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Quantitation of Enteroviral RNA by Competitive Polymerase Chain Reaction

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The polymerase chain reaction (PCR) is a new diagnostic technique for the detection of enteroviral infection; however, it currently provides only qualitative results. The aim of this study was to adapt PCR for the accurate quantitation of enteroviral RNA in clinical specimens. For this purpose, we designed a standard RNA which was homologous to sequences at the 5′ end of the coxsackie B3 enterovirus genome but contained a single-base-pair mutation which created a novel internal restriction site. Serial dilutions of this standard template RNA were mixed with a fixed concentration of coxsackie B3 enterovirus RNA. The viral and standard templates were reverse transcribed to cDNA and coamplified by PCR, and a comparison of the radioactive PCR products was made. Since the templates were both present in a single reaction tube and competed for the same primers, the ratio of products remained proportional throughout the amplification process. By this approach, a fourfold difference in viral titer was clearly distinguishable. Moreover, we were able to accurately quantitate as few as 15 50% tissue culture infectious doses, which reflects common clinical viral titers. This study lays the foundation for quantitation of enteroviral RNA in clinical specimens and establishes a technique that can readily be applied to the diagnosis of enteroviral infection.

The genus *Enterovirus* consists of positive-strand RNA viruses and is subdivided into five different groups: poliovirus, coxsackievirus group A (CVA), coxsackievirus group B (CVB), echovirus (EV), and numbered enterovirus. There are 68 serotypes which have been associated with disease in humans. While often characterized by subclinical syndromes, enteroviral infection has also been reported for a number of serious diseases. Clinical manifestations range from minor febrile illness to life-threatening conditions such as myocarditis, dilated cardiomyopathy, meningitis, and poliomyelitis (19).

A significant recent advance in the diagnosis of enteroviral infection has been the application of polymerase chain reaction (PCR) technology (3, 10, 14, 22, 35). Computer-assisted alignment of enteroviral nucleotide sequences has revealed a highly conserved 5′ nontranslated region (NTR) (12, 13, 16–18, 29). Amplification of this region of the enteroviral genome has made it possible to detect a broad array of serotypes with a degree of sensitivity and rapidity unequalled by the conventional techniques of cell culture, immunoassay, and nucleic acid hybridization.

Although PCR diagnosis provides notable qualitative results, it has not been used for quantitative information on enteroviruses in clinical specimens. Quantitation could provide valuable insight into patient prognosis and response to treatments and into the pathogenesis of enteroviral infection.

The principle constraint in quantitation of PCR products is inherent in the amplification process. That is, minute variations in PCR can make comparisons of product yield unreliable (7, 30). The purpose of this study was to develop a technique which utilizes the diagnostic PCR method while also allowing accurate quantitation of the original enteroviral RNA. To validate this approach, we have quantitated coxsackie B3 enterovirus (CVB3) RNA constituted in cerebrospinal fluid (CSF). A known quantity of a synthetic panenteroviral standard RNA, which differs from wild-type viral RNA by a single mutated restriction site, was added to purified viral RNA, reverse transcribed to cDNA, and amplified by PCR in the same reaction tube. After restriction enzyme digestion, the final products were distinguished by electrophoresis on ethidium bromide (EtBr)-stained agarose gels. The relative amounts of viral and standard RNA were determined by scintillation counting of [α-^{32}P]dCTP incorporated in the amplified DNA product, and the original starting quantity of viral RNA was extrapolated from these counts. The method described in this study is directly applicable to PCR diagnosis of clinical enteroviral infection.

MATERIALS AND METHODS

Viruses. The enteroviruses (CVB3 and EV11) used in this study were supplied by the Department of Microbiology, Hospital for Sick Children, Toronto, Canada. Viruses were propagated in primary AGMK (African green monkey kidney) cell culture (Viromed Laboratories, Inc.). Titers were determined as 50% tissue culture infectious doses ($TCID_{50}$) (28), and the endpoint was calculated by the method of Reed and Muench (21).

