JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1993, p. 395–399
0095-1137/93/020395-05$02.00/0

Vol. 31, No. 2

Rapid Detection of Poliovirus by Reverse Transcription and Polymerase Chain Amplification: Application for Differentiation between Poliovirus and Nonpoliovirus Enteroviruses

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Received 17 June 1992/Accepted 19 November 1992

This report describes a rapid method of detection of poliovirus from viral isolates of clinical specimens using a single set of primers selected from the conserved 5′ noncoding region of the poliovirus genome. Of the 144 clinical viral isolates examined, 81 were positive for polioviruses and 50 were positive for nonpoliovirus enteroviruses by tissue culture neutralization and infectivity. All 81 (100%) of the viral isolates identified as poliovirus by tissue culture infectivity were also positive by polymerase chain reaction. Of 50 nonpoliovirus enterovirus isolates found to be negative for poliovirus by tissue culture neutralization and infectivity, 48 were also negative by polymerase chain reaction. The high sensitivity (100%) and specificity (96%) of the primer set indicate that this assay has potential clinical applicability in the diagnosis of nonpoliovirus enterovirus infection.

The enteroviruses are among the most common and important viral pathogens of humans. These include polioviruses, coxsackieviruses, echoviruses, and the more recently numbered enteroviruses (types 68 through 72) (10). Polioviruses became less clinically significant in the United States and other developed countries with the effective introduction of poliovirus vaccines in the late 1950s. In developing countries, however, 4 of every 1,000 children born annually have paralytic disease caused by polioviruses (1).

In the United States, nonpoliovirus enteroviruses have gained increased attention because they cause a wide variety of infections, particularly in children. The spectrum of disease ranges from benign febrile illness to meningitis, myocarditis, and neonatal sepsis (14). Recovery of an enterovirus from clinical specimens still relies largely on cell culture techniques (2, 17). In general, the cytopathic effect (CPE) produced by enteroviruses in standard cell cultures is quite distinctive and can be recognized fairly early with accuracy by experienced technicians (2). However, the CPE caused by a vaccine poliovirus or a nonpoliovirus enterovirus pathogen cannot be distinguished. Enterovirus typing by neutralization methods using intersecting virus antiserum pools (8) is tedious and expensive and is therefore not available in most diagnostic virology laboratories. Differentiation between poliovirus and nonpoliovirus enteroviruses by limited neutralization using three types of poliovirus antisera, a somewhat simpler procedure, is still time consuming. A rapid method to differentiate vaccine strains of polioviruses from nonpoliovirus enteroviruses immediately after the appearance of the enterovirus CPE could aid the clinician in patient management, improve the accuracy of diagnosis, and help in infection control.

The polymerase chain reaction (PCR), a rapid method for the sensitive detection of specific nucleic acid sequences, has recently been used for rapid detection of viruses from clinical specimens (13). Numerous attempts to apply the PCR assay to the rapid detection of nonpoliovirus enteroviruses in clinical specimens have recently been made (5, 13). However, there have been no attempts to differentiate poliovirus from nonpoliovirus enteroviruses by the PCR technique.

In this study, we selected two specific oligonucleotide primers from the conserved 5′ noncoding region of polioviruses to investigate the potential use of PCR for the rapid differentiation of polioviruses from nonpoliovirus enteroviruses. We demonstrate here the utility of these oligonucleotide primers in specifically detecting polioviruses in 144 rand

MATERIALS AND METHODS

Specimen collection and virus isolation. The collection and processing of samples were carried out as described earlier (2). Briefly, throat and rectal specimens were collected with swabs and placed in 2 ml of viral transport medium (veal infusion broth with 0.1% gelatin and antibiotics). Nasal wash specimens were obtained by the methods described by Hall and Douglas (3). Cerebrospinal fluid and stool specimens were collected in sterile containers and transported to the laboratory without placement in viral transport medium. Respiratory tract and fecal specimens were vigorously vortexed and clarified by centrifugation or filtration in 0.45-μm-pore-size disposable filters before cell culture inoculation. Cerebrospinal fluid was inoculated directly into cell cultures. Tube cultures of primary cynomolgus or rhesus monkey kidney cells and human fibroblasts (MRC-5) were obtained from commercial sources. Buffalo green monkey (BGM) kidney cells were prepared in our laboratory. Cell cultures were inoculated with 0.2 ml of specimen, or the inoculum

