

## Comparison of Rapid Centrifugation Assay with Conventional Tissue Culture Method for Isolation of Dengue 2 Virus in C6/36-HT Cells

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**A rapid centrifugation assay was compared with conventional tube cell culture for dengue virus isolation in both sera and autopsy samples from dengue and dengue hemorrhagic fever/dengue shock syndrome fatal cases. The rapid centrifugation assay allowed isolation of virus from 16.6% more samples than the conventional method, and it shortened the time for dengue virus detection. Finally, it allowed the isolation of dengue 2 virus in 42.8% of tissue samples from five fatal cases. Our results suggest that the rapid centrifugation assay may be useful for detection of dengue virus in clinical specimens.**

With the expanding geographic distribution and increased disease incidence in recent years, active disease surveillance has become an important component of dengue and dengue hemorrhagic fever (DHF) prevention programs. The goal of these programs is to predict dengue epidemic transmission. The role of the public health laboratory is to provide diagnostic support in virology and serology, to specifically determine the circulating serotypes, to monitor the potential introduction of any new serotypes, and to characterize the isolates as required (6).

Dengue diagnosis depends on isolating the virus or detecting viral antigen, viral RNA, or specific antibodies in patient samples. Four viral isolation systems have been used for dengue viruses: intracerebral inoculation of suckling mice (10, 12, 14), mammalian cell cultures (7, 26), mosquito cell cultures (9, 23, 24), and mosquito inoculation (13, 22), the last two systems being the most sensitive. Although mosquito cell culture is less sensitive for dengue virus isolation than mosquito inoculation, it is the method of choice for routine virologic surveillance and it allows the processing of a large number of samples in a relatively short time. *Aedes albopictus* (C6/36) and *Aedes pseudoscutellaris* cell lines are the most frequently used.

The rapid centrifugation or shell vial assay has greatly improved the isolation rate for some viruses. This assay has revolutionized virus culturing, notably decreasing the time required for rapid laboratory detection of many viruses in clinical specimens (1–5, 16, 17, 19–21, 25).

The aim of the present study was to apply the shell vial assay for dengue virus isolation as part of an effort to improve the rate of isolation of these viruses from field samples.

A total of 30 acute-phase serum specimens were obtained from patients with a clinical diagnosis of dengue or DHF (18). Autopsy samples (28 samples from 10 fatal cases) were delivered to the reference laboratory at 4°C. A 10% suspension of the tissue specimens was prepared in minimal essential me-

dium containing 10% calf serum, 500 U of penicillin per ml, and 500 µg of streptomycin per ml.

Dengue 2 virus (A15 Cuban strain) with 16 passages in suckling mice was used in the study (7).

The *A. albopictus* cell line (C6/36-HT) was grown at 33°C in minimal essential medium supplemented with 10% heated fetal bovine serum (HFBS) (56°C for 30 min), 1% nonessential amino acids, and 1% glutamine solution (200 mM). A total of 100 µl of a mixture of dengue 2 virus (A15 strain) with a multiplicity of infection of 0.1, patient sera (diluted 1/30), and patient tissue supernatants was inoculated onto monolayers of cells grown in either 24 plastic plate wells or screw-cap tubes. After 1 h of viral adsorption at 33°C, 1 ml of culture medium with 2% HFBS was added to inoculated cells grown in screw-cap tubes. For the rapid centrifugation assay, inoculated cells grown on 24 plastic plate wells were centrifuged for 1 h at 1,000 × *g* at 33°C; supernatants were discarded and 1 ml of culture medium with 2% HFBS was added to each well. Inoculated cells were kept at 33°C and observed daily for viral cytopathic effect (CPE). Once CPE was detected, or after incubation for 11 days (in those cases without CPE), inoculated cells were mechanically detached and fixed with cold acetone, and dengue virus antigens were tested with an indirect immunofluorescence assay (IFA), using an ascitic fluid hyperimmune to dengue 2 virus as the primary antibody. After 1 h of incubation at 37°C, the fixed cells were washed with phosphate-buffered saline and then incubated with an anti-mouse antibody-fluorescein conjugate for 30 min at 37°C. Positive samples were retested by IFA for final identification using specific monoclonal antibodies against four dengue virus serotypes kindly donated by D. Gubler of the Centers for Disease Control and Prevention, Atlanta, Ga. The negative samples obtained were passaged twice in cell culture and retested by IFA.

The infective titer of dengue 2 virus (A15 strain) inoculated on C6/36-HT cells grown in screw-cap tubes or 24 plastic plate wells was determined by plaque titration in the BHK21 clone 15 cell line according to the method of Morens et al. (15).

