JOURNAL OF CLINICAL MICROBIOLOGY, June 1998, p. 1741–1745 Vol. 36, No. 6
0095-1137/98/$04.00+0

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

FRANCISCO POZO,[1]* INMACULADA CASAS,[1] ANTONIO TENORIO,[1]
GLORIA TRALLERO,[2] AND JOSE M. ECHEVARRIA[1]†

Diagnostic Microbiology Service[1] and Virology Service,[2] Centro Nacional de Microbiología, Majadahonda, Madrid, Spain

Received 10 November 1997/Returned for modification 15 January 1998/Accepted 18 March 1998

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, enteroviruses are responsible for a wide variety of clinical syndromes ranging from a mild febrile illness to severe paralysis, aseptic meningitis, myocarditis, bronchiolitis, conjunctivitis, and a broad spectrum of other manifestations (12). Aseptic meningitis (AM) is by far the most common and clinically vexing. Enteroviruses are involved in at least 85% of the cases of AM for which an etiology can be determined, particularly among children and infants (2, 21). Clinical criteria alone are not enough to distinguish between enteroviral AM and other, more serious, central nervous system (CNS) infections caused by other neurotropic viruses and some bacteria. Thus, because patient management and outcomes can be completely different, establishing a rapid and reliable enteroviral diagnosis in the early course of meningitis may both eliminate unnecessary treatment and shorten hospitalization periods (6).

Enteroviral infections of the CNS have been diagnosed by isolation of viruses from cerebrospinal fluid (CSF) specimens in appropriate cell cultures, requiring 4 to 8 days for positive identification. Moreover, cell culture is frequently unsuccessful due to low viral loads in some clinical specimens and because some enterovirus serotypes do not grow in routine cell cultures, particularly group A coxsackieviruses (7). Enterovirus serological assays have been developed, but they are impractical for routine diagnosis due to the large number of antigens required to cover the 66 different known serotypes and because the applicability of these reactions to populations with medi-

* Corresponding author. Mailing address: Servicio de Microbiología Diagnóstica, Centro Nacional de Microbiología, Ctra. de Pozuelo Km 2, 28220 Majadahonda, Madrid, Spain. Phone: 34 1 509 79 01. Fax: 34 1 509 79 66. E-mail: jmecheva@isciii.es.

† Investigator representing the European Union Concerted Action on Virus Meningitis and Encephalitis group. Other group members are G. M. Cleator, Department of Pathological Sciences, University of Manchester, Manchester, United Kingdom; Maria Ciardi, Universita di Roma 'La Sapienza,' Rome, Italy; Paola Cinque, Ospedale San Raffaele, Milan, Italy; José Manuel Echevarria, Instituto de Salud Carlos III, Madrid, Spain; Marianne Forsgren, Huddinge Hospital, Stockholm, Sweden; Giuseppe Gerna, IRCCS Policlinico San Matteo, Pavia, Italy; Monica Grandien, Swedish Institute for Infectious Disease Control, Stockholm, Sweden; Frauke Harms, Universität Würzburg, Würzburg, Germany; Tapani Hovi, National Public Health Institute, Helsinki, Finland; Paul Klapper, Manchester Royal Infirmary, Manchester, United Kingdom; Marjaleena Koskiniemi, University of Helsinki, Helsinki, Finland; Pierre Lebon, Hôpital Saint Vincent de Paul, Paris, France; Annika Linde, Swedish Institute for Infectious Disease Control, Stockholm, Sweden; Anton van Loon, Academic Hospital Utrecht, Utrecht, The Netherlands; Volker ter Meulen, Universität Würzburg, Würzburg, Germany; Philippe Monteyne, Université Catholique de Louvain, Brussels, Belgium; Peter Muir, UMDS Guys & St. Thomas' Hospitals, London, United Kingdom; Elisabeth Puchhammer-Stöckl, University of Vienna, Vienna, Austria; Floré Rozenberg, Hôpital Saint Vincent de Paul, Paris, France; Christian Sindic, Université Catholique de Louvain, Brussels, Belgium; Clive Taylor, Newcastle General Hospital, Newcastle-upon-Tyne, United Kingdom; Bent Vestergaard, Statens Seruminstitut, Copenhagen, Denmark; Thomas Weber, Marienkrankenhaus Hamburg, Hamburg, Germany; and Benedikt Weissbrich, Universität Würzburg, Würzburg, Germany.