Oligonucleotides. Oligonucleotide panenteroviral primers A, B, C, and D and the probe P1 were prepared by a solid-phase phosphotriester method on a Gene Assembler Plus (Pharmacia LKB Biotechnology) according to the manufacturer's directions. Concentrations of oligonucleotides suspended in sterile distilled water were determined by spectrophotometry.

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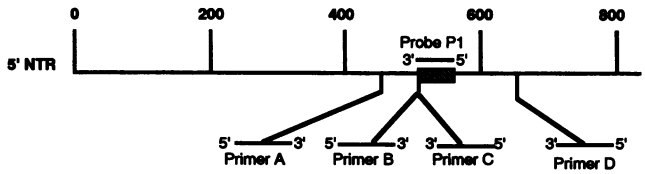

Primer A : 5'– TCCGGCCCCTGAATG–3'
Primer B : 5'– TGCAGCGGATCCGACTAC–3'
Primer C : 3'– ACGTCGCCTAGGCTGATG–5'
Primer D : 3'– ACCTAACCGGTAGGCCAC–5'

Probe P1 : 3'– CCTTGGCTGATGAAACCCACAGGCACA–5'

FIG. 1. Schematic representation of the positive-strand 5' NTR of the enteroviral genome and the sequences and relative positions of the synthetic oligonucleotide primers and probes. Primer A (bases 448 to 462 of the CVB3 genome), primer D (complementary to bases 625 to 642 of the CVB3 genome), and the probe P1 (complementary to bases 535 to 561 of the CVB3 genome) were described in reference 3. Primer B (bases 529 to 546) differs from the CVB3 genomic sequence by a single-base-pair change at position 538 (underlined). Primer C is complementary to primer B.

The oligonucleotides were derived from the conserved sequences in the 5' NTR of the enteroviral genome. Primers A and D and probe P1 were previously described as E2, E1, and E3, respectively (3). Primers B and C are novel. Primer B is identical to positions 529 to 546 in the CVB3 genomic sequence, with the exception of a single-base-pair change of an A to a T at position 538. Primer C is complementary to primer B. The sequence of each oligonucleotide is shown in Fig. 1.

RNA purification. Normal CSF was generously supplied by the Department of Hematology, Hospital for Sick Children, Toronto, Canada. The CSF was tested by PCR for enteroviral infection. Enterovirus-negative CSF from several patients was pooled.

CVB3 obtained from cell culture supernatants was added to 0.1-ml aliquots of the pooled CSF at titers ranging from 10^6 to 10^1 $TCID_{50}$, depending on the experiment. Viral RNA was then extracted by the acid guanidinium thiocyanate-phenol-chloroform method (4).

Synthesis of first-strand cDNA. The conversion of viral RNA to cDNA was carried out in a final volume of 40 μl containing RNA obtained from the extraction procedure (discussed above). The method used was as described previously (31), with two modifications. First, 0.4 μg of pdN6 random hexamers (Pharmacia) were used as primers. Second, samples were precipitated following cDNA synthesis by adding 4 μl of 3 M sodium acetate (pH 6.5) and 70% ethanol for 1 h at −70°C, centrifuged at 12,000 × *g*, washed in 70% ethanol, dried, resuspended in sterile distilled water, and stored at −20°C.

Generation of PCR products. Amplification of cDNA was carried out in a final volume of 50 μl. The cDNA (15 μl) was added to 18.5 μl of sterile distilled water, 1 μM upstream primer, 1 μM downstream primer, and the following reagents from the Gene Amp Kit (Perkin-Elmer Cetus): 1× reaction buffer, 1.5 mM $MgCl_2$, and 0.2 mM each deoxynucleoside triphosphate. The samples were denatured at 96°C for 10 min and cooled on ice. *Taq* DNA polymerase (2.5 U) was added, followed by a 50-μl overlay of light mineral oil (Fischer). Samples were then subjected to amplification in a DNA thermal cycler (Perkin-Elmer Cetus) for 39 cycles of annealing (2 min at 55°C), extension (3 min at 72°C), and denaturation (1 min at 94°C).

