Usefulness of Real-Time Reverse Transcription-Polymerase Chain Reaction for the Diagnosis of Echovirus Aseptic Meningitis Using Cerebrospinal Fluid

Tsuguto Fujimoto1*, Hiroyuki Izumi2, Nobuhiko Okabe1, Miki Enomoto1,3, Masami Konagaya1, Masatsugu Chikahira1, Tetsuya Munemura1, and Kiyosu Taniguchi1

1National Institute of Infectious Diseases, Tokyo 162-8640; 2Itabashi Medical Association Hospital, Tokyo 175-0082, and 3Hyogo Prefectural Institute of Public Health and Environmental Sciences, Hyogo 652-0032, Japan

(Received March 27, 2009. Accepted August 28, 2009)

SUMMARY: Quantitative real-time reverse transcription-polymerase chain reaction (q-RT-PCR) was used to diagnose echovirus infection and the results were compared to those obtained with the viral culture rate. Cerebrospinal fluid (CSF) from a total of 40 aseptic meningitis patients was used. Positive CSF samples, determined by viral culture (n = 29), contained significantly higher echovirus genome copy numbers (mean, 329 copies/μL) than did culture-negative CSF samples (n = 11) (mean, 34.2 copies/μL; P < 0.05). Echoviruses were identified as echovirus serotype 9 (E-9) (n = 21); E-30 (n = 16); and E-5, E-7, and E-18 (n = 1 each) by neutralization and/or conventional PCR-sequencing techniques. Viral culture-positive samples were collected at 1.41 ± 1.27 days after the onset of illness, and culture-negative samples were collected at 4.91 ± 3.34 days. Samples from which virus could be isolated were collected significantly earlier than were samples from which virus could not be isolated. These results strongly suggest the importance of early collection of CSF for echovirus isolation, and demonstrate the high sensitivity of q-RT-PCR for the detection of echoviruses in CSF.

Echoviruses are a major cause of aseptic meningitis, and echovirus serotypes 9 (E-9) and 30 (E-30) are two particularly important causative agents of this disease. According to the Infectious Agents Surveillance Report, National Institute of Infectious Diseases (Tokyo, Japan), enteroviruses were detected in 6,199 cases among aseptic meningitis patients in Japan between 2000 and 2008. During this period, E-30 was detected in 899 cases, and E-9 in 321 cases. These cases account for 14.5 and 5.2% of all enterovirus-detected cases, respectively (1).

Echovirus isolation from the cerebrospinal fluid (CSF) is the most reliable diagnostic method of determining the etiological agent of aseptic meningitis. However, viral culture methods are time-consuming, and the sensitivity of such approaches is lower than that of polymerase chain reaction (PCR) analyses. It has been reported that at least two-thirds of CSF specimens were enterovirus culture-negative, but reverse transcription (RT)-PCR-positive (2). One possible reason for this discrepancy could be the lack of CSF samples taken at an early stage after the onset of aseptic meningitis. Other groups have reported that CSF collection within 2 days is important for RT-PCR (3), and RT-PCR is a more sensitive test than viral culture for determining the causative agent of aseptic meningitis (4).

In this study, we compared the virus isolation rate with the quantitative real-time RT-PCR (q-RT-PCR) rate, and we compared conventional RT-PCR applications (Table 1) for the detection of echoviruses from CSF samples. The sample collection date after the onset of illness was considered to determine a possible relationship to the diagnostic results. We also compared the 50% tissue-culture infective dose (TCID₅₀) and the detection limits of PCR applications using an E-9 prototype strain.

The day of onset of aseptic meningitis (day zero in this study) was defined as the date when fever, vomiting, or headache was observed. Statistical analyses in this study were performed using Mann-Whitney’s U test. A P-value of <0.05 was considered significant.

CSF samples (n = 40) were obtained from aseptic meningitis patients in whom echovirus infection was suspected both clinically and epidemiologically. A sample was obtained from each of a total of 40 patients: 22 patients during an E-9 outbreak (in 1993, Hyogo Prefecture), and 18 patients during an E-30 outbreak (in 2008, Tokyo Metropolitan area). The virus was isolated using RD-18S and HEp-2 cells cultured in a monolayer on 24-well microplates. The cells were cultured for at least 2 weeks, during which time one blind passage was performed. If a cytopathogenic effect was observed, the sample was considered to be viral culture-positive. The isolated virus was identified by a neutralization test using neutralization antiserum specific to each echovirus and/or pool serum for the echovirus.

