DETECTION OF DENGUE VIRUS RNA BY REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION IN THE LIVER AND LYMPHOID ORGANS BUT NOT IN THE BRAIN IN FATAL HUMAN INFECTION

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Abstract. Autopsy tissues from 18 children believed to have died of dengue hemorrhagic fever were tested for the presence of dengue virus RNA by reverse transcription–polymerase chain reaction (RT-PCR). Such RNA was found in 14 of 18 liver specimens, 13 of 18 spleen specimens and 7 of 16 mesenteric lymph node specimens. No dengue virus RNA was detected in 44 samples of brain tissue from 15 individuals, 1 or more of whose other tissues yielded such RNA. All tissues had been tested previously for dengue virus by mosquito inoculation. In those tests, virus was recovered from 5 of 18 liver and 2 of 18 spleen specimens. Thus, the RT-PCR is more sensitive than the most sensitive virus isolation technique for detecting dengue virus or its components in human tissue. Failure to isolate virus from most of spleen and all mesenteric lymph node specimens may indicate that those tissues contained primarily degraded virus undergoing inactivation.

The 4 serotypes of dengue virus (genus flavivirus, family Flaviviridae) as a group are now the most important viruses transmitted to humans by arthropods, whether measured in terms of morbidity or mortality. The clinical manifestations of dengue range from a self-limited acute febrile illness to severe hemorrhagic manifestations and shock from leakage of plasma from the vascular compartment. The determinants of these severe manifestations and the relative importance of factors related to the virus compared with those of the host are unknown. Dengue viral antigen has been detected in cells of reticulo-endothelial origin in the spleen, thymus, lymph nodes, lung, and liver. However, despite the high levels of viremia that usually accompany dengue infections in humans, the cells in which dengue viruses replicate are not known.

It has become increasingly apparent in recent years that the liver may be an important site of dengue virus replication and the source of at least some of the physiologic aberrations that occur during dengue infections. In a previous study of tissues obtained at autopsy from children suspected of dying of such infections, dengue viruses were recovered by the mosquito inoculation technique from the liver of 5 of 17 cases studied, but rarely from other tissues. However, despite the high levels of viremia that usually accompany dengue infections in humans, the cells in which dengue viruses replicate are not known.

In view of the potential increased sensitivity offered by a reverse transcription–polymerase chain reaction (RT-PCR), we retested the liver of most of the patients for the presence of dengue virus RNA using that technique. We found that such RNA could be detected in the liver of 11 of the 15 cases analyzed, and we then retested all available tissues from the autopsies by a RT-PCR technique. The results of these studies are the basis of this report.

MATERIALS AND METHODS

Study material. The source of the study material and some of the characteristics of the patients have been described previously. Briefly, with the exception of 1 Vietnamese child who died shortly after arrival on Guam in 1975, all other patients were Burmese children who died in Rangoon in 1976 with a suspected diagnosis of dengue hemorrhagic fever. In addition to the patients described earlier, 1 additional Burmese patient (no. 1251) is included in this report; a 10-year-old boy whose acute-phase serum was collected on the fourth day of illness and who died on the seventh day. Nine hours elapsed between the time of death and autopsy. This child had received 250 ml of whole blood during hospitalization. With few exceptions, material available for study included serum collected soon after admission to the hospital and samples of heart blood, liver, spleen, mesenteric lymph node, kidney, cerebrum, cerebellum, and mesencephalon collected at autopsy with the informed consent of the relatives of the deceased under the Burmese and U.S. regulations in effect at that time. The study carried out in France were in accordance with the policy of the Clinical Review Committee of the Institut Pasteur based on the “Loi Huriet” no. 88–1138 of December 20, 1988. All specimens had been preserved at approximately –70°C for more than 20 years.

Detection and identification of dengue virus RNA in serum and autopsy samples by RT-PCR. The RNA was extracted from serum and autopsy samples following the procedure previously described. Briefly, using a mechanical shaker (Mini-beadbeater; Biospec Products, Bartlesville, OK) samples were homogenized 3 times (20 sec/homogenization) in a 2-ml tube (Quality Scientific Plastics, Petaluma, CA) containing 1 ml of Hanks’ balanced salt (Gibco-BRL Life Technologies, Gaithersburg, MD) solution, 4 units of RNAsine (Promega, Madison, WI), 0.2% NP40, and 0.6 ml of glass beads (0.5-mm diameter; Sigma, St. Louis, MO). After centrifugation at 10,000 rpm for 15 min at 4°C, 0.2 ml of the supernatant was mixed with 0.2 ml of lysis buffer (4 M guanidium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, 100 mM β-mercaptoethanol), 0.04 ml of 2 M sodium acetate, pH 4.0, 0.44 ml of water-saturated phenol (pH 8.0, Bioprobe; Quantum, Montreal, Quebec, Canada), and 0.175 ml of chloroform (Carlo Erba, Nanterre, France). The mixture was maintained on ice for 15 min and centrifuged at 10,000 rpm for 15 min at 4°C. The aqueous phase was collected and RNA was precipitated with an equal volume of ice-cold isopropanol.