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 samples prospectively 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 samples, including two echovirus type 4 (Echo-4), one Echo-7, three Echo-9, one Echo-11, one Echo-17, four Echo-30, one 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), buffalo green monkey kidney cells, and rhabdomyosarcoma cells. Cultures were grown 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).

AMPLICOR EV test. The AMPLICOR test was used according to the manufacturer's instructions after a period of training and performance of a validation study with simulated specimens. In this way, the instruments and reagents involved in the PCR procedure, as well as the technicians performing the assay, were validated before processing of clinical specimens. The AMPLICOR EV test procedure has been described elsewhere (9). In short, viral RNA from specimens was extracted by mixing 100 μl of CSF with 400 μl of lysis solution, with incubation at room temperature for 10 min. RNA was precipitated by the addition of 500 μl of isopropanol and centrifugation at 16,000 × *g* for 10 min. The pellet was washed with 750 μl of 70% ethanol and resuspended in 200 μl of diluent containing manganese acetate and potassium acetate in a bicine buffer. A 50-μl aliquot of this material was added to an equal volume of master mix, for which reverse transcription of target RNA and amplification of cDNA by *Thermus thermophilus* (*Tth*) DNA polymerase occurs in a single reaction tube. Amplification was performed in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Norwalk, Conn.). An initial step of reverse transcription at 60°C for 30 min was followed by 35 cycles of denaturation (at 94°C, with the first cycle for 70 s and the remaining 34 cycles for 10 s each), annealing (58°C, 10 s), and extension (72°C, 10 s). The PCR products were detected by hybridization in microwell plates coated with an enterovirus-specific oligonucleotide probe. The optical density of the wells was read at 450 nm (OD_{450}), and the results were scored as positive if the OD_{450} was ≥0.500, equivocal if the OD_{450} was between 0.250 and 0.500, and negative if the OD_{450} was <0.250. However, in the commercially available kit, the equivocal range has been eliminated and the cutoff has been set at 0.350. PCR testing of each extracted sample was performed in duplicate (two amplifications and one detection well per amplification; that is, two PCR results were generated for each specimen). Both negative and positive control tubes were processed in each PCR run. The enzyme uracil-*N*-glycosylase (AmpErase; Roche), which recognizes and catalyzes the destruction 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 ($TCID_{50}$)/0.1 ml but was only 16% for titers of 0.1 $TCID_{50}$/0.1 ml.

In-house RT-nested PCR. CSF specimens were also tested with a previously reported in-house RT-nested PCR method (5), but only primers for enteroviruses and specific pseudorabies virus primers (as an internal control) were used in order to detect exclusively enteroviral RNA. Briefly, viral RNA from 50 μl of CSF specimen was extracted according to Casas et al. (4) by mixing it with 200 μl of a guanidinium thiocyanate lysis buffer, which includes 100 molecules of a cloned and purified genome fragment of pseudorabies virus DNA as an internal control for extraction and amplification steps. Cold (−20°C) isopropyl alcohol was added to precipitate nucleic acids, which were pelleted at 14,000 × *g* for 10 min at 4°C. The pellet was washed with 70% ethanol and dissolved in 10 μl of sterile double-distilled water. RT and the first PCR amplification of the target RNA were performed in a single reaction tube by using the Access RT-PCR System (Promega, Madison, Wis.). This system uses reverse transcriptase from avian myeloblastosis virus for first-strand DNA synthesis and the thermostable DNA polymerase from *Thermus flavus* (*Tfl* DNA polymerase) for DNA amplification. This simplifies the procedure and reduces the chance of contamination. After extraction, 5 μl of the dissolved pellet was added to 45 μl of an RT-PCR mixture composed of 2 mM $MgSO_4$, 0.2 mM (each) deoxynucleoside triphosphate, 10 pmol of each downstream and upstream primer, 5 units of avian myeloblastosis virus reverse transcriptase, and 5 units of *Tfl* polymerase; all of these reagents were used in a buffer compatible for both enzymes. Amplifications were carried out in a PTC-200 Peltier thermal cycler (MJ Research, Watertown, Mass.). Samples were subjected to an initial cycle of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and extension at 72°C for 1 min, followed by 45 cycles for 30 s and a final incubation at 72°C for 5 min. A nested PCR was then performed by adding 1 μl of amplified product from the first reaction to 49 μl of a PCR mixture containing 60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate, 10 pmol of each sense and antisense primer, and 1.25 units of *Taq* polymerase (Perkin-Elmer). Amplification was carried out under the same conditions except for the annealing temperature (47°C), and only 30 cycles were performed. The nested amplification product was analyzed by electrophoresis through 2% agarose in a Tris-borate-EDTA gel stained with ethidium bromide (0.5 μg/ml).

