

Dengue Virus Isolation from Human Plasma Inoculated Into Mosquitoes

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OBJECTIVE: To compare the results of three techniques of dengue virus isolation from human plasma.

BACKGROUND: An earlier report (1) showed preliminary infection of *Aedes aegypti* mosquitoes with dengue seed virus followed by incubation for 10 days yielded 1 to 3 logs more virus per pool of mosquitoes than was inoculated. Pools of mosquitoes were ground and inoculated into LLC-MK₂ tissue culture for virus isolation and identification by a standard plaque assay. The preliminary results suggested this combined mosquito inoculation/tissue culture (MI/TC) assay was more sensitive than tissue culture alone, but information was needed on the usefulness of this technique for the isolation of dengue virus from human blood.

DESCRIPTION: A collaborative prospective study was done to compare virus isolation results at SEATO Medical Research Laboratory (SMRL) to those at the Pacific Research Section (PRS) of the National Institute of Allergy and Infectious Diseases (NIAID) in Hawaii. Acute plasma samples collected as part of the overall studies of dengue hemorrhagic fever (DHF) at Children's Hospital, Bangkok in 1974 were divided into three identical aliquots and numbered sequentially. Aliquots were frozen promptly at -70°C and not thawed until virus isolation was attempted by: 1) standard plaque assay in LLC-MK₂ tissue culture (TC) cells at SMRL, 2) inoculation of pools of *A. aegypti*, incubation for 10 days, then standard plaque assay in LLC-MK₂ cells at SMRL (MI/TC), and 3) sent to PRS for isolation attempts by inoculation into *A. albopictus* followed by a plaque assay of individual mosquitoes in LLC-MK₂ cells. Virus isolates were identified at SMRL by plaque reduction neutralization tests (PRNT) using type specific hyperimmune mouse ascitic fluid (HMAF). Only the isolates from the standard plaque assay have been completed so far. Identification of isolates at PRS was accomplished by PRNT and complement fixation (CF) using inoculated mosquitoes as the CF antigen.

PROGRESS: There was a striking difference in the number of isolates by the two laboratories. The SMRL TC yielded 11 (14%) isolates from 76 patients compared to 28 (37%) at PRS by MI/TC. Only three SMRL isolates were recovered from plasmas with dengue HI antibody titers of 1:40 or greater (Table 1). On the other hand, fully half of the PRS isolates came from plasmas with HI antibody titers of 1:320 to 1:5120. The results suggest that one reason the MI/TC technique used at PRS recovered more virus isolates was that mosquitoes may be able to disassociate neutralizing antibody from infectious virus particles in the plasma. SMRL did not obtain any isolates by TC that were missed by PRS. There was some difference between the clinical diagnosis of the patients yielding virus isolates to either laboratory (Table 2). Many more isolates were obtained by PRS from patients with DHF grade 2 and 3 than SMRL; patients in these categories generally had higher levels of antibody in the acute plasma samples.

A comparison of the results from MI/TC at SMRL can only be made for 58 plasmas which were carried to completion. Of the 58 plasmas, 6 (10%) were positive by TC, 14 (24%) by MI/TC at SMRL and 18 (31%) by MI/TC at PRS (Table 3). Five plasmas yielded isolates by the SMRL MI/TC only; their identity is still in doubt. One plasma was positive by SMRL TC and at PRS but negative by MI/TC at SMRL. On the other hand, four plasmas yielded isolates by both mosquito inoculation techniques. Some of the differences in results are probably due to technical problems at SMRL that still need to be solved.

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DISCUSSION AND SUMMARY: A collaborative study for the comparison of three techniques for dengue virus isolation was carried out with PRS/NIAID. Techniques using mosquitoes as amplifying hosts for dengue replication prior to isolation of virus in LLC-MK₂ cells yielded a greater number of isolates than a standard tissue culture plaque assay method. The greatest improvement in isolation results was found in plasmas containing dengue HI antibody at titers of 1:40 or higher. It is presumed that inoculation of plasma into mosquitoes permits dissociation of neutralizing antibody from some infectious virus particles. The benefit of mosquito inoculation is two-fold: 1) the mosquito may strip off interfering antibody; 2) the mosquito allows small amounts of virus to multiply to levels that can be more easily detected in a LLC-MK₂ tissue culture system. The mosquito inoculation step will be included in future attempts at dengue virus isolation.

REFERENCES:

1. Bancroft, W.H., Valthanomsat, S., Gould, D.J. and Scott, R.M.: SEATO Medical Research Laboratory Annual Report, March 1974.

Table 1. Dengue HI Antibody Titers of Plasma Specimens Yielding Virus Isolates

Dengue Antibody Titer ^a	No. Tested	No. of Virus Isolates	
		SMRL ^b LLC-MK ₂	PRS ^c MI/LLC-MK ₂
<20	7	6	6
20	2	2	2
40	2		1
80	3		2
160	3	2	3
320	7		6
640	11		2
1280	7		1
2560	15		3
5120	11	1	2
10240	5		
≥ 20480	3		
TOTAL	76	11	28

^a Reciprocal plasma dilution

^b Virus isolates made at SEATO Medical Research Laboratory by direct inoculation of LLC-MK₂ tissue culture

^c Virus isolates made at the Pacific Research Section by inoculation of mosquitoes before attempting virus isolation in LLC-MK₂ tissue culture

Table 2. Diagnosis of Patients Yielding Virus Isolates

Diagnosis	No. Patients	No. of Virus Isolates	
		SMRL ^a LLC-MK ₂	PRS ^b Mosquito/LLC-MK ₂
Dengue Fever	5	2	3
DHF grade 1	6	2	3
grade 2	31	4	11
grade 3	23	1	7
grade 4	10	2	4
unspecified	1	0	0
Total dengue	76	11	28
Non-dengue	25	0	0

a Virus isolates made at SEATO Medical Research Laboratory by direct inoculation of LLC-MK₂ tissue culture

b Virus isolates made at the Pacific Research Section by inoculation of mosquitoes before attempting virus isolation in LLC-MK₂ tissue culture

Table 3. Relative Number of Dengue Virus Isolates Obtained from 58 Human Plasmas by Three Techniques

PRS MI/TC ^a	SMRL MI/TC	SMRL TC ^b	No. Dengue Isolates
+	0	+	1
+	+	+	5
+	+	0	4
+	0	0	8
0	+	0	0 ^c
18	9	6	18

+ means test positive; 0 means test negative

a Mosquito inoculation followed by tissue culture isolation technique

b Tissue culture only

c Five isolates obtained only by SMRL MI/TC have not yet been identified