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was divided equally among tubes if smaller quantities were available. Enteroviral cultures were incubated at 36°C in the stationary position and observed for CPE daily for 10 days. When maximum CPE was observed in standard cell culture tubes, supernatant fluid (2 ml) was collected and saved as the viral isolate. Final identification of the other viruses was performed by standard methods in our laboratory (7). Typing of enteroviruses by neutralization was kindly provided by the Medical Virology Branch, Bureau of Laboratories, Texas Department of Health, Austin.

All viral isolates were kept frozen at −70°C until tested by PCR using a poliovirus-specific primer set. The 144 viral isolates tested were obtained from clinical specimens of feces (n = 63), nasal wash (n = 10), rectal swab (n = 24), cerebrospinal fluid (n = 17), throat swab (n = 22), throat and nasal wash (n = 6), and lung biopsy tissue (n = 2). The results were then compared to determine whether the oligonucleotide probes were specific in distinguishing polioviruses from nonpoliovirus enteroviruses and other viral isolates.

Viral RNA extraction. RNA was extracted from 400 μl of the viral isolate by using 1% sodium dodecyl sulfate in 1× proteinase K buffer (0.01 M Tris [pH 7.8] and 0.005 M EDTA). Proteinase K (10 mg/ml; Sigma) was then added (final concentration, 100 μg/ml), and the suspension was incubated for 1 h at 65°C. Nucleic acids were purified by successive extraction with phenol, phenol-chloroform-isoamyl alcohol (24:24:1), and chloroform-isoamyl alcohol (24:1) followed by ethanol precipitation (9). Purified nucleic acids were collected in 10 μl of distilled water, and the RNA quantity was determined by measuring optical density at 260 nm. The RNA concentrations in samples quantified by measuring optical density at 260 nm ranged from 300 to 500 ng/μl.

Primers. Primer sequences were selected from the highly conserved parts of the 5′ noncoding regions, nucleotides 513 through 533 (GG ACT TGC GCG TTA CGA CAG G-3′) and 297 through 317 (CA ACC CCG GAG TGT AGC TTG G-3′), respectively, of poliovirus RNA. The two primers had short 5′ extensions (CCGGTGC [primer 1] and CGATTTC [primer 2]) to incorporate cohesive termini (18) for cloning of PCR products. The primers were synthesized on a DNA synthesizer (Milligen BioSearch 8600) by the methoxyphosphoramidite method. The primers consisted of a 7-base adaptor plus 21 bases with homology ranging from 90 to 100% for the three serotypes of Sabin poliovirus RNA sequence (20). These two primers produced a 248-bp PCR product. Specificities of primer sequences were checked by using GenBank. No homology with other nonpoliovirus enteroviruses for which sequences were available was noted.

Reverse transcription and PCR. The cDNA was first synthesized and then amplified as described previously (6). Briefly, a 20-μl reaction mixture containing the following was prepared: 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM dithiothreitol, 1.0 mM each deoxynucleoside triphosphate (Pharmacia), PCR primer 1 (20 pmol), 30 U of ribonuclease inhibitor (RNasin; Promega), 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and 1 μl (~300 to 500 ng) of RNA isolated from the viral isolate. After incubation at 37°C for 45 min, the PCR mixture was added. The 100-μl PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.9), 1.5 mM $MgCl_2$, PCR primers 1 and 2 (30 and 50 pmol, respectively), and 2.5 U of *Taq* DNA polymerase (Perkin Elmer-Cetus). RNA-cDNA hybrids were denatured at 94°C for 4 min. The amplification was performed in 35 cycles in a DNA thermal cycler (Perkin Elmer-Cetus); each cycle consisted of denaturing for 1 min at 94°C, primer annealing for 2 min at 55°C, and elongation for 2 min at 72°C. Amplification reaction mixtures (10 to 15 μl) were mixed with 2 μl of 6× gel loading buffer (1× buffer is 0.25% bromophenol blue, 40% sucrose [final concentration]) and loaded onto 2% agarose gels. After electrophoresis, gels were soaked in 5-μg/ml ethidium bromide for 5 min and destained for 1 h, and the bands were visualized and photographed.

