JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1994, p. 2590–2592
0095-1137/94/$04.00+0

Vol. 32, No. 10

Diagnosis of Enteroviral Meningitis by Using PCR with a Colorimetric Microwell Detection Assay†

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Received 5 May 1994/Returned for modification 6 July 1994/Accepted 25 July 1994

A 5-h, user-friendly PCR assay for the diagnosis of enteroviral meningitis was developed. Reverse transcription and amplification were performed in a one-step reaction using rTth polymerase. Carryover contamination was prevented with dUTP and uracil *N*-glycosylase. Detection was performed colorimetrically on a microwell titer plate. Sensitivity, specificity, positive predictive value, and negative predictive value were 94.7, 97.4, 94.7, and 97.4%, respectively.

The enteroviruses (EVs) comprise 68 distinct serotypes of human pathogens, and are collectively responsible for 10 to 15 million symptomatic infections in the United States each year (10). EVs cause ≥85% of the cases of aseptic meningitis (1, 9) and may be difficult to distinguish from meningitis due to bacteria and herpes simplex virus, particularly in young infants and children. This results in unnecessary hospitalization and treatment, with antibiotics and/or antiherpesvirus medications, of thousands of children each year. Cell cultures of cerebrospinal fluid (CSF) for EVs take an average of 6 or 7 days for identification of growth (2), are typically observed for 2 or 3 weeks before being reported as negative, and may be only 65 to 75% sensitive. Even with those drawbacks, cell culture has a significant impact on the management of patients with EV meningitis (2), and a more rapid and more sensitive technique would be expected to produce an even greater impact.

Using separate reverse transcription (RT) and PCR steps and radioactive or chemiluminescent detection systems, several investigators have reported successful detection of EVs in CSF (6, 8, 9). While these assays have been more rapid and more sensitive than cell culture, they require opening the reaction tubes and adding new reagents between the RT and PCR steps, increasing the risk of carryover contamination. Furthermore, only one of the earlier studies (9) incorporated uracil *N*-glycosylase to prevent carryover contamination, a recent improvement in PCR quality control (5). Finally, studies to date have used cumbersome, noncolorimetric detection schemes. This report describes the application of a new PCR assay that utilizes a single enzyme for both RT and PCR, incorporates uracil *N*-glycosylase, and detects the amplified product in a simple, microwell colorimetric assay.

Twenty-seven EV serotypes (coxsackieviruses A3, A7, A9, A16, and B1 through B6; echoviruses 2 through 7, 9, 11, 14, 16, 18, 21, 24, 30, and 31; and EVs 70 and 71) were obtained from the American Type Culture Collection (Gaithersburg, Md.); these include the most commonly reported serotypes from patients with meningitis (10). Archival CSF specimens, some of which had been stored for as long as 9 years at −70°C, were collected from two cities, with some of the samples having been stored for several months at −20 prior to −70°C storage. The clinical histories of all patients were obtained by chart review.

CSF specimens were cultured for viruses by routine methods (3), with at least 1 ml of CSF inoculated onto three cell lines (≥0.33 ml per cell line) which were observed for cytopathic effect characteristic of the EVs (3); negative cultures were held for at least 2 weeks and, in some cases, frozen, thawed, blindly passed, and observed for an additional 2 weeks.

The PCR primers used were downstream primer EV1b (5′-biotinyl-CAATTGTCACCATAAGCAGCCA) and upstream primer EV2b (5′-biotinyl-GGCCCCTGAATGCGGCT AAT), and the PCR probe used was EV3 (5′-GAAACACGG ACACCCAAAGTA), all three of whose sequences were identical to or nearly identical to previously reported EV PCR sequences (7). The procedure and reagents were similar to those previously reported for one-step RT-PCR (12). One hundred microliters of sample (either clinical CSF specimens or EV serotypes suspended in culture medium) was mixed with 400 μl of lysis solution containing guanidine thiocyanate, glycogen, and dithiothreitol in Tris buffer, with incubation at room temperature for 10 min. To that mixture, 500 μl of isopropanol was added prior to centrifugation at 16,000 × *g* in a tabletop centrifuge at room temperature for 10 min. The pellet was washed with 750 μl of 70% ethanol and resuspended in 200 μl of diluent solution containing manganese acetate and potassium acetate in a bicine buffer. Fifty microliters of the resuspended specimen was added to an equal volume of master mix containing uracil *N*-glycosylase (AmpErase, Roche Molecular Systems, Branchburg, N.J.), biotinylated primers, deoxynucleotide triphosphates (with dUTP in place of dTTP), and rTth enzyme (Roche Molecular Systems) in a bicine buffer. With each PCR run, one negative control tube (containing buffer only) and one positive control tube (containing poliovirus RNA) were processed identically with tubes containing samples, as described above. Amplification was performed in a thermal cycler (Perkin-Elmer PCR System 9600) on a MicroAmp base (Perkin-Elmer). Initial settings at 50°C for 2 min, to allow uracil *N*-glycosylase inactivation of any carryover product, were followed by 60°C for 30 min of RT and then 35 cycles of denaturation (94°C; 1st cycle for 70 s and the remaining 34 cycles for 10 s), annealing (58°C; 10 s), and

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† Presented in part at the 93rd General Meeting of the American Society for Microbiology, Las Vegas, Nevada, May 1994.

TABLE 1. Results of PCR testing of CSF for EVs

PCR result and testing sequence	No. of specimens[a]	
	Cult +	Cult −
EV PCR +		
Initial	33	5
Final	34	3
EV PCR −		
Initial	2	74
Final	1	76

[a] n = 114 paired specimens. Cult +, culture positive; Cult −, culture negative.