Viral RNA was extracted from 200 μL of CSF using a High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany), and it was eluted in 50 μL of elution solution. For the cDNA synthesis, 4 μL of the 50 μL eluents were used. Thus, the original CSF samples were estimated to contain 3.1 times (50/200 × 50/4) the number of echovirus genomes obtained by q-RT-PCR.

Complementary DNA synthesis was performed using a PrimeScript 1st strand cDNA synthesis kit (Takara, Shiga, Japan). In addition to the q-RT-PCR, conventional RT-PCR (5) and nested PCR using 5’-untranslated (UTR)/VP4/VP2 complementary DNA synthesis.
(6) coding regions for enterovirus identification were performed for molecular identification of the echoviruses. Briefly, RT-PCR (product size, 753 bp) was performed using the primers P-2 (7) and E31 (5). Nested PCR (6) (product size, 653 bp) was performed using the primers EVP-4 and OL68-71R (8). In addition to these PCR applications, RT-PCR (product size, 753 bp) was performed using the primers EVP-4 and OL68-71R. These results revealed q-RT-PCR to be a more sensitive test than viral culture. Among 11 CSF samples from which echoviruses could not be isolated, 2 samples were RT-PCR (5′-UTR/VP4/VP2)-positive (5), and 5 were RT-nested PCR (5′-UTR/VP4/VP2) (6)-positive. These samples were identified as harboring E-9 (n = 6) or E-30 (n = 1) by sequencing analysis, as described by Ishiko et al. (6). The remaining 4 CSF samples tested negative on RT-nested PCR (5′-UTR/VP4/VP2). Among these 4 negative samples, 2 were found to be positive by RT-PCR (5′-UTR) using the primers E1 and E2 (9) (Table 2, nos. 38 and 40). The remaining 2 samples were RT-nested PCR (5′-UTR)-positive using the primers R1 and E2 (9) (Table 2, nos. 21 and 26). In these 4 RT-nested PCR-negative samples, E-9 (n = 2), E-30, and E-7 were identified by comparison of the sequences to those of isolated echoviruses in this study. It was possible to isolate E-7 from a feces specimen (no. 26), which was identified by neutralization. As a result, 40 echoviruses were identified as follows: E-9 (n = 21); E-30 (n = 16); and E-5, E-7, and E-18 (n = 1 each).

The detection of echovirus in CSF is an important step in the diagnosis of aseptic meningitis, as it is difficult to rule out the possibility that any echovirus isolated from the feces or throat of aseptic meningitis patients may be a mere passenger virus. Echovirus detection in the CSF strongly suggests the detected virus as the causative agent of a case of septic meningitis. It has already been noted that specimens for enterovirus culture should be collected as early as possible in the course of disease (11). The present results support such practice, as culture-positive specimens collected earlier contained higher copy numbers of echovirus genomes than culture-negative specimens. Using q-RT-PCR, we were able to detect echovirus genomes from all samples, including the viral culture-negative specimens. Conventional RT-nested

Table 1. PCR applications and primers

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>PCR application</th>
<th>Product size (bp)</th>
<th>Detection limit (copy) for E-9 prototype strain (in this study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5′-UTR</td>
<td>Real-time RT-PCR (F + R, probe)</td>
<td>156</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>5′-UTR/VP4/VP2</td>
<td>RT-PCR (P-2 + E31)</td>
<td>753</td>
<td>1.3 × 10^2</td>
</tr>
<tr>
<td>3</td>
<td>5′-UTR/VP4/VP2</td>
<td>RT-PCR (EVP-4 + OL68-71R)</td>
<td>653</td>
<td>1.3 × 10^2</td>
</tr>
<tr>
<td>4</td>
<td>5′-UTR</td>
<td>RT-PCR (E1 + E2)</td>
<td>197</td>
<td>1.3 × 10^2</td>
</tr>
<tr>
<td>5</td>
<td>5′-UTR</td>
<td>RT-PCR (R1 + E2)</td>
<td>154</td>
<td>1.3 × 10</td>
</tr>
<tr>
<td>6</td>
<td>5′-UTR/VP4/VP2</td>
<td>Nested PCR (No2 + No3)</td>
<td>653</td>
<td>1.3 × 10</td>
</tr>
<tr>
<td>7</td>
<td>5′-UTR</td>
<td>Nested PCR (No4 + No5)</td>
<td>154</td>
<td>1.3</td>
</tr>
</tbody>
</table>

1) Mismatch between primer and E-9 prototype strain is underlined.
PCR techniques are labor-intensive and the results less sensitive (Table 1) than those of q-RT-PCR. Thus, q-RT-PCR is a sensitive method expected to be highly useful in the diagnosis of aseptic meningitis caused by echoviruses.

**REFERENCES**