Analysis of PCR products. Appropriate PCR amplification was confirmed by Southern analysis of these fragments. A 14-mer located between the PCR primers with the sequence GAA TGC GGC TAA TC (nucleotides 472 through 485) was tagged by 5′ end labeling with a commercial kit (Stratagene Cloning Systems, La Jolla, Calif.) (16).

Aliquots (4 μl) of the PCR products were separated by electrophoresis on 2% agarose gels as described above. The separated fragments were transferred to nylon membranes (Nytran; Schleicher & Schuell) by using the Optiblot system (Stratagene) and then were UV cross-linked. The blot was prehybridized for 3 h at 42°C and hybridized overnight at 30°C with the labeled 14-mer. After probing, the filters were washed three times in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–1% SDS (16) for 10 min each time at room temperature, three times in 6× SSPE–1% SDS at 37°C for 3 min each time, and finally once in 1× SSPE–1% SDS at 42°C for 5 min. The filters were exposed to Kodak X-Omat AR film for 2 h.

RESULTS

The distribution of different viruses isolated from the clinical specimens by tissue culture infectivity is shown in Table 1. Of these, 81 were identified as polioviruses and 50 were identified as nonpoliovirus enteroviruses. All 81 (100%) viral isolates identified as polioviruses by tissue culture neutralization and infectivity were also positive by the PCR (Table 1). With primer sets 1 and 2, an amplification product of the expected size (248 bp) was obtained with all the isolates positive for poliovirus but not for any other virus cultures (Fig. 1A). Dideoxy sequencing (11) of several PCR products confirmed that the 248-bp product was from the poliovirus genome (unpublished data). Hybridization of the PCR amplification products with the labeled 14-mer probe (nucleotides 472 through 485) identified a band that correlated with the amplified 248-bp PCR product seen on the agarose gel (Fig. 1B). This confirmed that the fragments are indeed from the poliovirus. However, lower stringency during the hybridization washes revealed a slight cross-reaction in other nonpoliovirus enterovirus lanes. This did not, however, affect our ability to differentiate polioviruses from nonpoliovirus enteroviruses. Of 50 isolates typed as nonpoliovirus enteroviruses, 48 were also negative by the PCRs (Table 1). However, two nonpoliovirus enterovirus isolates gave positive reactions and the poliovirus-specific 248-bp amplification product. The specificity (19) of the oligonucleotide primer set with respect to other nonpolioviruses enteroviruses was 96%. The specificity with respect to all viruses examined was 97% (Table 1). The overall predictive value of the positive result was 98%.

Our primer pairs were further tested for their ability to yield specific products with the purified RNA of three common reference wild polioviruses; type 1 (Mahoney), type 2 (Lansing), and type 3 (Leon) strains. Templates of type 1 (Mahoney) and type 3 (Leon) reference strains yielded PCR products that had mobilities identical to those of the

TABLE 1. Sensitivity and specificity of poliovirus-specific probes for detection of poliovirus in clinical isolates by using PCR

Virus	No. of:		Sensitivity (%)	Specificity (%)
	Isolates	Positive tests		
Polioviruses				
Type 1	39	39		
Type 2	14	14		
Type 3	28	28		
Total	81	81	100	
Nonpoliovirus enteroviruses				
Echovirus type:				
2	1	0		
6	2	0		
7	3	0		
9	1	0		
11	16	1		
14	1	0		
16	1	0		
20	1	0		
26	1	0		
30	2	0		
32	1	1		
Coxsackievirus				
A9	5	0		
B1	5	0		
B2	5	0		
B3	1	0		
B4	2	0		
R1	1	0		
Total	50	2		96
Other viruses[a]	13	0		
Total	144	83		97

[a] Other viruses include rhinovirus (three isolates), cytomegalovirus (three isolates), adenovirus (two isolates), influenza virus (two isolates), rotavirus (one isolate), parainfluenza virus (one isolate), and respiratory syncytial virus (one isolate).

corresponding Sabin strain products (Fig. 2). In contrast, no amplification products were obtained with the genomes of type 2 (Lansing) strain. The RNA purified from Vero cells used to grow the wild strains of virus was used as a control and exhibited no amplification.