The RT-PCR was performed as previously described using oligonucleotide primers specific for flavivirus consensus

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sequences and for actin (Stratagene, La Jolla, CA), a ubiquitously expressed cDNA that was used to correct for between-sample differences in RNA content. The flavivirus oligonucleotide primers used were consensus VD8 [5'-GGGT-CTCCTCTAACCTTAG-3'] and EMF1 [5'-TGGATGAC-C(G)/AC/G/TGA(A/G)/GA(C/T)ATG-3']. Briefly, RNA was resuspended in 9 μl of sterile distilled water and mixed with 1 μl (100 ng) of VDB8 primer, heated at 95°C for 2 min, and placed on ice. The cDNA was synthesized in 20 μl of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM MgCl₂, 10 mM dithiothreitol, 200 μM of the four dNTPs, 40 units RNAse (Promega), and 2 units of reverse transcriptase from avian myeloblastosis virus (Boehringer-Mannheim, Mannheim, Germany). The reaction mixture was incubated for 1 hr at 42°C. For the PCR, 4 μl of the cDNA were added to 46 μl of a mixture containing Thermus aquaticus (Taq) polymerase buffer (provided by the manufacturer; Perkin-Elmer Cetus, Norwalk, CT), 2 mM MgCl₂, 0.5 mM of each of the 4 dNTPs, 300 ng of VD8 and EMF1 primers, and 0.5 units of Taq polymerase. After 5 min of denaturation at 95°C, the mixture was subjected to 30 cycles at 95°C for 30 sec, 53°C for 90 sec, and 72°C for 60 sec, followed by a final 10-min polymerization step at 72°C. Controls were included in each experiment to standardize the amount of viral RNA in similar organs among the patients. Positive cDNA controls for dengue were obtained by RT-PCR of dengue-infected human hepatoma HepG2 cell RNA using the VDB8-EMF1 primer set. Actin-specific cDNA controls were obtained by RT-PCR of noninfected HepG2 cells. Negative controls were introduced in each test by replacing RNA from sample extracts with distilled water.

**Dot-blot hybridization.** Dot-blot hybridization was carried out as previously described. Briefly, samples of cDNA corresponding to one-tenth of the PCR products were denatured in 0.1 M Tris-HCl, pH 7.4, 0.2 N NaOH, 6× SSC (0.9 M NaCl, 0.09 M sodium citrate) for 10 min at 80°C, treated with 0.2 M Tris-HCl, pH 7.4, and dotted onto nitrocellulose membranes. Positive hybridization controls for dengue viruses and actin were generated by dotting 100 ng of each amplified control cDNA on nitrocellulose membranes. Membranes were heated for 2 hr at 80°C in hybridization buffer (Rapid Hyb Buffer; Amersham, Les Ulis, France) and incubated with labeled probes. In a preliminary test, a first set of dengue probes was prepared to identify the virus serotypes present in the liver samples. These probes corresponded to recombinant plasmids containing a dengue sequence derived from VD8-EMF1 amplified cDNAs altered to remove highly conserved fragments, as previously described. This was done to improve specificity of the hybridization for each dengue serotype. Plasmid DNA was labeled with 32P-dATP (Amersham) using a nick-translation kit (Boehringer-Mannheim). A second set of dengue probes was prepared by PCR using as templates plasmids containing dengue-1, -2, -3, or -4 virus VD8-EMF1-framed undeleted sequences and primers VD8 and EMF1. The actin probe was prepared by RT-PCR of RNA extracted from HepG2 cells and actin-specific primers. The PCR products were purified by gel exclusion chromatography (Promega) and 200 ng of DNA was labeled with 32P-dATP by nick translation. Hybridization was performed with heat-denatured probes at 42°C overnight in hybridization buffer. After the membranes were washed in 1× SSC for 30 min at 65°C, they were dried, exposed to a storage phosphor screen, and signals were recorded using the phosphorimager system (Storm; Molecular Dynamics, Sunnyvale, CA). The presence of viral RNA in tissue samples was estimated using the formula

\[
\frac{[SD] - [CD]}{[CD+] - [CD]} / \frac{[SA] - [CA]}{[CA+] - [CA]}
\]

where [SD] and [SA] correspond to the values of radioactivity recorded for each sample tested for dengue virus and actin, [CD−] and [CA−] correspond to the values of radioactivity recorded for negative controls hybridized with dengue virus and actin probes, and [CD+] and [CA+] correspond to values recorded for positive controls dotted on the membranes (see above). A positive sample was defined as having a radioactive signal > 3 times that of the negative control.