In order to evaluate the rapid centrifugation assay, C6/36-HT cells were inoculated with dengue 2 virus (A15 strain). CPE, IFA results, and viral titers determined by plaque formation were recorded from day 2 to day 4 after inoculation of cells grown in either 24 plastic plate wells or tubes (Table 1).

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TABLE 1. Replication of dengue 2 virus (A15 strain) in C6/36-HT cells in conventional tube cell culture and rapid centrifugation assay

Day	Tube cell culture			Rapid centrifugation assay		
	CPE	IFA-positive cells (%)	PFU/ml	CPE	IFA-positive cells (%)	PFU/ml
2	-	0	$1.4 \times 10^4$	-	80	$1.8 \times 10^5$
3	+	50	$8.0 \times 10^4$	++	50	$2.6 \times 10^5$
4	+	80	$2.4 \times 10^5$	+++	30	$1.3 \times 10^6$

CPE and dengue 2 virus antigen detection by IFA were observed earlier in those cells inoculated by the rapid centrifugation method. Also, viral titer was higher using the rapid centrifugation assay.

The results obtained for dengue 2 virus inoculated by the rapid centrifugation method suggested the possible extension of the method to clinical specimens. To test this possibility, 30 acute-phase sera from clinically suspected dengue patients were examined for viral isolation using the tube cell culture and rapid centrifugation assay methods in parallel. The results are summarized in Table 2. On the second day postinoculation, 5 of 30 (16.6%) sera tested by rapid centrifugation assay were identified as positive for dengue 2 virus. This increased to 53.3% at day 3. Conventional tissue culture demonstrated 0% positive samples at day 2 and only 13.3% at day 3. At day 5, a total of 17 (56.6%) isolates were obtained by the conventional method and 22 (73.3%) were obtained by the rapid centrifugation assay. After 11 days of incubation, the eight negative samples were passaged twice under the same conditions (rapid centrifugation assay or conventional method), but no positive samples were identified. All isolates were identified as dengue 2 virus by IFA.

Viral isolation from autopsy samples was done using only the rapid centrifugation assay. Dengue 2 virus was identified in 12 (42.8%) tissue samples from five cases. Virus was detected in several tissues, including liver, spleen, and lung. It is important to note that one isolate was obtained from a brain sample.

Using a laboratory-adapted dengue 2 virus strain, we demonstrated the usefulness of the rapid centrifugation assay for dengue virus in terms of rapid antigen and CPE detection and increased production of the virus.

In order to test its usefulness on field specimens, the studied sera were processed in parallel, both in the conventional manner and by rapid centrifugation assay. The rapid centrifugation assay allowed the isolation of five (16.6%) more samples than conventional tubes. Also, it shortened the detection time for dengue virus infection. In 48 to 72 h postinoculation, 16 (53.3%) isolates were obtained, compared with 4 (13.3%) at 72 h by the conventional way.

The low frequency of isolation, the longer time required,

TABLE 2. Comparison of detection of dengue 2 virus in acute-phase serum samples by IFA in conventional C6/36-HT tube cell culture and rapid centrifugation assay

Day	No. (%) of IFA-positive samples (n = 30)	
	Tube cell culture	Rapid centrifugation assay
2	0 (0)	5 (16.6)
3	4 (13.3)	16 (53.3)
4	11 (36.6)	18 (60.0)
5	17 (56.6)	22 (73.3)
11	17 (56.6)	22 (73.3)

and the poor replication observed in some samples (and the consequent need for one or two passages in cell culture before IFA identification with monoclonal antibodies) are some of the major problems associated with dengue virus isolation. In addition, the rate of virus isolation from autopsy samples of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) cases is low, probably because death almost always occurs during the convalescent phase of the disease when most of the viral particles are associated with antibodies. The percentage of isolation from autopsy samples (42.8%) obtained in our study represents a high rate of isolation for tissue samples from fatal cases. The use of the rapid centrifugation assay allowed us to isolate dengue 2 virus from a brain sample of a fatal DHF/DSS case. This represents one of the few isolates obtained from this tissue and suggests that the virus replicates there (11).

In summary, our study has demonstrated that the application of a rapid centrifugation assay for dengue virus isolation improves the rate of isolation of these viruses, even in tissue samples. This assay represents an advance since it shortens the time needed to obtain results compared with the time required for the conventional tissue culture isolation method, it is easy to perform, and it is available in most diagnostic virology laboratories. Finally, this approach could be useful not only for increasing the virus isolation rate but also for obtaining a higher viral titer. We recommend the extension of the rapid centrifugation assay to the isolation of the other dengue virus serotypes.

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