Evaluation of a Commercially Available Reverse Transcription-PCR Assay for Diagnosis of Enteroviral Infection in Archival and Prospectively Collected Cerebrospinal Fluid Specimens

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A commercially available reverse transcription (RT)-PCR method (AMPLICOR EV; Roche Diagnostic Systems, Inc., Branchburg, N.J.) was evaluated for detection of enteroviruses in cerebrospinal fluid from patients with neurological disease. This assay was compared with virus isolation in cell culture and an in-house RT-PCR method designed with a nonoverlapping region of the enteroviral genome. A panel of 200 cerebrospinal fluid specimens prospectively collected from patients with a wide variety of neurological symptoms, including 50 patients involved in three different outbreaks of acute aseptic meningitis, was assayed. A second panel of 97 archived cerebrospinal fluid specimens, stored for 2 to 5 years, from patients with aseptic meningitis associated with several enterovirus outbreaks was also studied. From the first panel, enteroviruses were detected in 13 of 50 specimens by cell culture (26%), in 43 of 50 specimens by AMPLICOR EV (86%), and in 46 of 50 specimens by the in-house assay (92%) from patients with aseptic meningitis associated with outbreak and 1 of 29, 3 of 29, and 4 of 29 specimens, respectively, from sporadic cases of aseptic meningitis. The remaining 121 cerebrospinal fluid specimens from patients with other neurological syndromes were negative by all tests. From the second panel, enteroviral RNA was detected by the AMPLICOR test (31 of 97 specimens, 32%) and the in-house assay (39 of 97 specimens, 40%). According to our results, patients with aseptic meningitis should be analyzed for enteroviral infection in cerebrospinal fluid by RT-PCR methods, and the AMPLICOR EV test is a suitable tool for performing such studies. Archival cerebrospinal fluid specimens are less suitable for evaluation of the performance of RT-PCR methods designed for enterovirus detection.

The enterovirus group includes 68 distinct serotypes of positive single-stranded RNA viruses which are human pathogens (poliovirus types 1 to 3, coxsackievirus groups A and B, echoviruses, and enteroviruses 68 to 71). Most enteroviral infections progress without clinical symptoms. However, enterovi-
um-high rates of enteroviral infection has not been validated (1, 20).

Several methods of enzymatic RNA reverse transcription (RT) followed by cDNA amplification (RT-PCR) have recently been introduced and used to obtain rapid diagnoses of enteroviral infection (3, 14, 15, 17, 22, 23). These new techniques showed greatly improved sensitivity compared to isolation in cell culture; however, typing of the virus strains, an important issue for epidemiological purposes, is not possible.

We have evaluated a commercially available RT-PCR test (AMPLICOR EV; Roche Diagnostic Systems, Branchburg, N.J.) for establishing the diagnosis of enteroviral infection of the CNS by comparison with isolation in cell culture and an in-house RT-PCR assay designed with a nonoverlapping region of the enteroviral genome. The three methods were applied to CSF specimens collected from patients with diverse neurological symptoms for which lumbar puncture was routinely performed and to archival CSF samples from patients involved in several identified outbreaks of AM caused by enteroviruses.

MATERIALS AND METHODS

CSF specimens and patients. Two hundred consecutive CSF specimens, from the same number of patients with neurological symptoms for which lumbar puncture was routinely performed, were prospectively collected. After collection, 400 μl of each CSF specimen was immediately inoculated on appropriate cell lines for virus isolation. The remainder was subsequently aliquoted and frozen at −80°C for later detection of enteroviral RNA. For 106 specimens, the volume collected was not enough to perform the complete study. These specimens were diluted fourfold prior to aliquoting and culture.

Epidemiological data were highly suggestive of an acute enterovirus infection in 50 patients, because they were associated with three outbreaks of AM in Spain during the time of the study (November 1995 to May 1996). The remaining 150 patients presented with a wide variety of neurological symptoms, including 37 cases of neurological disorders associated with human immunodeficiency virus (HIV) infection and 7 cases associated with other causes of immunosuppression, 7 cases of suspected congenital infection, 29 cases of non-outbreak-associated or sporadic AM, and 29 cases of encephalitis; finally, a miscellaneous group of 41 patients presented with other neurological syndromes.

Ninety-seven archived CSF specimens, stored at −20°C for 2 to 5 years, from patients with AM who had been associated with several identified enterovirus outbreaks in Spain within a period of four years (1991 to 1994) were retrospectively selected and tested. All of these specimens had previously been cultured, and the results of virus isolation tests were recovered from our records. Enteroviruses had been isolated in 22 of these CSF specimens, including two echovirus type 4 (Echo-4), one Echo-7, three Echo-9, one Echo-11, one Echo-17, four Echo-19, four coxsackievirus B6, and nine nonpoliovirus, untyped samples. The remaining 75 specimens were negative.

