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Rapid and Sensitive Detection of Enteroviruses in Specimens from Patients with Aseptic Meningitis

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A 5-h PCR assay (Amplicor enterovirus test) was compared with viral culture for the detection of enteroviruses in cerebrospinal fluid. Of the cerebrospinal fluid specimens collected during a summer outbreak of aseptic meningitis, 34% were positive by viral culture whereas 66% were positive by the Amplicor PCR, suggesting that this technique improves the diagnosis of enteroviral meningitis.

Nonpolio enteroviruses, including echovirus and coxsackie A and B viruses, are the common etiologic agents of aseptic meningitis (16, 18). During summer outbreaks of meningitis, children are frequently infected with enteroviruses, and in this age group, clinical features can overlap those of bacterial origin (1, 15, 17). Laboratory diagnosis of enteroviral infection relies on virus isolation in cell cultures. However, this technique has several limitations, which include a period of days to weeks to obtain results and the limited sensitivities of cell cultures to certain enteroviruses (13). The rapid availability of results may improve a patient's care (3, 12). The detection of enteroviral RNA in clinical samples by PCR has recently been developed (4, 7, 8, 11). By taking advantage of the conserved sequences in the 5′ end of the enteroviral genome, single PCR assays for the detection of the majority of enteroviruses which infect humans have been developed (5, 9, 10). However, the use of in-house PCR assays for the routine diagnosis of enteroviral infections is limited by time-consuming procedures and by the lack of standardization. In this study, we have compared the ability to detect enteroviruses in cerebrospinal fluid (CSF) specimens of a commercially available PCR assay with that of the cell culture method using specimens of patients with suspected aseptic meningitis.

Thirty-eight children with a meningitis syndrome were monitored prospectively during a summer outbreak of aseptic meningitis (June to September 1994) at the Department of Pediatrics, Geneva University Hospital (14). Lumbar punctures revealed a pleiocytosis ($\geq$6 cells per mm^3) and negative Gram stain. Cultures of CSF were negative for bacteria and fungi. All of these children were considered to have an aseptic meningitis. After testing CSF on viral cultures, aliquots of CSF were stored at −75°C for later use in detecting enteroviral RNA by PCR. PCR assays were performed by a technician unaware of culture results. All discrepant results caused us to repeat in duplicate the entire Amplicor PCR procedure with new CSF aliquots. For enterovirus isolation, 200 μl of CSF was inoculated on human fibroblasts, A549 cells, and Vero cells under standard conditions. Cultures were regularly observed for cytopathic effects up to 14 days after inoculation. Typing positive culture was performed by neutralization with specific antibodies (6). PCR was performed with the Amplicor enterovirus test (prototype; Roche Molecular Systems, Basel, Switzerland) according to the manufacturer's instructions. Specimens with an optical density at 450 nm greater than or equal to 0.35 were considered positive. One positive and three negative controls were included in each run.

