

Isolation of dengue virus from dengue hemorrhagic fever patients

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PURPOSE: To increase the efficiency of isolation of dengue viruses from patients with dengue hemorrhagic fever (DHF)

BACKGROUND: This Laboratory's standard method of isolating dengue viruses from patients uses serum inoculated into MK-2 cells in the direct and delayed plaque system. Although this procedure permits isolation of dengue viruses from 40-50% of patients with primary dengue infections and from a similar proportion of patients with secondary infections sampled before the fifth day of disease, isolation rates from children with DHF approximate 15% (see previous section). Future studies of the role of specific dengue antigens and antibodies in the pathogenesis of DHF and DSS are likely to be critically dependent upon identification of the dengue serotype currently infecting the patient. Such identification is not currently possible by serologic means, but can only be accomplished by isolation of the agent. Thus we elected to study patients with DHF to see if a number of different isolation techniques might increase isolation efficiency in these patients who are often admitted late in the course of their infection with high dengue antibody titers.

I. Attempts to isolate dengue and chikungunya viruses from throat swabs of children with dengue fever.

Children with DHF have early and rapid rises in serum IgG antibodies to dengue which may result in in vivo neutralization of circulating virus. Neutralization of viruses in respiratory tract secretions appears dependent upon locally formed IgA rather than IgG antibody. Although the kinetics of local IgA dengue antibody rises in secondary dengue infections is unknown, we hypothesized that it likely was slower than serum IgG antibody rises and that dengue virus, if present in the nasopharynx, might be still unneutralized when it could no longer be detected in serum.

Ten patients presenting to the outpatient department of Children's Hospital with fevers of unknown origin and shown retrospectively to have serologic evidence of dengue or chikungunya infection were studied.

Throat swab and serum were obtained from each patient and were inoculated onto MK-2 monolayers in the direct and delayed plaque system. Viruses were isolated from serum of 5 patients (3-dengue 2, 1-dengue 1 and chikungunya) but were not isolated from throat washings of any patient.

II. Dengue isolation attempts from serum or plasma of DHF patients.

Studied were 57 patients admitted to Children's Hospital with a diagnosis of DHF. Serum was drawn on admission and on discharge for dengue HI antibody determinations. Serum (0.3 ml) obtained on admission was inoculated freshly onto MK-2 monolayer for isolation by the direct and delayed plaque method. Plasma was prepared from heparinized blood (100 µg/ml). Adult female Aedes aegypti mosquitoes,

obtained from the Department of Medical Entomology, were inoculated intrathoracically with needles dipped in freshly obtained heparinized blood. Additionally A. aegypti mosquitoes were allowed to feed on fresh heparinized blood from patients through a Baudrache membrane and engorged mosquitoes were collected. Fifteen mosquitoes of each group were kept in the insectary for 14 days when they were triturated and tested for virus by the direct and delayed plaque method using MK-2 cells. An additional volume of plasma was allowed to settle for 3 hours in the refrigerator or centrifuged at 600 RPM in an International Centrifuge for 3 minutes. The top layer containing WBC and platelets was collected. The remaining specimen was centrifuged at 5,000 or 10,000 RPM for 3 minutes and the remaining plasma collected. WBC and platelet counts were performed on the 2 plasma samples and then 0.3 ml volumes were inoculated onto monolayers of MK-2 and Aedes albopictus cells (Fluid Phase Cultures). Maintenance medium from these cultures were harvested at 7, 12, and 14 days after inoculation and titered for virus in direct MK-2 plaques.

At the conclusion of this study, serum stored at -70°C from patients with an isolate by any technique was inoculated onto fluid cultures of MK-2 cells as above, and centrifuged plasma samples from these patients were inoculated onto MK-2 cells in the direct and delayed plaque system.

PROGRESS: Of the 57 patients studied, 33 had serologic evidence of recent dengue infection and are included in the following data. No isolates were obtained from any of the 15 patients without serologic evidence of dengue infection, and one isolate was obtained from the 9 patients without convalescent serum samples on whom serologic confirmation of infection was not possible.