Analysis by electrophoresis, Southern transfer, and hybridization. A 10-μl aliquot of the PCR product was electrophoresed at 100 V onto 1.6% EtBr-stained agarose gels in 1× TAE running buffer (40 mM Tris acetate, 1 mM EDTA, 0.0002 μg of EtBr per μl). DNA was detected by illumination under a UV light (302 nm).

To confirm specificity, Southern hybridization was performed by the method described previously (31). Briefly, the DNA bands were transferred and denatured onto Hybond N+ nylon membranes (Amersham Canada Ltd.) for 2 h in 0.4 M sodium hydroxide. The probe P1, 5' end labelled with [γ-^{32}P]ATP (3,000 Ci/mol; DuPont), was used for hybridization.

Site-directed mutagenesis of CVB3 by overlap extension. A synthetic panenteroviral standard was constructed for use in competitive PCR (1, 8, 9). The standard was designed so that it was identical to the CVB3 genome between primer sites A and D, with the exception of a single internal base pair mutation. This mutation created a unique restriction site for *Bam*HI enzyme in the standard. To create this standard, CVB3 fragments were generated from one PCR mixture containing primers A and C and from a second reaction mixture containing primers B and D. The fragments were isolated from their reaction mixtures by size-fractioning electrophoresis on an agarose gel. The bands of appropriate size were cut from the gel and purified by using the Sephaglas Band Prep Kit (Pharmacia) according to the manufacturer's specifications. The two purified fragments (A-C and B-D) were combined and then amplified by PCR with outside primers A and D.

Cloning of the standard PCR fragment. The synthetic standard DNA fragment was first cloned into a TA cloning vector (Invitrogen Corp.) according to the manufacturer's directions. This kit allowed direct insertion of the PCR product into the vector. The insert was then subcloned from the *Spe*I and *Not*I vector restriction sites into the plasmid pBluescript II KS$^+$ (Stratagene), which contained a T7 RNA polymerase recognition site. The resulting plasmid, pBlue CVB3, was transformed into *Escherichia coli* cells (Invitrogen), which were then plated onto Luria-Bertani agar plates containing ampicillin (50 μg/ml) and 25 μl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 mg/ml; Boehringer Mannheim).

The presence of a standard insert containing the *Bam*HI mutation was verified by sequencing by the one-step reaction protocol for automated DNA sequencing with T7 DNA polymerase (Pharmacia LKB Biotechnology) and by restriction enzyme digestion.

RNA transcript of the standard. The plasmid pBlueCVB3 was purified from the transformed *E. coli* cells by Midiprep column chromatography (Qiagen) according to the manufacturer's directions. The insert containing the mutated standard DNA was cut from the plasmid at the flanking *Bss*HI (Boehringer Mannheim) restriction sites and transcribed to RNA by T7 RNA polymerase (Promega Corp.) according to the transcription protocol of Promega Corp. The resulting positive-sense RNA standard was purified from the original DNA template by digestion with 20 U of RNase-free DNase (Promega Corp.) for 15 min at 37°C followed by phenol-chloroform extraction. The RNA standard was stored at −70°C in a 20-μl solution of 5 M ammonium acetate–70% ethanol–diethylene polycarbonate-treated water.

For use in quantitative PCR, an aliquot of the standard

RNA was removed, centrifuged at 12,000 × *g*, washed in 70% ethanol, dried, and resuspended in sterile distilled water. The concentration of standard RNA was measured by absorbance spectrophotometry at 260 nm.