DISCUSSION

We have shown that with a single set of primers selected in the conserved 5′ noncoding region it is possible to identify polioviruses rapidly and with high degrees of specificity and sensitivity. The need for a rapid diagnostic assay for the enteroviruses cannot be overemphasized. The diversity of clinical manifestations often leads to confusing and lengthy differential diagnosis, which in turn results in significant personal and financial hardships on the patient and family (14). With available tissue culture methods, an isolate can be identified as an enterovirus with reasonable certainty only on the basis of characteristic CPE (2). It must be considered, however, that the isolate may be a nonpoliovirus enterovirus or a vaccine poliovirus. The differentiation between the two

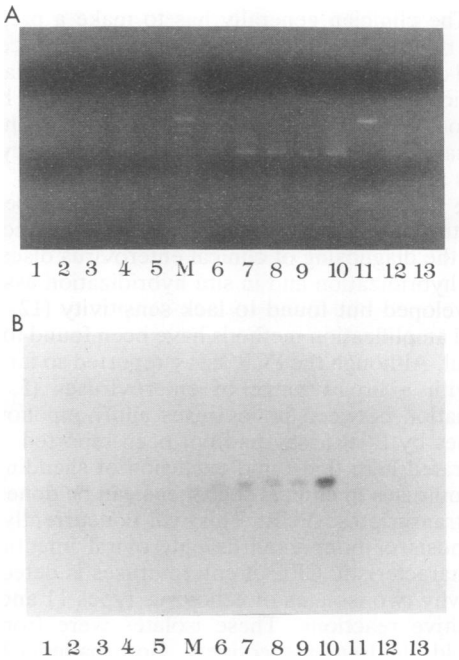

FIG. 1. (A) PCR products of nonpoliovirus and poliovirus isolates in an ethidium bromide-stained 2% agarose gel. Lanes 1 through 5, nonpoliovirus enteroviruses; lane M, 1-kb DNA ladder (Bethesda Research Laboratories); lanes 6 through 10, polioviruses; lane 11, positive nonenterovirus control, 500 bp (lambda phage DNA from Perkin Elmer-Cetus PCR kit); lane 12, negative control respiratory syncytial virus; lane 13, negative control rotavirus. (B) Southern blot hybridization. Lanes correspond to those in panel A.

requires virus neutralization using three types of poliovirus antisera, which is somewhat time-consuming. The final typing of the virus by neutralization using intersecting antiserum pools is even more cumbersome and is often not available until weeks or months after the onset of acute

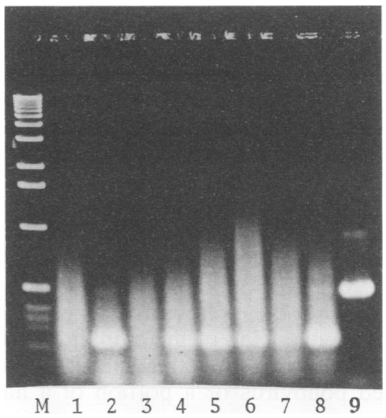

FIG. 2 PCR products of wild reference strains and vaccine strains of poliovirus in an ethidium bromide-stained 2% agarose gel. Lane M, 1-kb DNA Ladder (Bethesda Research Laboratories); lane 1, reverse-transcribed RNA from Vero cells; lane 2, poliovirus 1 Mahoney; lane 3, poliovirus 2 Lansing; lane 4, poliovirus 3 Leon; lane 5, poliovirus 3 Sabin; lane 6, poliovirus Sabin; lane 7, poliovirus 2 Sabin; lane 8, poliovirus 1 Sabin; lane 9, positive control (500 bp) (Perkin Elmer-Cetus PCR kit). Note that lanes 1 and 3 are negative for the 248-bp product.

illness. The clinician generally has to make a presumption based on other information such as specimen source, time of year, and clinical history of the patient (4). Early diagnosis of a nonpoliovirus enteroviral disease can therefore help physicians to eliminate unnecessary tests, reduce the use of unnecessary antibiotics, and improve the accuracy of their diagnosis and prognosis (21).