Cell culture and typing. Virus isolation was performed for each CSF sample (100 μl/tube) on human embryo lung fibroblasts, human lung carcinoma cells (A549), human kidney cells, and monkey HeLa cells. Culture media were maintained at 37°C and observed daily for cytopathic effect during 15 days. If, after this time, no cytopathic effect had been observed, cultures were discarded. Enteroviruses isolated were typed by the standard method of virus neutralization (Lim-Banyesh-Melnick immune serum pools). If reverse transcription of target RNA and amplification of cDNA by the AMPLICOR EV test; (ii) concordant duplicate PCR results with the AMPLICOR test; (iii) discordant duplicate PCR results with the AMPLICOR test; and (iv) specimens with discordant results by both PCR methods that were repeated by both PCR assays.

In-house RT-PCR assay. CSF specimens were also tested with a two PCR results were generated for each specimen). Both negative and positive control wells were processed in each PCR run. The enzyme uracil-N-glycosylase (AmplErase; Roche), which recognizes and catalyzes the deamination of deoxyuridine-containing DNA, was included in the AMPLICOR master mix. This is a novel improvement designed to prevent false-positive amplification by carryover contamination (11).

The sensitivity of this test, as reported by Lina et al. (10) in a multicenter evaluation, ranged from 67 to 98% for viral titers of 1 to 10 50% tissue culture infective doses (TCID50)/0.1 ml but was only 16% for titers of 0.1 TCID50/0.1 ml. In-house RT-PCR results of 106 specimens were analyzed by electrophoresis through 2% agarose in a Tris-borate-EDTA gel stained with ethidium bromide (0.5 μg/ml).

The in-house PCR method had been shown to be highly sensitive (5), capable of detection of between 0.2 and 0.02 TCID50/0.1 ml with poliovirus type 1, coxsackievirus B1, coxsackievirus A16, Echo-4, Echo-9, and Echo-30.

Study design. All tests (cell culture, typing, AMPLICOR, and the in-house PCR) were performed at the Centro Nacional de Microbiología laboratories, which receive clinical specimens from a high number of different hospitals all over Spain.

The 200 prospectively collected CSF specimens were not specially selected for this study, but all CSF specimens sent to our laboratory for virological diagnosis were enrolled. Just after receipt, CSF specimens were cultured on appropriate cell lines and immediately aliquoted and frozen at −80°C for subsequent detection of enteroviral RNA. All AMPLICOR testing of each extracted sample was performed in duplicate; i.e., RNA from each extracted specimen was amplified twice and one detection per amplification was performed. In this way, each sample generated two PCR results. The in-house RT-PCR assay was performed in parallel on each CSF specimen. In order to avoid false-positive PCR results by carryover contamination, four distinct areas for reagent preparation, nucleic acid extraction and first amplification, nested PCR, and detection of products were established. Blinding of the study was guaranteed because cell culture, the AMPLICOR test, and the in-house assay were performed separately by different technicians without knowledge of the results obtained with the other assays.

Any discrepant result was resolved or reaffirmed by further PCR testing on a new frozen aliquot. Discrepant results included (i) discordant duplicate PCR results with the AMPLICOR test; (ii) concordant duplicate PCR results with the AMPLICOR test failing between 0.250 and 0.500 absorbance unit (equivocal range); (iii) AMPLICOR PCR results for which the corresponding culture was discordant, i.e., PCR-negative and culture-positive or PCR-positive and culture-negative specimens; and (iv) specimens with discordant results by both PCR methods that were repeated by both PCR assays.

Statistical analysis. Comparisons between AMPLICOR test and cell culture isolation results were evaluated by McNemar's test.

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was positive for all tested specimens. Thirteen of them (13 of 50, 26%) gave positive results by cell culture and both RT-PCR methods. Thirty additional cases were detected by the AMPLICOR test; thus, a total of 43 positive specimens (86%) were identified. All of these 43 positive samples were also amplified, and therefore confirmed, by our in-house RT-PCR, an alternative method which amplifies a nonoverlapping region of the enteroviral genome. In addition, three further cases were detected by the in-house assay (46 of 50, 92%). All viral isolates were typed as Echo-30.

In only one of the 29 patients with sporadic AM was an enterovirus detected by cell culture and both RT-PCR methods; it was typed as coxsackievirus B5. Two further cases were identified by both RT-PCR methods, and one additional case was detected by the in-house assay. Thus, three CSF samples were found to be positive for enteroviral RNA by AMPLICOR (10%) and four samples were found positive by the in-house assay (14%). These four samples came from two infants, 1 month and 5 days old, and two adults, 33 and 49 years old.