The competitive PCR assay. CVB3 (~3.8×10^5 $TCID_{50}$) was suspended in 100 μl of CSF. The RNA was purified and resuspended in 140 μl of sterile distilled water. The purified RNA was divided equally into several sample tubes (6.6 μl per tube, or ~18,000 $TCID_{50}$). Twofold dilutions of the standard RNA (from 6,250 to 0.25 pg) were then added to the sample tubes. Viral and standard RNA were reverse transcribed together to cDNA, purified, and resuspended in 45 μl of sterile water. One-third of this cDNA (15 μl, or ~6,000 $TCID_{50}$) was amplified by PCR in the presence of 0.5 μl of [α^{32}P]dCTP (3,000 Ci/mmol; Dupont) with primers A and D. Following PCR, the samples were digested with 50 U of *Bam*HI enzyme for 1 h at 37°C and the electrophoresed into a 1.6% EtBr–agarose gel. This allowed differentiation of the viral product (uncut upper band) from the standard product (*Bam*HI-digested lower band). Bands corresponding to the viral and standard products were individually excised from the gel, and the amount of radioactivity in each sample was determined by scintillation counting.

The quantity of viral RNA in the original sample was determined from these counts. To do this, the counts obtained from each upper band (the viral PCR product) had to first be corrected for heterodimer formation. Since >30 PCR cycles were used, the PCR had reached its plateau phase (20, 27), resulting in the formation of heterodimeric DNA fragments consisting of one strand each of viral template and standard template. These heterodimeric DNA fragments resist the enzyme digestion, leading to an overrepresentation of the uncut upper band. To compensate, the samples can be diluted prior to the last PCR cycle or the observed values can be corrected by an experimentally determined factor of 0.3 (1). In this study, we chose the latter method of correction, since it required less handling of the samples and allowed for analysis of the PCR products directly on the EtBr-agarose gels.

For each sample, the ratio of viral to standard product was determined and plotted against the known concentration of standard as a scattergraph. Linear regression analysis was used to generate a line of best fit with Cricket Graph 1.2 (Cricket Software). The point where the viral/standard product ratio was equal to one (and the quantity of viral RNA was equal to the known quantity of standard RNA) was determined by extrapolation on the graph.

Level of sensitivity and fidelity. To determine the level of sensitivity and fidelity of the competitive PCR method, the cDNA samples prepared as described above were diluted 10-fold and 100-fold in sterile distilled water. These samples were then subjected to PCR and analyzed by the method described above.

Detection of a fourfold difference in viral titer. To demonstrate the ability of competitive PCR to distinguish a fourfold difference in viral titer, a 1:4 dilution experiment was also performed. For this, 1.65 μl of the purified viral RNA was used (~4,500 $TCID_{50}$, as opposed to the 18,000 $TCID_{50}$ used for the competitive assay described above). These samples were spiked with serial dilutions of standard template (6,250 to 0.25 pg) and reverse transcribed to cDNA. Approximately 1,500 $TCID_{50}$ were then amplified by PCR and analyzed by the methods described above. Sensitivity testing (10-fold and 100-fold dilutions) was also performed on these samples.

Specificity of the PCR method. To demonstrate specificity, all experiments described above were repeated in the same manner, except that nonradioactive dCTP was used. Following PCR, the products were digested with *Bam*HI enzyme and subjected to electrophoresis. Southern hybridization analysis with the radiolabelled probe P1 was performed.

RESULTS

Specificity of primers and probes. We first confirmed the specificity of the primers A and D and the probe P1. Enteroviral RNA purified from CVB3- and EV11-infected cell culture supernatants was reverse transcribed to cDNA and then amplified by PCR with primers A and D. The DNA products were visualized on EtBr-stained agarose gels and by Southern hybridization to the probe P1 (results not shown). Our findings were similar to those reported previously (3) and clearly demonstrated the specificity of the oligonucleotides.

The competitive PCR assay. To demonstrate the use of the synthetic panenteroviral standard for the quantitation of enteroviral RNA in clinical specimens, titered CVB3 virus suspended in CSF was used. The competitive PCR assay was conducted, and following digestion with *Bam*HI enzyme, the products were analyzed on EtBr-agarose gels. Results for samples amplified without [^{32}P]dCTP can be seen in Fig. 2. The CVB3 product, which does not contain a *Bam*HI site, migrated as the upper band at the anticipated 194-bp size. The standard contained the introduced *Bam*HI restriction site and was clearly distinguished as the lower band of comigrating cleaved product. The samples containing an excess of internal standard showed a stronger lower band. Following dilution of the standard, the intensity of this band gradually decreased and the intensity of the upper band indicative of CVB3 fragment gradually increased.