All CSF specimens were confirmed by an in-house PCR protocol. Briefly, RNA from 100 μl of CSF was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (2). Total RNA was reverse transcribed in a volume of 20 μl containing 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM each deoxynucleoside triphosphate, 25 U of RNase inhibitor (Boehringer, Mannheim, Germany), 100 pmol of dN6 random primers (Boehringer) and 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, Md.). Samples were incubated for 10 min at room temperature and for 1 h at 42°C. After the addition of 0.1 μg of proteinase K per ml, samples were incubated for 30 min at 55°C and then for 15 min at 95°C. Total cDNA was amplified in 100 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 10% glycerol, 0.25 μg each of primer EV1 (10) and primer Hyn2n (5′ CAAGCACTTCTGTTTCCCC, modified from reference 5), and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The reaction mixture was cycled once at 94°C for 70 s, at 50°C for 10 s, and at 72°C for 20 s and then cycled again four times under the same conditions. Next, the reaction mixture was cycled 30 times at 94°C for 10 s, 55°C for 10 s, and 72°C for 20 s with a 9600 DNA thermal cycler (Perkin-Elmer). The PCR products were analyzed by gel electrophoresis in a 2% Nu-Sieve agarose gel (FMC Bioproducts, Rockland, Maine) stained with ethidium bromide and checked for the presence of a 438-bp product. To confirm the specificities of the PCR products, Southern blot analysis was done. Briefly, the agarose gel was denatured for 30 min in a 0.5 M NaOH–1 M NaCl solution and then neutralized for 30 min in a 0.5 M Tris-HCl (pH 7.5)–1.5 M NaCl solution. The PCR products were transferred to a positively charged nylon membrane (GeneScreen Plus; NEN, Boston, Mass.) by overnight blotting in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membrane was backed at 80°C for 1 h and incubated at 50°C for 1 h in prehybridization solution (4× SSC, 5× Denhardt's solution, 5% sodium dodecyl sulfate [SDS], 20 mM sodium phosphate [pH 7], 100 μg of sheared and denatured salmon sperm DNA per ml). Then, the ^{32}P-labeled enterovirus-specific probe (EV2 from reference 10) was added, and after overnight hybridization at 50°C, the membrane was

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TABLE 1. Comparison of enterovirus detection in CSF specimens by viral culture and PCR

Cell culture result	No. of specimens with Amplicor PCR result	
	Positive	Negative
Positive	12	1[a]
Negative	13	12

[a] Of the 13 culture-positive specimens, 1 specimen was negative by Amplicor PCR but positive by the culture assay (this specimen, however, was positive by repeated [twice] Amplicor PCR).

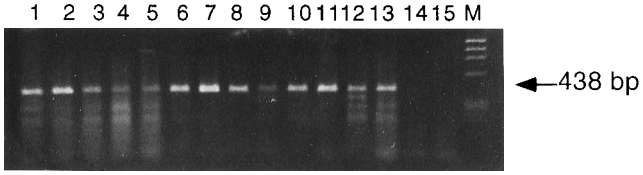

FIG. 1. Visualization of enteroviral RNA by gel electrophoresis after the alternative PCR protocol. Lanes 1 to 13, Amplicor PCR-positive and culture-negative CSF samples. Lanes 14 to 15, negative controls. M, molecular weight standard (ϕX174 digested with *Hae*III).

washed for 15 min at 50°C, once with 3× SSC–0.1% SDS, once with 0.5× SSC–0.1% SDS, and once with 0.1× SSC–0.1% SDS, and exposed to X-Omat film (Kodak, Lausanne, Switzerland).

Precautions to prevent carryover contamination were the following. The samples were processed and reagents were prepared in a room different from that used for PCR amplification and a third room was used for analysis of PCR products. Dedicated reagents, disposable sterile tubes, and filtered pipette tips were used. Negative and positive controls were included in each experiment and processed with patient specimens.

Thirty-six of 38 patients presented with the complaint of a stiff neck, 35 presented with fever, and 33 complained of a headache. Vomiting and photophobia occurred in 33 and 14 patients, respectively. The ages of these children were between 2 months and 15 years (mean, 6 years). CSF pleiocytosis was present in all patients with a total nucleated cell count ranging from 6 to 1,803 cells per mm^3 (mean, 297). A predominance of lymphomonocytic cells was observed for 17 patients. The protein concentration was higher than normal (>0.4 g/liter) in 11 patients, and glucose concentration was within normal limits in CSF specimens.

Thirteen (34%) of 38 CSF specimens were positive for enteroviruses in cell cultures after 2 to 6 days. The numbers of cultures with the following types of identified viruses were as follows: four echovirus 20, four echovirus 30, one echovirus 14, one coxsackie B2 virus, one coxsackie B3 virus, one coxsackie B5 virus, and one poliovirus 2. Enteroviral RNA was detected by the Amplicor PCR assay in 25 (66%) of the 38 CSF specimens. The Amplicor PCR yielded positive results in all cases except one, in which an enterovirus was recovered by culture (Table 1). The discrepant sample was positive both times after repeating the whole Amplicor PCR procedure twice with new aliquots of CSF, indicating a false-negative result for the initial assay. Twenty-two of 25 positive samples had an optical density of >3.0. Lower optical densities were observed with three samples, but these values were at least twice above the cutoff value. The Amplicor PCR was positive with 13 of 25 culture-negative CSF specimens. All of these samples were positive upon repeating the whole Amplicor PCR procedure with new aliquots.