## RESULTS

**Identification of dengue virus RNA in liver samples of human fatal cases.** To assess the utility of the RT-PCR for detecting dengue RNA in tissues taken at autopsy, a preliminary test was performed on liver samples of 15 Burmese children who died in 1976 with a clinical diagnosis of dengue. The RT-PCR products were tested in a molecular hybridization test using DNA probes corresponding to the 4 dengue serotypes due to extensive homologies between serotypes, common dengue sequences in recombinant plasmids were deleted. All tests were carried out without prior knowledge of the previous virus isolation results. The data from this preliminary study are shown in Table 1. It should be noted that with 2 exceptions (patients no. 430

<table>
<thead>
<tr>
<th>Patient identification</th>
<th>Age in years and sex</th>
<th>Virus isolation*</th>
<th>Detection of virus RNA in liver†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guam</td>
<td>9 M</td>
<td>D2 (L)</td>
<td>NT</td>
</tr>
<tr>
<td>262</td>
<td>4 F</td>
<td>D3 (S + L)</td>
<td>D3</td>
</tr>
<tr>
<td>294</td>
<td>7 F</td>
<td>D3 (S)</td>
<td>—</td>
</tr>
<tr>
<td>430</td>
<td>6 F</td>
<td>D3 (S)</td>
<td>—</td>
</tr>
<tr>
<td>439</td>
<td>10 F</td>
<td>D2 (S)</td>
<td>D2</td>
</tr>
<tr>
<td>450</td>
<td>5 F</td>
<td>—</td>
<td>D2</td>
</tr>
<tr>
<td>617</td>
<td>4 F</td>
<td>D3 (L)</td>
<td>D3</td>
</tr>
<tr>
<td>629</td>
<td>8 F</td>
<td>D2 (S)</td>
<td>D2</td>
</tr>
<tr>
<td>843</td>
<td>3 F</td>
<td>D3 (S)</td>
<td>—</td>
</tr>
<tr>
<td>915</td>
<td>3 M</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>959</td>
<td>4 M</td>
<td>D2 (L)</td>
<td>D2</td>
</tr>
<tr>
<td>1028</td>
<td>2 M</td>
<td>—</td>
<td>D3</td>
</tr>
<tr>
<td>1034</td>
<td>3 M</td>
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<td>3 M</td>
<td>—</td>
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</tr>
<tr>
<td>1152</td>
<td>5 M</td>
<td>—</td>
<td>D3</td>
</tr>
<tr>
<td>1251</td>
<td>10 M</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Dengue virus type 2 (D2) or 3 (D3) was detected in admission sera (S) or extracts of liver (L) by the inoculation of mosquitoes. NT = not tested.
† Dengue virus cDNA obtained by reverse transcription-polymerase chain reaction of liver RNA extracts was identified by dot-blot hybridization with dengue serotype-specific DNA probes (see Materials and Methods).
and 843), each of the liver specimens from patients whose liver or admission serum had yielded virus also yielded dengue virus RNA. In each case, the serotype of the RNA matched that of the virus isolated beforehand. Among the 7 liver specimens found negative for virus isolation, 5 were positive (3 dengue-2 and 2 dengue-3) by RT-PCR and 2 (no. 915 and 1151) were also negative by RT-PCR.

Detection of dengue virus RNA in various tissues. To assess the distribution of dengue virus in various tissues of fatal cases and to identify the presence of viral RNA in the samples, a study was carried out in 18 children, including 3 additional cases (1 Vietnamese and 2 Burmese) not tested in the preliminary trial. To control for the quality of the RNA extracted from different organs of each patient, we also probed for the expression of actin mRNA since it is ubiquitously expressed in all tissues. The signal obtained for each dengue-specific RT-PCR product was adjusted by comparison with that obtained from the same sample for actin using the formula described in the Materials and Methods. An example of results obtained for dengue-positive cases is shown in Figure 1. The data obtained are presented in Table 2. Dengue virus RNA was found in 1 or more specimens from 16 of the 18 patients studied. In every instance viral RNA was found in each specimen that had yielded virus.

