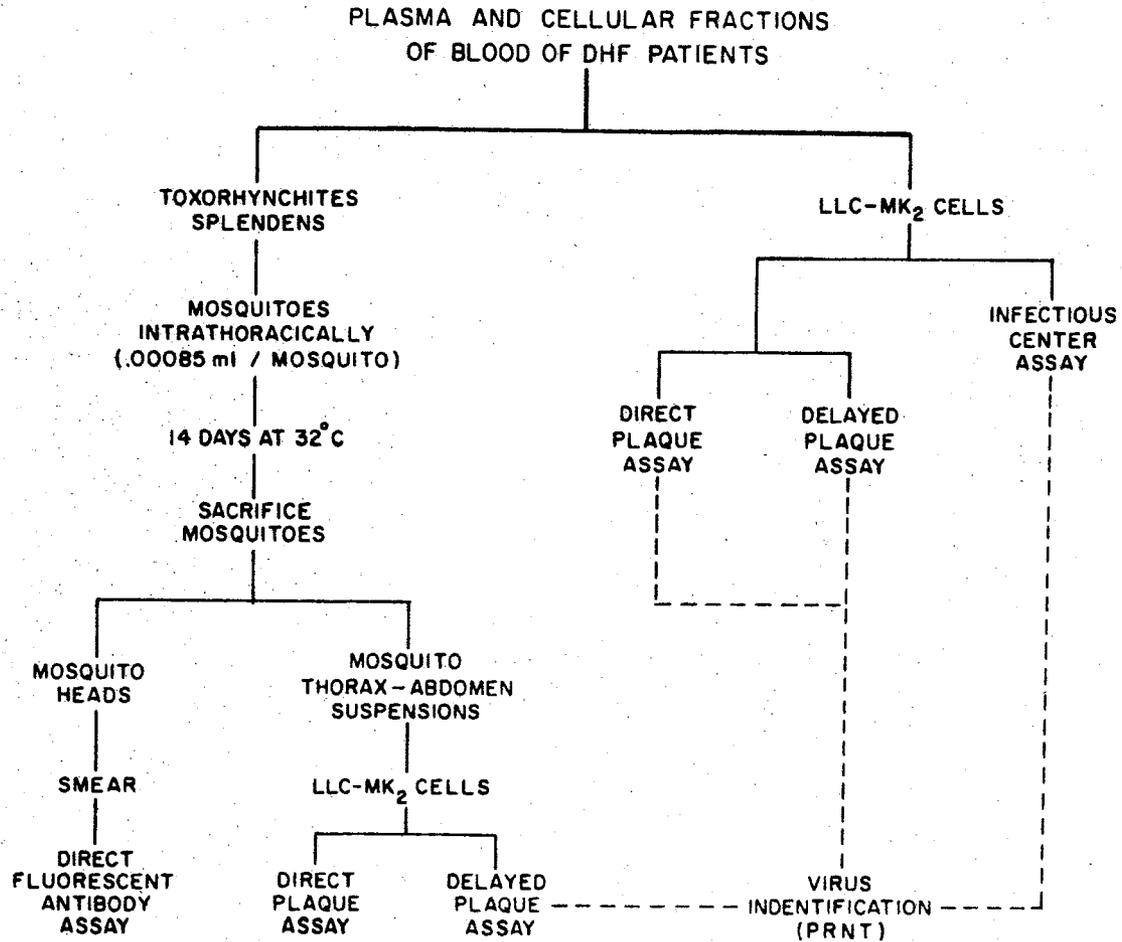


Figure 1. Procedure used to assay fractions of the blood of dengue patients for virus.

Table 1. Dengue Virus Isolations from Plasma and Cellular Components of the Blood of Dengue Patients.

Blood Fractions	Virus Assay Technique				Total ¹
	Plaque Assay (%)		Tx. splendens		
Plasma	18/116	(16) ²	24/116	(21)	25/116 (22)
Mononuclear Cells	21/103	(19)	24/103	(20)	27/103 (26)
Polymorphonuclear Cells	11/71	(15)	11/71	(15)	14/71 (20)
Platelets	09/64	(14)	10/64	(16)	12/64 (19)

¹ Number of virus isolations obtained by both assay techniques.