This in-house PCR method has been shown to be highly sensitive (5), capable of detection of between 0.2 and 0.02 $TCID_{50}$/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 falling 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.

TABLE 1. Results of cell culture and RT-PCR tests for 156 CSF samples from immunocompetent patients included in the prospective study

Neurologic syndrome	No. of CSF specimens	No. negative by all tests	No. positive by[a]:			
			Cell culture and both RT-PCR methods	Both RT-PCR methods	AMPLICOR RT-PCR	In-house RT-PCR
Outbreak-associated AM	50	4	13	30	0[b]	3[b]
Sporadic AM	29	25	1	2	0[b]	1[b]
Other syndromes	77	77	0	0	0	0

[a] All specimens which yielded positive results by culture were successfully amplified by both RT-PCR methods.
[b] Result(s) confirmed by testing of a new aliquot of CSF by both RT-PCR methods.

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.

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 in all tested specimens.

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 (four specimens), 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.

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.).

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 neurological 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 neurological 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 2. Results of cell culture isolation and RT-PCR methods for CSF specimens from the retrospective study

AMPLICOR test result	Cell culture isolation			
	No. of positive specimens (n = 22) with in-house RT-PCR result:		No. of negative specimens (n = 75) with in-house RT-PCR result:	
	+	−	+	−
+	10	3	14	4
−	6	3	9	48

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

CSF specimen no.	AMPLICOR result(s) (OD_{450})[a]			In-house RT-PCR result		Cell culture result
	1st assay	2nd assay	RMS[b]	1st assay	2nd assay	
1	0.137, 0.414	0.198, 0.917	0.726	−	+	−
2	0.175, 0.798	0.213, 0.987	2.824	+	+	−
3	0.161, 0.332	0.158, 0.580	0.105	+	−	Echo-9
4	0.133, 0.135	0.155, 0.346	0.950	−	−	Echo-9
5	0.131, 0.544	0.151, 0.484	0.061	+	+	−
6	0.129, 0.518	0.159, 0.330	1.234	+	+	−
7	0.110, 0.127	0.184, 0.604	0.053	+	+	Echo-30
8	0.187, 0.521	0.156, 0.411	3.008	+	+	−

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

[b] Results obtained by Roche Molecular Systems, Somerville, N.J.

and 46 of 50 (92%) were positive for enteroviral RNA by the AMPLICOR test and the in-house RT-PCR assay, respectively. All of the culture-positive specimens were successfully amplified by both PCR methods, so no false-negative results were obtained. False-positive results with the AMPLICOR test were ruled out because all positive specimens were also amplified, and therefore confirmed, with the alternative RT-PCR assay, which amplifies a nonoverlapping region of the enteroviral genome. These data show that the AMPLICOR test is more sensitive by far than cell culture of CSF for diagnosis of enteroviral meningitis ($P = 0.22$ [McNemar's test, Yates corrected]).

Three additional specimens that were positive by the in-house assay could not be amplified by the AMPLICOR test. These three samples were each assayed again with a new frozen aliquot. Although the initial volume of CSF used in the AMPLICOR test is double the volume used in the in-house assay, the amount of target RNA put into the amplification reaction mixture is the same for both PCR methods.

