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Enzymatic RNA Amplification of the Enteroviruses

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Enteroviruses are among the most common causes of childhood infection. Current diagnostic techniques are often too slow and too insensitive to benefit the patient optimally. This report describes a modified polymerase chain reaction technique by which enteroviral RNA can be amplified, over a few hours, to a level detectable by agarose mini-gel electrophoresis or nucleic acid hybridization or both. Three oligomeric regions of great homology among the enteroviruses were identified and designated as a potential primer pair and probe. With this combination, all 11 of the enterovirus serotypes tested, representing the major subgroups of these pathogens, were successfully amplified and detected. The sensitivity and rapidity of this new assay speak to its potential clinical applicability in the diagnosis of enterovirus infections.

The enteroviruses (EVs) are RNA viruses in the family *Picornaviridae* and are among the most common causes of childhood infection (1). Since the effective control of polioviruses with the introduction of vaccines 30 years ago, the diseases caused by the more than 65 non-polio EVs have received increased attention. These range in severity from benign to fatal and include manifestations in all major organ systems. Distinguishing EV-related illness from that due to bacteria and other viruses is important for prognostic, therapeutic, and epidemiologic purposes. Current diagnostic techniques for the EVs are limited by their slow turnaround time and relative insensitivity, as well as by the serotypic diversity of the EVs (2, 3, 22–24). Our own attempts at EV diagnosis, using nucleic acid hybridization (12, 13, 17), have been hampered by the low viral titers in certain clinical specimens, particularly cerebrospinal fluid (CSF) (22).

Two recent developments have provided renewed opportunity for a potentially rapid and accurate nucleic acid diagnosis of EV infections. First, six EV serotypes have been fully sequenced (5, 8, 9, 21), confirming the intertypic genomic conservation demonstrated previously by dot blot hybridization experiments (4, 16). Second, the polymerase chain reaction (PCR) technique has made it possible to amplify nucleic acid by 5 to 6 logs in a few hours in vitro (10, 18). This report describes a modification of the PCR technique for the enzymatic amplification of EV RNA, potentially providing a new, more accurate, and sensitive diagnostic method for these pathogens.

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MATERIALS AND METHODS

Viruses. EV serotypes of known (poliovirus types Sabin 1 and 3; coxsackievirus B1) and unknown (coxsackieviruses B6, A9, and A16; echoviruses 2, 4, 6, 11, and 22) genomic sequence were obtained from Lederle Laboratories (Sabin polioviruses) or from the American Type Tissue Collection (all others). Viral stocks of known titer were prepared and purified by sucrose gradient centrifugation.

Control virus stocks (herpes simplex type 1, cytomegalovirus, and respiratory syncytial virus) were prepared from clinical isolates and purified, and their titers were determined.

Specimen preparation. Viruses were added to normal CSF or phosphate-buffered saline (PBS) at titers ranging from 10^3 to 10^6 50% tissue culture infective doses ($TCID_{50}$) depending on the particular experiment (see figure legends). Normal CSF was obtained from specimens remaining in the clinical laboratories at the university hospital after all routine tests had been performed.

Samples, 100 μl, of virus-containing CSF or PBS were treated with 40 U of RNasin (Promega, Madison, Wis.) to inhibit RNase activity (15). Viral RNA was extracted by the addition of sodium dodecyl sulfate to a final concentration of 0.5%, followed by 1 volume of phenol-chloroform (1:1 mixture). Specimens were centrifugated at 15,000 × *g* for 5 min in a tabletop Eppendorf centrifuge. The aqueous phase was removed to a new tube, and the remaining organic phase was "back extracted" by adding an equal volume of a solution consisting of 10 mM Tris hydrochloride (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 0.5% sodium dodecyl sulfate. This mixture was centrifuged as above, and the two aqueous phases were combined. Ammonium acetate was added (final concentration, 2 M), followed by 2.5 volumes of cold 100% ethanol. This preparation was stored overnight at −20°C and then centrifuged for 30 min. The supernatant was discarded.