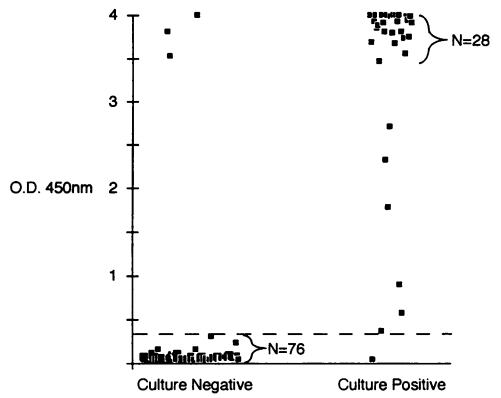

FIG. 1. Scattergram depiction of OD readings at 450 nm for the 114 CSF specimens reported in this study. The dashed line represents an OD of 0.35, below which the assay is interpreted as negative; OD readings ≥0.35 are positive in this PCR assay. Values for virus culture-negative and culture-positive samples are shown.

extension (72°C; 10 s). The tubes were not opened at any time following the initial distribution of the master mix. Triplicate amplifications of the extracted negative control, and a single amplification of the extracted positive control were performed with each PCR run. Following amplification, 100 μl of an NaOH denaturing solution was added to each tube and incubated for 10 min at room temperature.

Detection of amplification products was performed as previously reported for *Chlamydia trachomatis* by the Amplicor (Roche Molecular Systems) kit format (4) except that the microwell plates contained immobilized oligonucleotide probe EV3. Hybridization, incubation with conjugate, and addition of substrate were all done as reported previously (4). The optical density (OD) of the wells was read on an Emax plate reader (Molecular Devices Corporation, Menlo Park, Calif.) at 450 nm, and results were scored as positive if the OD was ≥0.35. When PCR results differed from culture results, another aliquot of the original CSF specimen was retested, with the identical extraction, amplification, and detection techniques used, as described above.

The results of inclusivity testing revealed successful detection of all 27 commonly isolated EV serotypes tested at a sensitivity of ≤1 50% tissue culture infective dose (except coxsackievirus A7, which was detected at 1 50% lethal dose) (data available on request). The test results for clinical CSF specimens are summarized in Table 1. Initial testing found sensitivity, specificity, positive predictive value, and negative predictive value of PCR versus culture to be 94.3, 93.4, 86.8, and 97.4%, respectively. Three of the five culture-negative specimens which were initially identified as positive by PCR were obtained from patients with summer aseptic meningitis for whom other etiologic diagnoses were not found. Hence, the sensitivity, specificity, positive predictive value, and negative predictive value of PCR versus combined culture and clinical diagnosis were 94.7, 97.4, 94.7, and 97.4%, respectively, in initial testing. Two culture-negative specimens from patients without meningitis were initially PCR positive but were PCR negative on final testing; one culture-positive specimen was initially PCR negative but was PCR positive on repeat testing (Table 1). The final sensitivity, specificity, positive predictive value, and negative predictive value of PCR versus culture plus clinical diagnosis were 97.4, 100, 100, and 98.7%, respectively. The actual OD values for clinical samples are shown in Fig. 1. A clean separation between positive and negative OD readings is evident.

PCR has been shown to be a rapid and sensitive alternative to cell culture for the diagnosis of EV infections of the central nervous system (6, 8, 9). The PCR assay described in the current report has several important advantages over other EV PCR assays; the incorporation of uracil *N*-glycosylase carryover prevention, the combination of RT and PCR performed by a single enzyme in a single tube, and a simple, user-friendly microwell colorimetric detection system. The sensitivity and specificity of this assay were comparable to those of previously reported EV PCR systems for aliquots of the same CSF specimens studied for this report (8, 9) (data not shown). Three initially discrepant specimens resolved (i.e., correlated with culture results) on repeat PCR testing, indicating a false result on the initial assay. Three such results in a study of 114 samples is a low rate for false positives of false negatives; in unpublished data we have accumulated on other (non-CSF) types of specimens tested with this assay, discordance between duplicate aliquots of the same specimen run by PCR occurred in only 5 of 194 samples. (In each of those five, one aliquot gave a positive PCR result and the other gave a negative result [data available on request].) Duplicate testing of specimens in a clinical setting is a potential way of reducing false results which are due to the technique itself, but duplicate testing will likely be unnecessary with the discordance rate so low.

Inclusivity testing with the PCR assay and colorimetric microwell detection described in this report detected all 27 EV serotypes tested at a sensitivity adequate to detect even cases of EV meningitis with very low CSF viral titers (11). This is consistent with the broad reactivity reported for EVs with the use of primers and a probe which are nearly identical to those in this study (8, 13). Extensive exclusivity testing has also been previously performed, with no cross-reactivity to other (non-EV) viral or bacterial causes of central nervous system disease found (8, 13) (data not shown).

Current management of children and, less commonly, adults with EV meningitis often includes hospitalization and treatment with intravenous antibiotics and, in some cases, acyclovir, pending the outcome of bacterial and viral cultures. The delay in detecting EVs by CSF culture results in clinical decision making based on the more-quickly-available results of bacterial and/or herpes simplex virus cultures. Even so, results of CSF EV cultures reduce the length of hospitalization and unnecessary therapies (3). This EV PCR assay should have a dramatic impact on both the quality and cost of caring for patients with aseptic meningitis.

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