During the past 6 years, great strides have been made towards the adaptation of nucleic acid-based detection systems for the diagnosing of clinical enterovirus diseases (14). Dot blot hybridization and in situ hybridization assays have been developed but found to lack sensitivity (12, 15). Nucleic acid amplification methods have been found to be more successful. Although the PCR assay reported so far has been tested with a broad range of enteroviruses (5, 13, 23), differentiation between polioviruses and nonpoliovirus enteroviruses by PCR assay had not been reported. We have demonstrated here that rapid exclusion of shedding of vaccine polioviruses in clinical specimens can be done by using reverse transcriptase PCR. This will concurrently confirm the diagnosis of nonpoliovirus enteroviral infections soon after a characteristic CPE of enteroviruses is detected. It is unclear why two isolates of echovirus types 11 and 32 gave false-positive reactions. These isolates were from 2- and 12-year-old children, respectively. Both patients had been immunized with poliovirus vaccines. It is possible that a vaccine strain of poliovirus given to the patients or to their contacts was shed from the patients simultaneously with echovirus. GenBank analysis of the primers used did not show any common sequence between polioviruses and nonpoliovirus enteroviruses which have been sequenced. However, these two echoviruses have not been sequenced.

The Southern blot confirms that the PCR procedure can be used to definitely identify the presence of poliovirus. Amplified samples containing this virus produce a clear signal that is easily recognizable. However, either prolonged exposure or low-stringency washes revealed weak signals in the nonpoliovirus enteroviral test samples. This can be explained by the presence of poliovirus-related sequences in these nonpoliovirus enteroviruses, dual shedding of viruses, or PCR contamination. PCR contamination, which cannot be completely excluded, is not likely, since the PCR control was totally negative (Fig. 1B, lane 11). Additionally, the PCR reactions were performed and set up away from areas involved in the RNA extraction and reverse transcription in an attempt to minimize contamination by using dedicated reagents and pipets. Related sequences cannot be completely excluded, but it seems unlikely that similar-size fragments would be found. A related sequence with reduced homology could account for the weak signal found upon prolonged exposure in some of the samples. However, the strength of the signal alone allows clear identification of the poliovirus. Another explanation of the cross-reaction may be dual shedding, with the poliovirus being present at low concentrations. Sequencing of the amplified product would confirm whether the cross-reacting species seen in the test lane were shed poliovirus or a portion of a nonpoliovirus enterovirus.

The results of our PCR assay with reference wild polioviruses compare well with those of Yang et al. (22). Templates of type 1 (Mahoney) and type 3 (Leon) reference strains yielded PCR products with mobilities identical to those of Sabin 1 and 3 products (Fig. 2). In contrast, no amplification product was obtained with genomes of the type 2 strain (Lansing). These observations can be readily interpreted as indications of genetic relationships between the reference poliovirus strains (22). Mahoney and Sabin type 1 strains and Leon and Sabin type 3 strains share >99% nucleotide sequence homology. The genomic templates of the Lansing strain are highly different from those of Sabin 2. A common primer for Sabin 1, 2, and 3 would not therefore amplify the template of Lansing strain.

The results of this study indicate that reverse transcriptase PCR can potentially be used for specific detection of polioviruses and rapid differentiation of poliovirus and nonpoliovirus enteroviruses.

ACKNOWLEDGMENTS

We thank the Clinical Virology Laboratory at the Children's Hospital in Buffalo, N.Y., and the Clinical Virology Laboratory, Department of Pathology, University of Texas Medical Branch, for providing viral isolates; Jesse Lozano, Shirley Jacob, and Samuel Cunningham for technical assistance; and Sandra Fuentes for preparation of the manuscript.

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