Primers. With the use of a computer-assisted analysis of the genomic RNA sequences of the six EV serotypes which have been fully sequenced (5, 8, 9, 21), three 20- to 25-base regions of absolute (100%) sequence conservation were identified within a 154-base segment near the 5′ end of the viral genome (Fig. 1). The two flanking sequences were chosen as primers for PCR, and the middle sequence, which did not overlap with either of the primers, was chosen as a probe. These three oligomeric strands were synthesized as single-stranded DNA, using an automated synthesizer (Applied Biosystems, Foster City, Calif.). The downstream primer and the probe were synthesized "antisense" to genomic viral RNA, and the upstream primer was synthesized "sense" to genomic RNA.

Reverse transcription and enzymatic amplification. The following components were added to each pellet from the ethanol precipitation (see above) for a reverse transcription reaction: 40 U of RNasin, 2 μl of 5 × reverse transcription buffer (250 mM Tris hydrochloride [pH 8.3], 15 mM $MgCl_2$, 350 mM KCl, 50 mM dithiothreitol), 1 μl each of 10 mM ATP, CTP, GTP, and TTP, and 2 μl of diethylpyrocarbonate-treated H_2O. One microliter of the downstream primer

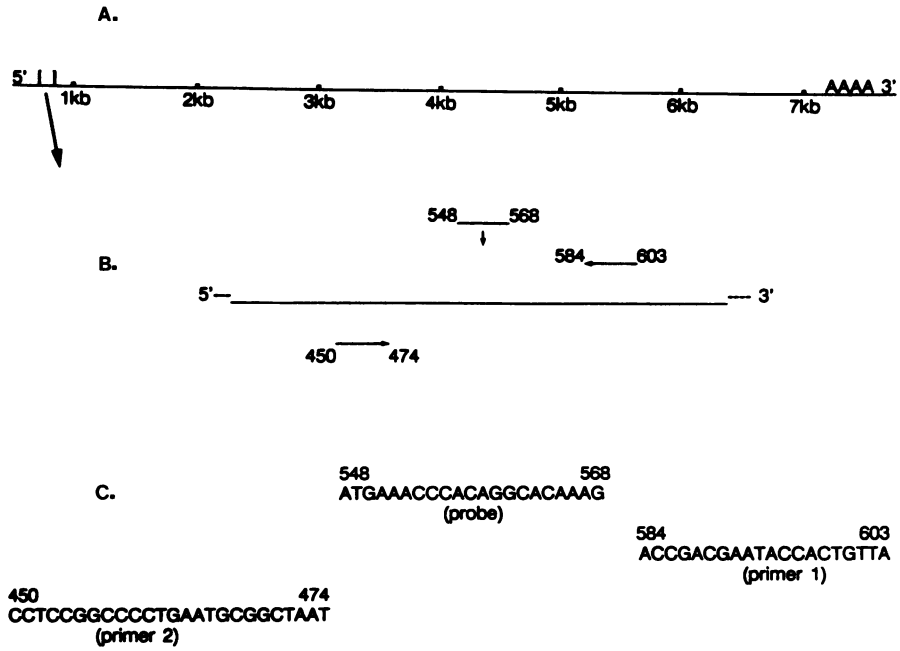

FIG. 1. Regions of absolute sequence conversion. (A) Schematic representation of the (approximately) 7,500-nucleotide, single-stranded, poly(A)-tailed RNA genome of the enteroviruses and the location, near the 5′ end, of the oligomeric primer pair and probe (region in brackets). (B) Enlargement of the bracketed area depicts the downstream 20-nucleotide primer (bases 584 to 603 of the genome), the upstream 25-nucleotide primer (bases 450 to 474), and the nonoverlapping (with either of the primers) 21-nucleotide probe (bases 548 to 568). The small arrows indicate the direction of priming and that the probe will hybridize to amplified template in the region shown. (C) Specific sequences of primers and probe. kb, Kilobases.

(10 pmol/μl) and 5 U of avian reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) were added last, and the mixture was then incubated for 90 min at 37°C.