Specificity of the competitive PCR assay—Southern blots and controls. To demonstrate the specificity of the PCR assay, the samples were transferred from the gels to nylon membranes for Southern blot hybridization. Extraneous bands were not detected (Fig. 3). The Southern blot also demonstrated that the panenteroviral probe P1 was capable of hybridizing to the novel standard band. Product bands were slightly more distinct at the lower viral titers. This would be expected, since the use of radioactivity allows for better detection of small quantities of DNA than EtBr analysis.

In all experiments, exclusive sample controls were run (standard or viral RNA only) to demonstrate that the standard product band was distinct from the viral product band (Fig. 2 and 3, lanes 12 and 13). The efficiency of the restriction enzyme for homodimeric DNA digestion could also be monitored through these control lanes. Water controls monitoring sample contamination were clearly negative (Fig. 2 and 3, lanes 14).

Quantitation, sensitivity, and fidelity of the competitive PCR assay. Duplicate samples amplified by PCR in the presence of [α-^{32}P]dCTP were used for quantitation. Bands corresponding to CVB3 and the standard product were individually excised from gels, and the amount of radioactivity was determined. Figure 4 shows each product ratio (CVB3/standard) plotted against the quantity of standard present in the sample tube. Using the internal standard, we were able to quantitate the enteroviral RNA at all titers tested, from 6,000 to 15 $TCID_{50}$. Moreover, we could resolve a fourfold difference in viral RNA concentration, with high fidelity down to only 15 $TCID_{50}$.