Possible reasons for the low rate of enteroviral detection by cell culture (13 of 50 specimens, 26%) compared with results obtained by RT-PCR methods could be either a low number of infectious particles in CSF specimens or numerous replication-defective or antibody-neutralized viruses which cannot be propagated in cell culture (13). Nevertheless, it is possible that previous dilution of some specimens decreased the sensitivity of cell culture. If we considered only the specimens from outbreak-associated AM cases that were tested undiluted (20 of 50), the percentage of positive specimens in cell culture would rise to 45% (9 of 20), which is still far below the 90% (18 of 20) positive specimens with the AMPLICOR test ($P = 0.55$ [McNemar's test, Yates corrected]) or 95% (19 of 20) with the in-house assay. Prior studies comparing cell culture and PCR assays for enteroviral detection in prospectively collected CSF samples from patients with AM have demonstrated that PCR is more sensitive than viral cultures. The rates of enterovirus detection by Yerly et al. for 38 patients whose specimens were collected from June to September 1994 (24) were 34% by culture and 66% by the AMPLICOR test. Thorén and Widell (23) had rates of 22% by culture and 55% by PCR in a series of 27 patients with AM, with all except two patients enrolled from July to November 1994. Lina et al. (10) reported rates of 30 and 56% by cell culture and PCR, respectively, in a multicenter evaluation of the AMPLICOR test with a panel of CSF specimens which had been artificially infected with different loads of enteroviruses (10, 1, or 0.1 $TCID_{50}$/0.1 ml).

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 samples (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

F. Pozo is a predoctoral fellow funded by the Fondo de Investigación Sanitaria, Beca de Ampliación de Estudios (BAE 96/5231 and BAE 97/5098). I. Casas is a postdoctoral fellow funded by the Instituto de Salud Carlos III, Becas de Perfeccionamiento (97/4165). This work was partially supported by Fondo de Investigación Sanitaria grant FIS 96/0304 and by Spanish Ministry of Health grant MVP-613/95.

We thank M. Pedrocchi, Roche Diagnostics, Basel, Switzerland, for providing the AMPLICOR EV kits and A. Butcher, Roche Molecular Systems, Somerville, N.J., for repeat testing.