The following reagents were added directly to each of the reverse transcription mixtures for PCR: 10 μl of double-distilled H_2O, 4 μl of 10× PCR buffer (560 mM KCl, 100 mM Tris hydrochloride [pH 8.3], 15 mM $MgCl_2$, 0.1% [wt/vol] gelatin), 6.5 μl of diluted mix of deoxynucleotides (125 μl each of 10 mM dATP, dCTP, dGTP, and TTP diluted in 500 μl of double-distilled sterile H_2O), 4 μl of downstream primer (10 pmol/μl), 4 μl of upstream primer (10 pmol/μl), and 0.5 μl of *Taq*I polymerase (5 U/μl) (Cetus Corp., Emeryville, Calif.). A lambda phage template and primer pair control provided in the GeneAmp kit (Cetus Corp.) were run per the manufacturer's directions. The latter template and primer pair combination produces a distinct band of 504 bases in length. PBS containing no template was run as an additional control for possible contamination. Twenty-five 2-min cycles of denaturation (95°C), annealing (50°C), and primer extension (72°C) steps were then performed, followed by analysis of the amplification product with agarose mini-gel electrophoresis. To enhance the sensitivity of testing in certain samples, a second set of 25 PCR cycles was performed by removing 1 μl of the initial PCR mixture (following the first 25 cycles), diluting with 10 μl of H_2O, and adding a full new complement of PCR reagents. A gel electrophoresis band of 154 bases in length was considered presumptively positive for enteroviral RNA (109-base intervening sequence between the two primers plus the incorporated 25- and 20-base primers; Fig. 1). Confirmation of specificity and enhanced sensitivity was achieved by using the oligomeric probe, end labeled with ^{32}P, in a standard slot blot hybridization reaction performed as described previously (14). Briefly, hybridization was performed in a solution of 5× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate [pH 7.0]), 1% sodium dodecyl sulfate, and 0.5% bovine serum albumin at 60°C for 15 min. Three washes were performed in a solution of 1× SSC and 1% sodium dodecyl sulfate at 50°C for 5 min each. Blots were then exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens in sealed cassettes for 1 h. Lambda phage, non-EV, and saline control samples were considered negative when no 154-base bands were seen by electrophoresis and hybridization with the EV oligomeric probe was negative.

RESULTS

All 11 EV serotypes tested resulted in successful RNA amplification with the described primers. Bands of 154 bases in length were seen after the first 25 PCR cycles for all EV serotypes except echoviruses 2 and 22, both of which were detectable following an additional 25 cycles (Fig. 2 and 3). Hybridization with the ^{32}P-labeled oligomeric probe confirmed the gel results (Fig. 2 and 3). In contrast, the same procedure with the same primers and probe applied to herpes simplex virus, cytomegalovirus, and respiratory syncytial virus resulted in neither 154-base bands by electrophoresis nor positive hybridization (Fig. 4). A second set of 25 PCR cycles did not result in any false-positive bands or hybridization among the negative controls (not shown). To address the sensitivity of this RNA amplification method, log dilutions of echovirus 11 were added to either CSF or PBS (Fig. 5). Amplified RNA was detected at all dilutions tested in PBS (10^3 through 10^6 $TCID_{50}$) by both gel electrophoresis and hybridization. In CSF, the lowest titer of echovirus 11 (10^3 $TCID_{50}$) was detectable only by hybridization, but all other dilutions were detectable by both assays.

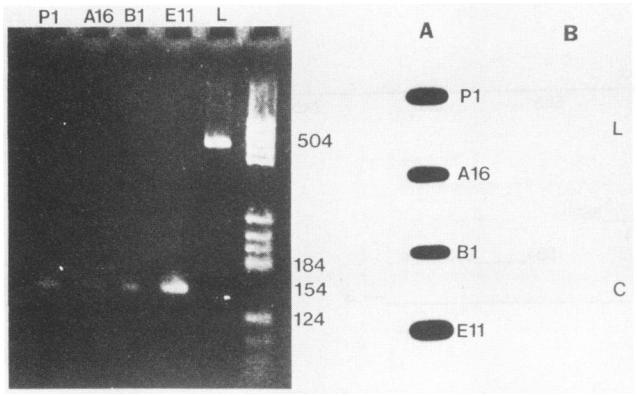

FIG. 2. Enzymatic amplification of four EV serotypes (10^5 $TCID_{50}$), poliovirus type 1 (abbreviated P1), coxsackievirus A16 (A16), coxsackievirus B1 (B1), echovirus 11 (E11), and the lambda phage control template (L). By mini-gel electrophoresis (left), bands of 154 bases in length are seen for the EVs, and a distinct band of 504 bases represents the amplified product of the control template. The lane on the far right is a sizing ladder. Hybridization with the oligomeric probe (right) confirms the presence of amplified product from the enteroviruses (lane A). The probe does not hybridize to the amplified product of lambda phage or to the saline control (C) (lane B).