Dengue virus RNA was detected in 10 of 12 admission sera that were available for testing compared with 5 of 14 previously found positive for virus. However, such RNA was found in only 3 of 16 samples of heart blood collected at

![Figure 1](image)

**Table 2**

Detection and identification of dengue virus RNA by reverse transcription–polymerase chain reaction in tissues obtained at autopsy*  

<table>
<thead>
<tr>
<th>Patient identification</th>
<th>Dengue 2</th>
<th>Dengue 3</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission serum</td>
<td>262</td>
<td>439</td>
<td>450</td>
</tr>
<tr>
<td>Liver</td>
<td>NA</td>
<td>+†</td>
<td>NA</td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heart blood</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mesencephalon</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*NA = not available.
† Dengue virus was recovered by the mosquito inoculation technique."
the time of autopsy. One of the 3 positive specimens (no. 959) had previously yielded virus.

Dengue virus RNA was found in 14 of 16 liver specimens examined in dengue-positive patients, including one patient (no. 843) who had not yielded such RNA in the preliminary trial. Surprisingly, 13 of 18 spleen specimens were also positive, although only 2 had yielded virus previously, one of which was from a patient (no. 959) whose heart blood was also positive for virus. Dengue virus DNA was found in 7 of 14 mesenteric lymph node specimens in dengue-positive patients.

A small amount of dengue virus RNA was found in the kidney of 2 of 6 patients known to have been infected with dengue types 2 or 3. No dengue RNA was found in 44 samples of brain tissue from 15 patients known to have been infected with dengue types 2 or 3. The single brain specimen previously found positive for virus isolation was not available for RNA analysis. It should be noted, however, that the virus-positive brain specimen was from a patient whose heart blood also yielded virus, raising the question of whether the brain tissue itself was the source of the virus.

**DISCUSSION**

A comparison of the results of our previous virus isolation attempts with those obtained with RT-PCR clearly shows that the latter technique is far more sensitive than the former in detecting the presence of dengue virus or one of its components in tissue obtained at autopsy. In no instance were the results of the RT-PCR negative when a specimen tested had been found to contain virus. The one instance in which a liver specimen (from patient no. 843) had been found negative by RT-PCR in the preliminary test and positive in the second assay may be explained by the absence of confirmation that RNA had indeed been successfully extracted in the first trial. The use of actin as an indicator of RNA extraction served to confirm successful extraction in the assays.

Among the different approaches to detect small amounts of dengue virus RNA in clinical samples, the most sensitive method provided is the nested or semi-nested PCR. However, the enhanced sensitivity of those tests is accompanied by an increased risk of contamination. We have shown previously that dot-hybridization using radiolabeled DNA probes enabled us to detect dengue virus RNA in samples from which we failed to isolate virus in tissue culture, suggesting that our technique is more sensitive than virus isolation. Moreover, the molecular hybridization used in the present study allowed a direct comparative analysis in the same blot among all samples of 1 individual and between patients.

The lack of a correlation between virus isolation and RNA detection may suggest that the information obtained from the two techniques may not be of equal significance. Virus and viral RNA were detected somewhat more frequently in liver specimens compared to other organs. However, the inability to isolate virus consistently from organs other than liver or admission sera and the lack of virus isolation from 7 mesenteric lymph nodes and 11 spleen specimens in which dengue virus RNA was detected may indicate that the latter organs contained primarily inactivated virus.

There is now little doubt that dengue viruses replicate in hepatocytes in liver tissue. Dengue antigens are observed in hepatocytes surrounding the necrotic foci in the liver of fatal cases. The replication of dengue virus in infected hepatocytes in vitro induces stress leading to apoptosis, which suggests that the elimination of apoptotic bodies by phagocytic cells may be a pathway of dengue virus clearance from infected tissues. Dengue virus antigen is also commonly observed in cells of the reticulo-endothelial system, but it is not known if such antigen represents replicating virus or phagocytized virus in the process of being destroyed. Our findings of dengue virus RNA in a large proportion of mesenteric lymph nodes and spleen specimens in the absence of infectious virus favor the latter explanation.

Dengue virus RNA was detected in 3 specimens of heart blood and we cannot exclude that organs in the same individuals found positive were contaminated with virus RNA present in the blood. No attempt was made to test for negative-stranded RNA of viral replicative forms in the positive organs. This could have discriminated between virus-infected cells and mature virus particles in the blood. Cross-contamination detectable by RT-PCR seems unlikely since lymph nodes and brain samples available for 2 of the children remained negative.

The absence of detectable dengue virus RNA in the brain specimens tested is especially noteworthy in the view of recent reports of the isolation of dengue virus from cerebrospinal fluid and the presence of dengue viral antigen and RNA in macrophage-like cells and neurons in the central nervous system. Encephalopathy without pleocytosis or signs of inflammation in the central nervous system is common in severe cases of dengue and indeed convulsions and/or encephalitis were noted before death in 12 of the 17 Burmese patients included in our study. Our negative RT-PCR data, and previous negative virus isolation attempts by the most sensitive technique available, indicate that if dengue virus replicates in the central nervous system, it does so only rarely.

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