All CSF specimens positive for enteroviruses by cell culture were also positive by both RT-PCR tests. No positive results, either by cell culture or RT-PCR, were obtained among the CSF specimens taken from patients with neurologic diseases other than AM nor among the 44 specimens taken from immunocompromised patients. False-negative results with the in-house PCR assay were discarded because the internal control was positive for all tested specimens.

In order to emphasize the erratic nature of the results obtained with archived CSF samples, Table 3 shows in detail the results obtained for eight specimens with discordant duplicate PCR results after the AMPLICOR test was performed in our laboratory. These discordant results were resolved by repeated AMPLICOR testing on new frozen aliquots sent to Roche Molecular Systems (Somerville, N.J.).

**RESULTS**

**Prospective study.** The results obtained with the 156 CSF specimens from immunocompetent patients included in the prospective phase are summarized in Table 1. A total of 50 CSF specimens from patients with AM associated with epidemic outbreaks caused by enteroviruses were assayed. Thirteen of them (13 of 50, 26%) gave positive results by cell culture and both RT-PCR methods. Thirty additional cases were detected by the AMPLICOR test; thus, a total of 43 positive specimens (86%) were identified. All of these 43 positive samples were also amplified, and therefore confirmed, by our in-house RT-PCR, an alternative method which amplifies a nonoverlapping region of the enteroviral genome. In addition, three further cases were detected by the in-house assay (46 of 50, 92%). All viral isolates were typed as Echo-30.

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There was a clear segregation between positive and negative specimens with the AMPLICOR test, since none of the results from the 200 prospective specimens analyzed fell between 0.250 and 0.500 absorbance unit (equivocal range).

**Retrospective study.** Table 2 shows the results obtained with 97 retrospectively collected specimens. Totals of 31 of 97 and 39 of 97 specimens yielded amplification of enteroviral RNA by the AMPLICOR test and the in-house assay, respectively. Both RT-PCR methods yielded negative results for 51 specimens, including 3 specimens found previously by culture to be positive. Moreover, six additional culture-positive specimens failed to amplify with the AMPLICOR test. Enteroviruses isolated from these nine specimens were typed as Echo-4, Echo-7, Echo-9, Echo-11, Echo-30, coxsackievirus B6, and a nonpoliovirus, untyped enterovirus. Three additional culture-positive specimens were also negative by the in-house assay. The enterovirus types involved in these six specimens were Echo-4, Echo-9, Echo-11, Echo-30, coxsackievirus B6, and a nonpoliovirus, untyped enterovirus. False-negative results with the in-house PCR assay were discarded, because the internal control was positive for all tested specimens.

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**DISCUSSION**

We have evaluated the AMPLICOR EV test to determine the suitability of this method for detection of enteroviral RNA in CSF specimens by comparing it with isolation in cell culture and an in-house RT-PCR assay. Prospectively and retrospectively collected clinical samples from a wide variety of syndromes were assayed.

**Prospective study.** Despite testing of a broad spectrum of neurologic syndromes, including specimens from 29 patients with encephalitis, 37 patients neurological disorders associated with HIV infection and 7 patients with other causes of immunosuppression, 7 patients with suspected congenital infection, and 41 patients with other neurologic syndromes, enteroviruses were detected only in specimens from 79 patients with AM. Although some types of enteroviruses have been involved as etiological agents in some cases of encephalitis (12), such cases are uncommon and we did not find any positive results with patients who presented with this disease. In addition, none of the neurologic disorders of the immunosuppressed patients included in this study could be imputed to enteroviral infection; nevertheless, only one patient presented with agammaglobulinemia, while the immunosuppression of the remaining patients was caused by HIV infection or antioncogenic treatment. Several authors have suggested previously that enteroviral infection is probably an important cause of neurological disease in patients with antibody deficiencies (18, 19).

Fortunately, we possessed 50 CSF specimens from patients involved in enteroviral AM epidemics; therefore, a reliable comparison between isolation in cell culture and RT-PCR techniques could be done. Only 13 of 50 specimens (26%) yielded enteroviral growth in cell culture, while 43 of 50 (86%)

<table>
<thead>
<tr>
<th>Neurologic syndrome</th>
<th>No. of CSF specimens</th>
<th>No. negative by all tests</th>
<th>Cell culture and both RT-PCR methods</th>
<th>Both RT-PCR methods</th>
<th>AMPICLOR RT-PCR</th>
<th>In-house RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outbreak-associated AM</td>
<td>50</td>
<td>4</td>
<td>13</td>
<td>30</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Sporadic AM</td>
<td>29</td>
<td>25</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Other syndromes</td>
<td>77</td>
<td>77</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* All specimens which yielded positive results by culture were successfully amplified by both RT-PCR methods.