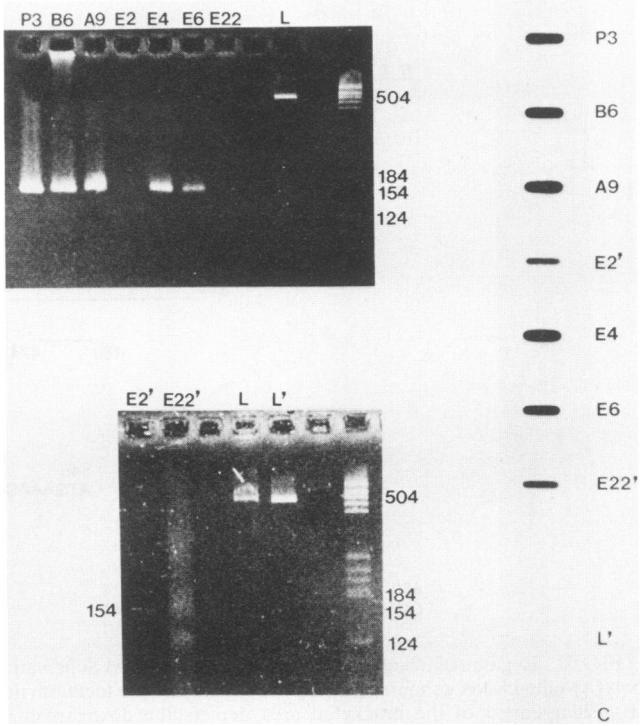

FIG. 3. Enzymatic amplification of seven additional EV serotypes (10^5 $TCID_{50}$): poliovirus type 3 (abbreviated P3); coxsackievirus B6 and A9; echoviruses (E) 2, 4, 6, and 22; and the lambda phage control template (L). Bands of 154 bases in length are seen for all EV serotypes except E2 and E22 after the first 25 cycles of the PCR (top gel). An additional 25 cycles (bottom gel) results in 154 base bands for those two serotypes as well (E2′ and E22′ indicate the amplified products following the second set of cycles). The lambda phage control template was also subjected to another 25 cycles (L′), resulting in a more prominent band than after the first set of cycles (L). Hybridization with the oligomeric probe (right) detects the amplified products of all enterovirus serotypes (five serotypes after the first set of amplification cycles, E2′ and E22′ after the second set), but not the lambda control template even after two sets of amplification cycles (L′) or the saline control (C).

DISCUSSION

The need for an accurate and rapid diagnostic test for EV infections is widely appreciated (2). Every summer and fall, thousands of children are unnecessarily hospitalized and treated with unnecessary antibiotics or antiherpes drugs or both while waiting for culture results which "rule out" bacterial and herpetic infections. Viral culture results which "rule in" EVs and, for the most part, would predict a good clinical outcome with no therapy are often delayed: the average time to culture isolation of EVs from CSF specimens in our laboratory is nearly 1 week (unpublished data); the reported range is 3.7 to 8.2 days (2, 7). Furthermore, only 65 to 70% of EV serotypes will grow in standard tissue culture cell lines (2). Attempts at developing widely applicable rapid immunoassay or serologic techniques have been thwarted by the great antigenic diversity among the many EV serotypes (3, 23, 24), although a recent report of a monoclonal antibody which reacts with an array of serotypes (25) may improve the chances for such immunology-based testing. The lack of an acceptable rapid test has impeded the development of antiviral agents for the EVs as well. Although several very effective compounds have been tested in vitro and in animals (11, 16; R. E. McKinney and C. M. Wilfert, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 126, 1986), clinical trials cannot proceed until a diagnostic method can rapidly confirm infection and, hence, the eligibility of a patient to be enrolled and studied.