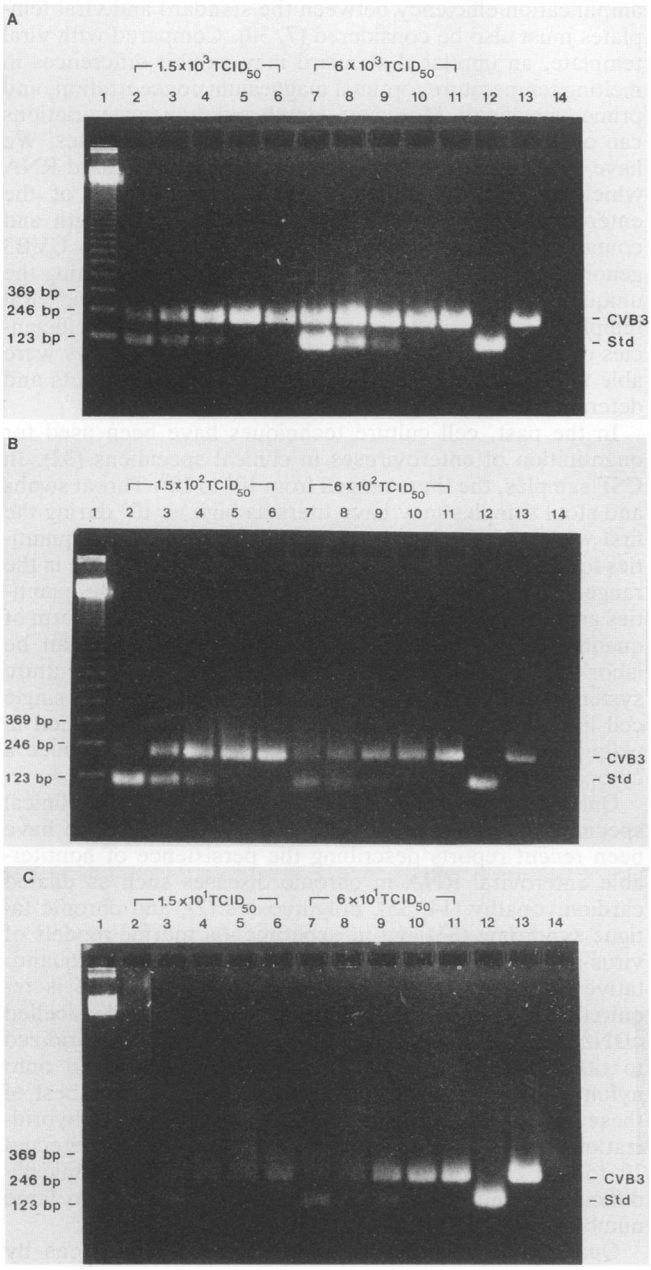

FIG. 2. Quantitation of CVB3 RNA by competitive PCR. Samples containing equal aliquots of CVB3 RNA were spiked with twofold dilutions of standard (Std) RNA. Samples were reverse transcribed to cDNA and amplified by PCR. After 39 cycles, 10% portions of the samples were digested with *Bam*HI and electrophoresed into 1.6% EtBr–agarose gels. (A) PCR products derived from 1,500 and 6,000 $TCID_{50}$ of CVB3. The final concentrations of the standard in each tube (lanes 2 to 11) were 1,560, 781, 195, 49, 25, 6,250, 1,560, 781, 195, and 49 pg, respectively. (B) PCR products derived from 150 and 600 $TCID_{50}$ of CVB3. The final concentrations of the standard in each tube (lanes 2 to 11) were 625, 78.1, 19.5, 4.9, 2.5, 625, 156, 78.1, 19.5, and 9.8 pg, respectively. (C) PCR products derived from 15 and 60 $TCID_{50}$ of CVB3. The final concentrations of the standard in each tube (lanes 2 to 11) were 62.5, 7.81, 1.95, 0.49, 0.25, 62.5, 15.6, 7.81, 1.95, and 0.98 pg, respectively. For all three gels, controls are shown in lanes 12 (standard only), lanes 13 (CVB3 only), and lanes 14 (normal CSF only). Lane 1 contains 123-bp ladder DNA marker.