² Number of virus isolations/number of fractions tested.

Table 2. Summary of Dengue Virus Isolations Obtained by Different Assay Techniques from Blood Fractions of Dengue Patients.

Blood Fractions	<i>Tx. splendens</i> and LLC-Mk2	<i>Tx. splendens</i> Only	LLC-Mk2 Only ¹	Direct and Delayed ²	Delayed Only	Total
Plasma	17	07(87.5)	01(12.5)	15(83)	03(17)	25
Mononuclear Cell	15	05(45)	06(55)	11(55)	09(45)	27
Polymorphonuclear Cell	08	03(50)	03(50)	05(45.5)	06(54.5)	14
Platelets	07	03(60)	02(40)	04(44)	05(56)	12
Total		18(60)	12(40)	35(60)	23(40)	

¹ Virus isolations obtained only by the delayed plaque assay.

² Virus isolations obtained by both direct and delayed plaque assay in LLC-Mk2 cells.

³ One virus isolation obtained by mosquito assay from specimen that was not tested by cell culture assay.

Table 3. Isolation of Dengue Viruses from Plasma of Dengue Patients by the Mosquito Inoculation Technique and by the Direct and Delayed Plaque Assay in Relation to Hemagglutination Inhibition Antibody and Neutralizing Antibody Titers.

Patients Number	Day of Disease	Technique		Antibody Titer	
		Mosquito Assay	Plaque Assay LLC-Mk2	HI ¹	PRNT ²
D78-017	4	+ ³	0 ⁴	640	ND
D78-042	4	+	0	5120	ND
D78-054	5	+	0	80	160
D78-059	5	+	0	2560	640
D78-108	4	+	0	80	180
D78-145	5	+	0	160	640
D78-168	5	+	0	80	ND
Ave. 4.6				GM 352 ⁵	
D78-080	6	0	+	2560	640
D78-025	4	+	+	20	ND
D78-044	4	+	+	20	32
D78-050	3	+	+	10	00
D78-051	5	+	+	320	040
D78-052	5	+	+	1280	096
D78-069	2	+	+	10	ND
D78-078	5	+	+	160	190
D78-099	6	+	+	10	35
D78-112	5	+	+	10	00
D78-114	2	+	+	10	ND
D78-117	3	+	+	10	130
D78-132	4	+	+	160	ND
D78-133	2	+	+	10	350
D78-135	2	+	+	10	ND
D78-136	4	+	+	1280	ND
D78-157	5	+	+	10	ND
D78-159	4	+	+	640	640
Ave. 3.8				GM 1:33 ⁵	
Total		24	18		

- 1 Homologous reciprocal hemagglutination inhibition antibody titer.
- 2 Homologous reciprocal plaque reduction neutralization antibody titers.
- 3 Virus negative.
- 4 Virus positive.
- 5 Geometric mean titer.

Table 4. Isolation of Dengue Viruses from Platelet Fractions of Dengue Patients by the Mosquito Inoculation Isolation Technique and the Direct and Delayed plaque Assay in Relation to the Hemagglutination Inhibition Antibody and Neutralizing Antibody Titers.

Patient Number	Day of Disease	Technique		Antibody Titers	
		Mosquito Assay	LLC-Mk2 Plaque Assay	HI	PRNT
D78-078	5	+ ¹	0 ²	160	190
D78-117	5	+	0	10	000
D78-044	4	0	+	020	032
D78-136	4	0	+	1280	ND
D78-117	3	+	0	10	130
D78-042	6	+	+	5120	ND
D78-050	3	+	+	10	00
D78-069	2	+	+	10	ND
D78-099	6	+	+	10	035
D78-114	2	+	+	10	ND
D78-133	2	+	+	10	350
D78-135	2	+	+	10	ND
Total		10	09		

¹ Virus positive

² Virus negative

Table 5. Isolation of Dengue Viruses from Polymorphonuclear Cell Fractions of Dengue Patients by the Mosquito Inoculation and Direct and Delayed Plaque Assay in Relation to Hemagglutination Inhibition Antibody and Neutralizing Antibody Titers.