The potential of nucleic acid hybridization for rapid diagnoses of EV infections is great because of the proven conservation of genomic regions across multiple, perhaps all, serotypes, the relative ease with which hybridization assays can be performed, and the recent availability of nonisotopic probe labeling techniques. Our attempts to apply cDNA (16, 17), RNA (13), and oligomeric DNA (14) probes to EV diagnosis have confirmed that one or a combination of two probes can be used to detect a broad range of serotypes. The limit of sensitivity we encountered, however, using the most sensitive of the probe reagents (single-stranded RNA probes, labeled with ^{32}P) and target EVs which were very homologous to the probes, was approximately 10 pg of RNA when tested in saline (15). In CSF, the sensitivity is 10-fold less (100 pg) because of the high levels of indigenous RNases in all body fluids (15). RNase inhibitors, while effective, restore the sensitivity only partially. With less homologous target EV serotypes, the sensitivity is lower still. Sensitivity of 10 pg translates to 10^6 EV RNA molecules. Estimating a particle/infectivity ratio of 100:1 for the EVs, the sensitivity of routine hybridization may be as poor as 10^4 titratable (i.e., can grow in tissue culture) viruses. While few studies exist which quantitate EVs in clinical CSF specimens, the titers are apparently in the range of 10^1 to 10^3 viruses (22).

For all of these reasons, PCR technology has the promise to be useful in EV diagnosis. The selection of primers based on the sequences of only six serotypes poses the potential risk that the homology required for the primers to anneal to additional serotypes would be insufficient. In fact, even at a relatively stringent annealing temperature (50°C), 11 of 11 tested serotypes were amplified in this study, including 8 of unknown RNA sequence. Echoviruses 2 and 22 required additional cycles of PCR for detection, perhaps because the

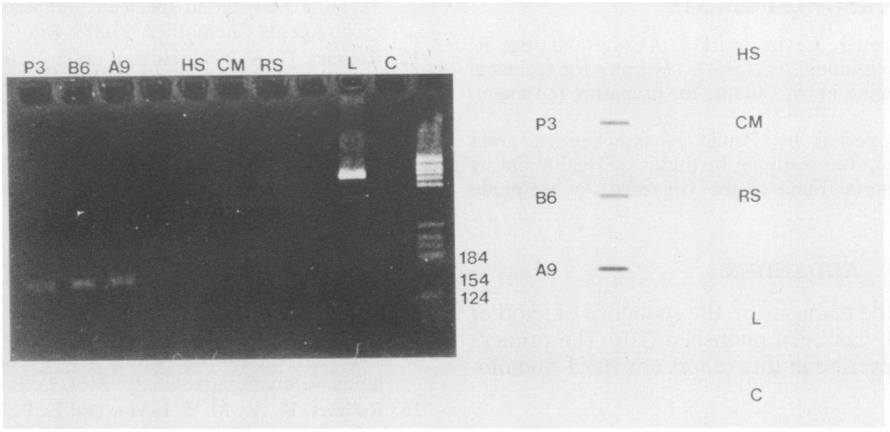

FIG. 4. Enzymatic amplification of 10^5 $TCID_{50}$ of EV and non-EV controls. P3, Poliovirus type 3; B6, coxsackievirus B6; A9, coxsackievirus A9; HS, herpes simplex virus type 1; CM, cytomegalovirus; RS, respiratory syncytial virus. The lambda phage (L) and saline controls (C) are also shown. Hybridization with oligomeric probe (right-hand strips) confirms the results by gel electrophoresis.

primers are less homologous to these serotypes and, therefore, less efficient in annealing and priming. Although the sequences of echoviruses 2 and 22 are not known, echovirus 22 is known to be dissimilar in many respects to the other EVs (19). We have shown weaker hybridization of poliovirus and coxsackievirus probes to echovirus 22 than to other serotypes (17).