REFERENCES

1. **Bell, E. J., R. A. McCartney, D. Basquill, and A. K. Chaudhuri.** 1986. Muantibody capture ELISA for the rapid diagnosis of enterovirus infections in patients with aseptic meningitis. J. Med. Virol. **19:**213–217.
2. **Berlin, L. E., M. L. Rorabaugh, F. Heldrich, K. Roberts, T. Doran, and J. F. Modlin.** 1993. Aseptic meningitis in infants <2 years of age: diagnosis and etiology. J. Infect. Dis. **168:**888–892.
3. **Casas, I., P. E. Klapper, G. M. Cleator, J. E. Echevarría, A. Tenorio, and J. M. Echevarría.** 1995. Two different PCR assays to detect enteroviral RNA in CSF samples from patients with acute aseptic meningitis. J. Med. Virol. **47:**378–385.
4. **Casas, I., L. Powell, P. E. Klapper, and G. M. Cleator.** 1995. New methods for the extraction of viral RNA and DNA from cerebrospinal fluid for use in the polymerase chain reaction assay. J. Virol. Methods **53:**25–36.
5. **Casas, I., A. Tenorio, J. M. Echevarría, P. E. Klapper, and G. M. Cleator.** 1997. Detection of enteroviral RNA and specific DNA of herpesviruses by multiplex genome amplification. J. Virol. Methods **66:**39–50.
6. **Chonmaitree, T., C. D. Baldwin, and H. L. Lucia.** 1989. Role of the virology laboratory in diagnosis and management of patients with central nervous system disease. Clin. Microbiol. Rev. **2:**1–14.
7. **Chonmaitree, T., C. Ford, C. Sanders, and H. L. Lucia.** 1988. Comparison of cell cultures for rapid isolation of enterovirus. J. Clin. Microbiol. **26:**2576–2580.
8. **Glimaker, M., B. Johansson, P. Olcén, A. Ehrnst, and M. Forsgren.** 1993. Detection of enteroviral RNA by polymerase chain reaction in cerebrospinal fluid from patients with aseptic meningitis. Scand. J. Infect. Dis. **25:**547–557.
9. **Kao, S. Y., T. M. Niemiec, M. J. Loeffelholz, B. Dale, and H. A. Rotbart.** 1995. Direct and uninterrupted RNA amplification of enteroviruses with colorimetric microwell detection. Clin. Diagn. Virol. **3:**247–257.
10. **Lina, B., B. Pozzetto, L. Andreoletti, E. Beguier, T. Bourlet, E. Dussaix, L. Grangeot-Keros, B. Gratacap-Cavallier, C. Henquell, M. C. Legrand-Quillien, A. Novillo, P. Palmer, J. Petitjean, K. Sandres, P. Dubreuil, H. Fleury, F. Freymuth, I. Leparc-Goffart, D. Hober, J. Izopet, H. Kopecka, Y. Lazizi, H. Lafeuille, P. Lebon, A. Roseto, E. Marchadier, B. Masquelier, B. Picard, J. Puel, J. M. Seigneurin, P. Wattre, and M. Aymard.** 1996. Multicenter evaluation of a commercially available PCR assay for diagnosing enterovirus infection in a panel of cerebrospinal fluid specimens. J. Clin. Microbiol. **34:**3002–3006.
11. **Longo, M. C., M. S. Berninger, and J. L. Hartley.** 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene **93:**125–128.
12. **Melnick, J. L.** 1990. Enteroviruses: polioviruses, coxsackieviruses, echoviruses and new enteroviruses, p. 549–605. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman, and R. E. Shope (ed.), Virology, 2nd ed. Raven Press, New York, N.Y.
13. **Muir, P., F. Nicholson, M. Jhetam, S. Neogi, and J. E. Banatvala.** 1993. Rapid diagnosis of enterovirus infection by magnetic bead extraction and polymerase chain reaction of enterovirus RNA in clinical specimens. J. Clin. Microbiol. **31:**31–38.
14. **Nicholson, F., G. Meetoo, S. Aiyar, J. E. Banatlava, and P. Muir.** 1994. Detection of enterovirus RNA in clinical samples by nested polymerase chain reaction for rapid diagnosis of enterovirus infection. J. Virol. Methods **48:**155–166.
15. **Olive, D. M., S. Almufti, W. Almulla, M. A. Khan, A. Pasca, and W. Alnakib.** 1990. Detection and differentiation of picornaviruses in clinical samples following genomic amplification. J. Gen. Virol. **71:**2141–2147.
16. **Rotbart, H. A., M. H. Sawyer, S. Fast, C. Lewinski, N. Murphy, E. F. Keyser, J. Spadoro, S.-Y. Kao, and M. Loeffelholz.** 1994. Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay. J. Clin. Microbiol. **32:**2590–2592.
17. **Rotbart, H. A.** 1990. Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol. **28:**438–442.
18. **Rotbart, H. A., J. P. Kinsella, and R. L. Wasserman.** 1990. Persistent enterovirus infection in culture-negative meningoencephalitis: demonstration by enzymatic RNA amplification. J. Infect. Dis. **161:**787–791.
19. **Rudge, P., A. D. Webster, T. Revesz, T. Warner, T. Espanol, C. Cunningham, and N. Hyman.** 1996. Encephalomyelitis in primary hypogammaglobulinaemia. Brain **119:**1–15.
20. **Samuelson, A., E. Skoog, and M. Forsgren.** 1990. Aspects on serodiagnosis of enterovirus infections by ELISA. Serodiagn. Immunother. Infect. Dis. **4:**395–406.
21. **Sawyer, M. H., D. Holland, N. Aintablian, J. D. Connor, E. F. Keyser, and N. J. Waecker, Jr.** 1994. Diagnosis of enteroviral central nervous system infection by polymerase chain reaction during a large community outbreak. J. Pediatr. Infect. Dis. **13:**177–182.
22. **Severini, G. M., L. Mestroiani, A. Falashi, F. Camerini, and M. Giacca.** 1993. Nested polymerase chain reaction for high-sensitivity detection of enteroviral RNA in biological samples. J. Clin. Microbiol. **31:**1345–1349.
23. **Thorén, A., and A. Widell.** 1994. PCR for the diagnosis of enteroviral meningitis. Scand. J. Infect. Dis. **26:**249–254.
24. **Yerly, S., A. Gervaix, V. Simonet, M. Caflisch, L. Perrin, and W. Wunderli.** 1996. Rapid and sensitive detection of enteroviruses in specimens from patients with aseptic meningitis. J. Clin. Microbiol. **34:**199–201.