* Result(s) confirmed by testing of a new aliquot of CSF by both RT-PCR methods.

<table>
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<tr>
<th>Table 2. Results of cell culture and RT-PCR tests for 156 CSF samples from immunocompetent patients included in the prospective study</th>
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<tr>
<td>Neurologic syndrome</td>
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<td>---------------------</td>
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<tr>
<td>Outbreak-associated AM</td>
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<tr>
<td>Sporadic AM</td>
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<td>Other syndromes</td>
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<tr>
<th>Table 2. Results of cell culture isolation and RT-PCR methods for CSF specimens from the retrospective study</th>
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<tr>
<td>AMPLICOR test result</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>+</td>
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<tr>
<td>−</td>
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</table>
Among our 29 patients with sporadic AM, the percentage of positive specimens detected by RT-PCR (3 of 29 [10%] with the AMPLICOR test and 4 of 29 [14%] with the in-house assay) was significantly lower than that found among the 50 outbreak-associated AM patients in this study or those reported in the studies cited above (23, 24). Nevertheless, neither of these two previous reports specified the origins of the CSF specimens as being from sporadic cases of AM or from cases involved in outbreaks, a well-established distinction in our study. Other epidemiologic factors, such as seasonal variations in the circulation of enteroviruses, year-to-year variations in the incidence of enteroviral infections, and cocirculation of other infectious agents causing AM at the time of the study, might also explain these differences. Note that based on the clear segregation of positive and negative results with the AMPLICOR test, in the commercially available kit the equivocal range has been eliminated and the cutoff has been set at 0.350.

**Retrospective study.** The analyses done on archived CSF specimens were less successful than those from prospectively collected samples. Only 31 of 97 specimens (32%) yielded a positive result by the AMPLICOR test; 39 of 97 (40%) did so by the in-house assay. In addition, both RT-PCR methods failed to detect three specimens which had been positive by culture, six specimens were AMPLICOR negative and culture positive, and three specimens were in-house negative and culture positive, for a total of 12 false-negative specimens. After typing, none of these 12 enteroviruses were found to be Echo-1, Echo-5, Echo-22, or Echo-23, four enteroviral types often missed by the AMPLICOR test (9). Moreover, the in-house PCR assay has previously been shown to be highly sensitive in detecting the enterovirus types involved in these false-negative results (5). Therefore, low sensitivity for detecting particular types of enteroviruses should be discarded as an explanation for these findings. The lack of reproducibility of the results obtained by the PCR assays in some of the archived specimens (see Table 3) suggests that degradation of enteroviral RNA, caused by freezing-thawing and long-term storage of specimens at −20°C (8), likely accounts for the low sensitivity of the PCR tests in the retrospective study and indicates that archived CSF samples are not suitable for evaluation of the performance of such tests in diagnosis. Nevertheless, Rotbart et al. (16) reported high sensitivity (94.7%) and specificity (97.4%) for the AMPLICOR test in a study performed with archival CSF specimens stored at −70°C.

In conclusion, the present study indicates that RT-PCR is a powerful tool for the diagnosis of AM syndromes due to enteroviral infection and shows that the AMPLICOR EV test is a reliable and standardized method for rapid and sensitive detection of enteroviruses in CSF. Long-term storage of enterovirus-containing CSF specimens is likely to lead to enteroviral RNA degradation that renders the specimen unsuitable for further testing. Archival CSF samples should not, therefore, be used for evaluation of PCR assays designed to detect enteroviral RNA in human CSF.

**ACKNOWLEDGMENTS**

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**TABLE 3. Discordant duplicate PCR results by the AMPLICOR test with archived CSF specimens**

<table>
<thead>
<tr>
<th>CSF specimen no.</th>
<th>AMPLICOR result(s) (OD&lt;sub&gt;450&lt;/sub)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>In-house RT-PCR result</th>
<th>Cell culture result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.137, 0.414</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>0.175, 0.798</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.161, 0.332</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>0.133, 0.155</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.131, 0.544</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>0.129, 0.518</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>0.110, 0.127</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>0.187, 0.521</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Positive, >0.500; indeterminate, 0.250 to 0.500; negative, <0.250. Each specimen was assayed in duplicate (two microtiter plate results) in the first and second assays with the AMPLICOR test.

<sup>b</sup> Results obtained by Roche Molecular Systems, Somerville, N.J.

<sup>c</sup> Sensitivity, 95% (19 of 20) with the AMPLICOR test (McNemar’s test, Yates corrected).

<sup>d</sup> Specificity, 95% (19 of 20) with the AMPLICOR test (McNemar’s test, Yates corrected).
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