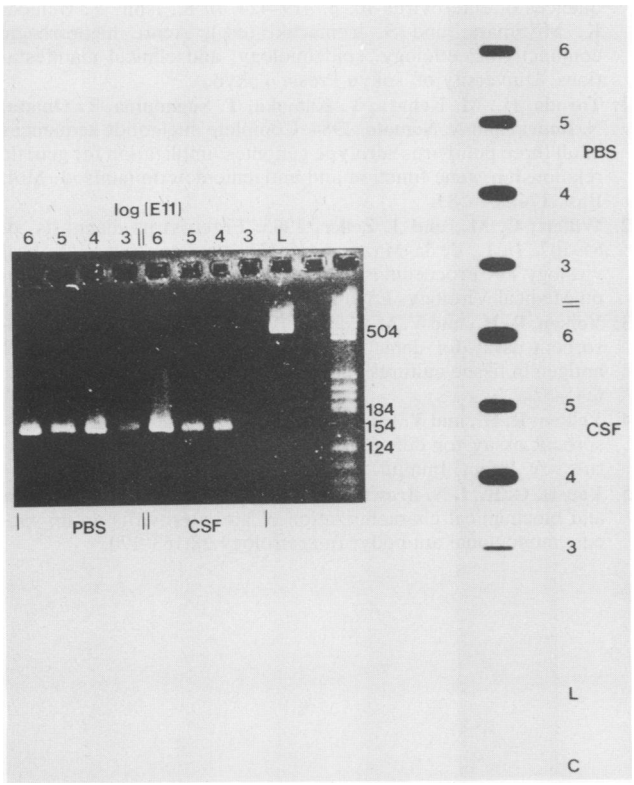

FIG. 5. Enzymatic amplification of log dilutions (10^3 to 10^6 $TCID_{50}$) of a single EV serotype, echovirus 11. Virus was added, in the titers shown, to either CSF or PBS. Mini-gel electrophoresis (left) reveals bands of 154 bases in length for all but the lowest titer of virus in CSF. Hybridization with the oligomeric probe detects all dilutions in both CSF and PBS. Lambda phage control template (L) and saline control (C) are negative.

The sensitivity of this RNA amplification assay is at least 10^3 $TCID_{50}$. That limit of sensitivity testing was chosen in these experiments because it represented a 2-log increase over the sensitivity my co-workers and I observed previously with direct hybridization to the echovirus 11 serotype (17). In fact, the actual sensitivity of PCR for echovirus 11 and other serotypes may be much greater than 10^3 $TCID_{50}$. The autoradiograph detecting 10^3 $TCID_{50}$ of echovirus 11 in CSF (Fig. 5) was exposed for only 1 h; lower titers (untested) might well be detected with longer exposures, and a second set of PCR cycles, as was performed for echoviruses 2 and 22 (Fig. 3), should further reduce the titer of target viruses which can be detected, albeit increasing the test performance time by 2 to 3 h. Additional primer pairs could readily be added to this system to detect a broader serotypic spectrum if necessary: such a modification would add no time and minimal effort to the performance of the testing. The theoretical limit to detecting virus, if enough PCR cycles are performed, is the presence of a single copy of intact viral RNA which can be reverse transcribed to form the first strand of cDNA. It is that reverse transcription reaction, coupled with the ubiquitous presence of RNases, which makes enzymatic amplification of RNA more complicated than PCR of DNA. As this study demonstrates, however, those difficulties are surmountable. The risk of contamination (or carry-over) in PCR reactions is high, necessitating meticulous technique and the inclusion of appropriate negative controls in every experiment. Both template-negative and non-EV controls were "clean" of detectable amplified product in this study.

The method described in this study can currently be performed in 6 to 8 h; however, incubation durations have not been optimized and might well be shortened. The availability of automated PCR heat cyclers greatly reduces the required "hands on" time so that a diagnostic laboratory could readily incorporate such a protocol. The possible applications of EV RNA amplification extend beyond diagnosis of acute infections. PCR can be performed on archival specimens which have been stored for many years and even those which have been Formalin fixed and paraffin embedded (6). New disease associations with the EVs and new insights into EV pathogenesis are likely outgrowths of this methodology.

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ADDENDUM

Since I submitted the manuscript, the sequence of another EV serotype, EV 70, has been published (20). The primers and probe which I describe in this report are 100% homologous to EV 70.

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