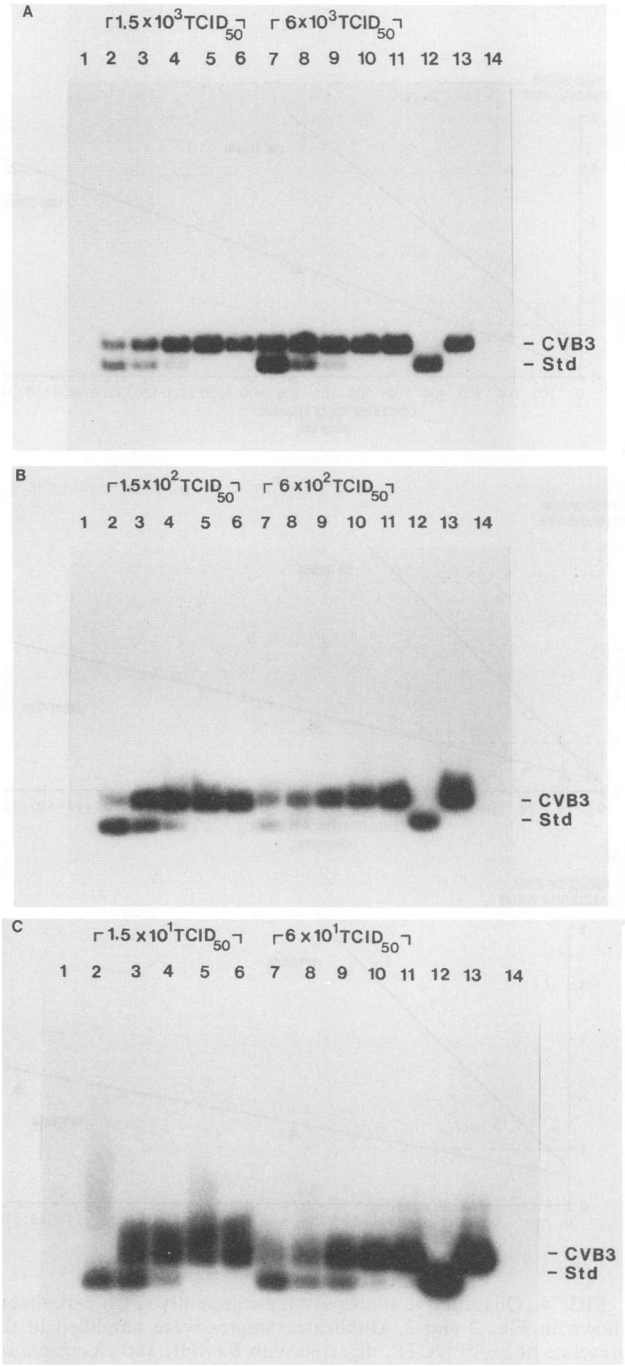

FIG. 3. Southern hybridization of the gels shown in Fig. 2 with the radiolabelled probe P1. Exposure was for 30 min at room temperature with intensifying screens.

DISCUSSION

The competitive PCR method described in this study can be used to quantitate very small amounts of enteroviral RNA (<1 pg) from limited amounts of sample. There are a number of difficulties associated with accurate quantitation with PCR-based technologies. The yield of final product can be affected by inhibitors present in some patient specimens, variability in sample preparation, inefficiency of reverse

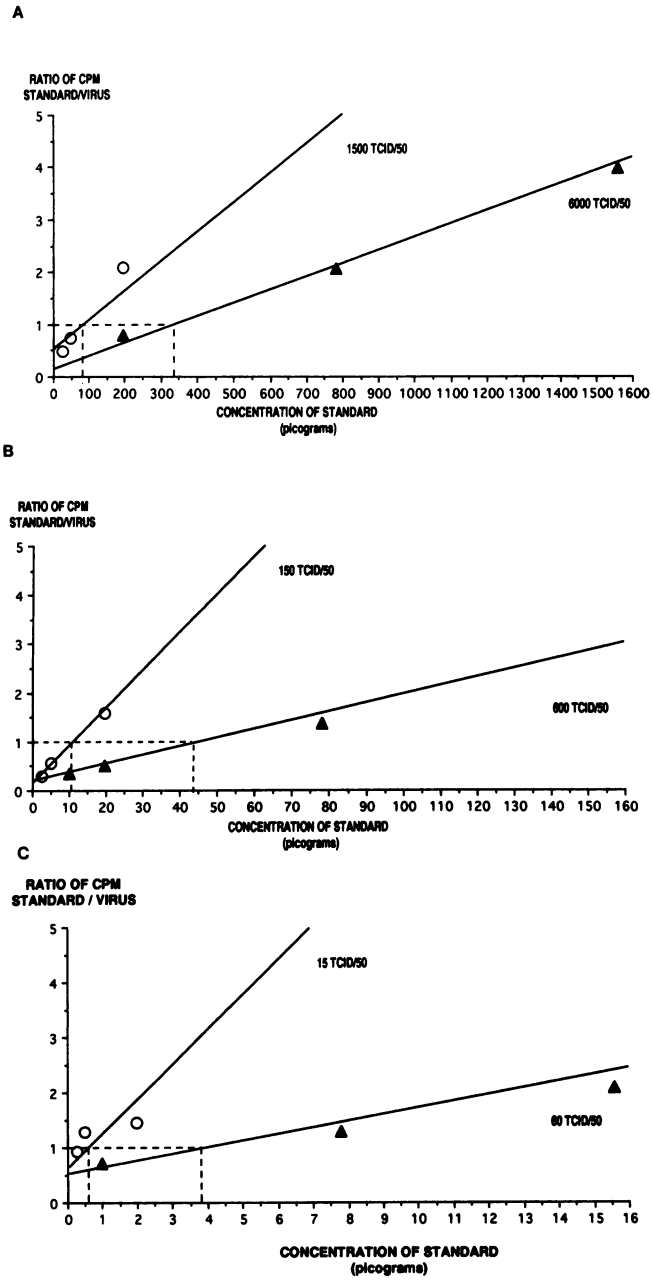

FIG. 4. Quantitative analysis of the competitive PCR experiment shown in Fig. 2 and 3. Duplicate samples were amplified in the presence of [α-^{32}P]dCTP, digested with *Bam*HI, and electrophoresed onto 1.6% EtBr–agarose gels. Bands corresponding to the viral and standard DNA were individually excised, and the amount of radioactivity was determined by scintillation counting. The ratio of radioactivity was plotted against the known concentration of the enteroviral standard in each sample tube.