The in-house PCR assay was performed on all CSF specimens. Products of the expected size (438 bp) were observed by ethidium bromide staining with all Amplicor PCR-positive CSF specimens (Fig. 1). The in-house PCR gave positive results for 3 of the 12 Amplicor PCR-negative samples by Southern blot analysis.

The percentage of Amplicor PCR-positive samples was independent of the delay between the onset of symptoms and CSF collection. In contrast, samples collected more than 24 h after the onset of symptoms were all culture negative (Fig. 2).

This study shows the Amplicor PCR assay was more sensitive than viral cultures for the diagnosis of enteroviral meningitis in children. All Amplicor PCR-positive and culture-negative CSF specimens were confirmed by repeating the Amplicor PCR twice, and these specimens were also determined to be positive by the in-house PCR protocol. Thus, the possibility of false-positive PCR results is remote. The more laborious in-house PCR assay detected enteroviral RNA in three additional samples. The higher sensitivity of the in-house assay might be related to the different method used for RNA purification or to the selection of alternative primers. Around 50% of the patients with negative viral cultures had enteroviral RNA detected in their CSF. The lower sensitivity of culture might be explained by the known low recovery rate of certain viral strains in culture systems, by a rapid loss of infectivity, and by the presence of neutralizing antibodies. We also observed that the interval between the onset of symptoms and CSF collection might play a role in the recovery of virus by culture but did not seem to affect the sensitivity of the PCR. Other PCR assays have shown sensitivities higher than those of viral cultures (7, 10, 11). However, the routine use of these assays is limited by their time-consuming procedures and by their limited availability. This commercial PCR assay has several advantages, including the incorporation of uracil *N*-glycosylase for carryover prevention, the one-step reverse transcription and PCR, a simple, user-friendly microwell colorimetric system, and the rapidity of the procedure (10).

These data suggest that PCR could be routinely performed to evaluate specimens from patients with meningitis syndrome whose initial CSF examination does not allow a differentiation between bacterial and viral meningitis. A quick diagnosis re-

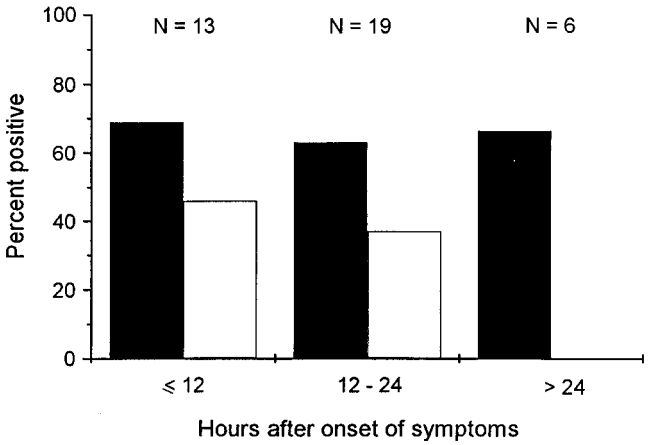

FIG. 2. Percentages of culture-positive and PCR-positive CSF specimens according to the time between the onset of symptoms and CSF collection. Filled bars, PCR; open bars, culture.

assures patients and doctors, since the clinical course of enteroviral meningitis is usually benign, in contrast to herpes meningoencephalitis or bacterial meningitis. In most cases, the rapid detection of enteroviruses by PCR would improve the management of patients and reduce cost, since many children with suspected aseptic meningitis are hospitalized and treated with antibiotics until bacterial infection can be excluded.

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