Dengue 2 virus was isolated from 6 of the 33 patients with confirmed dengue (18%). A summary of virus isolations by the various techniques from these 6 patients is shown in Table 1. Dengue 2 virus was isolated from plasma samples of all 6 patients inoculated into MK-2 fluid phase cultures. Virus was isolated from plasma from 3 of 5 of these patients in direct and delayed plaques. It should be noted that centrifuged plasma stored at -70°C for 3 months was used in this latter test. (Fresh centrifuged plasma inoculated into fluid phase cultures in these 5 patients yielded 4 isolates in fluid phase cultures). In contrast, fresh serum from the 6 patients yielded but 2 dengue strains in the direct and delayed MK-2 plaque system, while but one isolate was obtained from the 5 patients whose frozen serum was inoculated into MK-2 fluid phase cultures after storage for 3 months. No dengue isolations were obtained from fresh plasma inoculated into fluid phase cultures of A. albopictus cells. One of the 6 heparinized bloods yielded dengue 2 virus after intrathoracic inoculation and membrane feeding using A. aegypti mosquitoes.

Attempts were made to ascertain whether isolations from fresh plasma in fluid phase MK-2 cultures correlated with the presence of platelets or WBC in plasma. These results are shown in Table 2. The centrifugation technique used did not completely separate WBC or platelets from most plasma samples. Virus was recovered from both uncentrifuged and centrifuged specimens from 5 patients and only from uncentrifuged plasma in one patient. A correlation of virus recovery and platelet or WBC counts in either plasma specimen is not apparent.

We were initially disappointed that the most efficient isolation system in this study (plasma into MK-2 cell cultures) yielded an isolation rate of 18%, similar to the 15% obtained in the DSS study in which serum-MK2 plaques system was used. However, populations sampled in these 2 studies differed in at least one important aspect. A much higher proportion of patients in the DSS study (29%) had dengue 2 antibody titers of $\leq 1:80$ when studied than patients in this study (6%). In the DSS study, dengue was only isolated from patients' serum with dengue 2 HI antibody titers of $\leq 1:80$ (see Table 3—DSS study, this report). In the present study, dengue virus was isolated from the 2 patients with titers $\leq 1:80$, but also from 4 patients with HI antibody titers ranging from 1:160 or 1:1280 (Table 3). Only one of these latter 4 patients had an isolate from serum. This finding of dengue isolations from heparinized plasma containing relatively large quantities of dengue antibody suggests that heparinized plasma may be a more efficient sample for isolation of dengue virus from DHF patients. Reasons for this finding in the small number of patients with isolations studied is unclear and will require further investigation. A. aegypti mosquitoes or A. albopictus cell cultures were not effective isolation systems in this study.

Table 1.
Dengue isolates obtained from DHF patients employing different isolation techniques

Specimen #	MK-2 cells		A. albopictus cells		A. aegypti mosquitoes		Identity of isolate
	Fluid phase cultures (Plasma)	Delayed or direct plaque (Plasma)	Fluid phase culture (Plasma)	Intrathoracic Inoculation (Heparinized Blood)	Intrathoracic Inoculation	Membrane feeding	
49236	+	not done	+	0**	0	0	dengue 2
49649	+	0	0	0	0	0	dengue 2
49678	+	0	0	0	0	0	dengue 2
49693	+	+	+	0	+	+	dengue 2
49734	+	0	+	0	0	0	dengue 2
49786	+	0	+	0	0	0	dengue 2

* positive isolation

** no isolation

Table 2.
Relation of dengue virus recovery to presence of cellular elements in plasma

Specimen Number	Centrifugation Speed	Platelet Count (per mm ³)	WBC Count (per mm ³)	Virus Recovery in MK-2 Fluid Phase Cultures
49236	none or 600	53,000	0	+*
	5,000	3,000	0	+
49649	none or 600	63,000	700	+
	10,000	53,000	0	0**
49678	none or 600	141,000	4,400	+
	10,000	28,000	100	+
49734	none or 600	63,000	1,700	+
	10,000	18,000	0	+
49786	none or 600	50,000	4,800	+
	10,000	9,000	0	+

* + = isolate

** 0 = no isolate

Table 3.
Dengue isolations from plasma — MK-2 fluid phase cultures in relationship
to dengue 2 HI titer in plasma used for isolation

Reciprocal dengue 2 HI titer	Number of patients studied	Number of patients with isolates
≤20	1	1*
40	0	0
80	1	1
160	2	1
320	5	2*
640	4	0
1280	6	1
2560	9	0
5120	2	0
10240	1	0
≥ 20480	<u>2</u>	<u>0</u>
Total	33	6

* Includes 1 patient with isolation from serum.