Patient Number	Day of Disease	Techniques		Antibody Titers	
		Mosquito Assay	Plaque Assay	HI	PRNT
D78-050	3	+ ¹	0 ²	10	00
D78-055	5	+	0	2560	ND
D78-097	6	+	0	10240	ND
D78-042	6	0	+	5120	ND
D78-044	4	0	+	020	32
D78-048	5	0	+	640	ND
D78-089	6	+	+	2560	640
D78-099	6	+	+	10	035
D78-112	5	+	+	10	000
D78-114	2	+	+	10	ND
D78-117	3	+	+	10	130
D78-133	2	+	+	10	350
D78-135	2	+	+	10	ND
D78-136	4	+	+	1280	ND
Total		11	11		

¹ Virus positive

² Virus negative

Table 6. Isolation of Dengue Viruses from Mononuclear Cell Fractions of Dengue Patients by the Mosquito Inoculation Technique and Cell Culture in Relation to Hemagglutination Inhibition Antibody and Neutralizing Antibody Titers.

Patient Number	Disease	Technique		Antibody Titers	
		Mosquito Assay	Plaque Assay	HI	PRNT
D78-017	4	+ ¹	ND ²	1280	ND
D78-026	6	+	0	5120	ND
D78-050	3	+	0	10	00
D78-069	2	+	0	10	ND
D78-091	5	+	0	10	ND
D78-118	2	+	0	10	ND
D78-009	4	0 ³	+	320	ND
D78-014	4	0	+	2560	ND
D78-044	4	0	+	20	32
D78-074	6	0	+	5120	ND
D78-077	5	0	+	2560	640
D78-108	6	0	+	5120	ND
D78-025	4	+	+	1280	ND
D78-051	5	+	+	320	040
D78-078	5	+	+	160	190
D78-099	6	+	+	10	035
D78-112	5	+	+	10	00
D78-114	2	+	+	10	ND
D78-117	3	+	+	10	130
D78-132	4	+	+	160	ND
D78-133	2	+	+	10	350
D78-135	2	+	+	10	ND
D78-136	4	+	+	1280	ND
D78-145	5	+	+	160	ND
D78-157	5	+	+	10	ND
D78-159	4	+	+	640	640
D78-168	5	+	+	80	ND
Total		21	21		

¹ Virus positive

² Not Done

³ Virus negative

Table 7. Summary of Dengue Virus Isolations that were Obtained from Dengue Patients Employing Three Different Virus Assay Techniques

	Virus Assay Techniques			Total Isolations
	Tx. splendens	Plaque Assay LLC-Mk2	Infectious Center Assay	
Plasma	18/68(.26) ²	15/69(.22)	-	19/69(27)
Mononuclear Cell	15/59(.26)	16/57(.28)	-	21/59(36)
Polymorphonuclear Cell	11/64(.17)	10/64(.16)	-	14/64(22)
Platelet	9/58(.16)	10/58(.17)	-	11/58(19)
Adherent Cell	-	-	22/69(.32)	
Nonadherent Cell	-	-	17/69(.25)	

¹ Virus isolation rates were based on the total number of the same fractions that were assayed by all techniques.

² Number virus isolations/number of fractions tested (%).

Table 8. Results of Fluorescent Antibody Tests for Mosquito Head Smears, and Direct and Delayed Plaque Assays for Corresponding Thorax-abdomens of the Mosquito Heads.

Blood Fraction	Total Virus Isolations	Mosquito Heads Fluorescent Antibody	Thorax-abdomen Plaque Assay
Plasma	24	21 (88)	24
Mononuclear	21	19 (95)	21
Polymorphonuclear	10	09 (90)	10
Platelets	10 ²	09 (90)	09
Total	65	58 (89)	64 (98)

¹ Percent of total virus isolations

² One platelet fraction was virus positive by the F.A. mosquito head smear but the corresponding thorax-abdomen suspension was negative for virus by the direct and delayed plaque assays.