transcription, and the conditions of PCR (7, 30). To circumvent these problems, we have used an RNA standard which is coamplified with the viral RNA. Introducing the standard template to the same reaction tube allowed us to internally control the variables associated with PCR detection of viral RNA in clinical specimens.

Although an internal standard can control for variables affecting the reaction mixture, the potential differences in amplification efficiency between the standard and viral templates must also be considered (7, 30). Compared with viral template, an unrelated standard may exhibit differences in melting temperature, optimal magnesium concentration, and primer specificity. Moreover, length and sequence variations can contribute to differential declines in reaction rates. We have avoided these problems by designing a standard RNA which is closely related to the amplified region of the enteroviral genome. Our standard is the same length and contains the same primer binding sites A and D as CVB3 genomic RNA, but it differs internally by 1 bp, creating the unique *Bam*HI restriction site. Since the standard and viral templates are virtually identical, the amplification efficiencies of the two should be equal. By this approach, we were able to compare the final yields of both PCR products and determine the starting quantity of viral RNA.

In the past, cell culture techniques have been used for quantitation of enteroviruses in clinical specimens (32). In CSF samples, the titers ranged from 10^1 to 10^3. Throat swabs and stool samples may have titers as high as 10^5 during the first week of infection; however, the most common quantities found during the following weeks of infection were in the range of 10^1 to 10^2 titerable viruses. Although these quantities are easily detected by growth in cell culture, this form of quantitation poses some difficulties. Quantitation can be laborious and slow, since there is no single cell culture system capable of detecting all enteroviruses. Within a single cell line, enteroviruses can show considerable variation in plaquing efficiency and the CVA viruses fail to produce a cytopathic effect in most culture systems.

Quantitation by using cell culture is also limited to clinical specimens containing titerable virus. However, there have been recent reports describing the persistence of nontiterable enteroviral RNA in chronic diseases such as dilated cardiomyopathy (14, 15), polymyositis (2), and chronic fatigue syndrome (33) and in experimental murine models of virus-induced heart disease (5, 31), and for these, a quantitative technique capable of detecting viral genome is required. In an elegant series of experiments, radiolabelled cDNA, RNA, and oligomeric DNA probes were hybridized to titered enterovirus or clinical specimens blotted onto nylon membrane filters (23–26); however, even the best of these methods detected only 10 pg of RNA. In situ hybridization, a more sensitive technique, has reportedly detected 10 to 100 enteroviral genomes (34), but it is technically difficult and not appropriate for rapid application to large numbers of clinical samples.

Quantitation of enteroviruses in clinical specimens by competitive PCR offers many advantages over the techniques listed above. In contrast to cell culture methodology, competitive PCR allows quantitation of both infectious virions and persistent but unculturable viral RNA and it can easily be performed within a single day. Moreover, the level of sensitivity surpasses that of immunoassay or nucleic acid probe hybridization.

In this study, we were able to quantitate CVB3 at titers as low as 15 $TCID_{50}$ (<1 pg). Our level of detection was consistent with the most common levels of virus reported for clinical specimens. We were also able to clearly distinguish between fourfold differences in viral titer, even at the level of 15 $TCID_{50}$. However, the method did appear to be limited to viral titers of at least 10^1 $TCID_{50}$, which may reflect the limits of PCR with an internal standard.

Quantitation of cellular RNA by competitive PCR has been reported previously (1, 7, 30). However, quantitation of

enteroviral RNA by competitive PCR requires consideration of several additional factors. First, although the standard can serve as a control during cDNA synthesis and PCR, it cannot control for the loss of enteroviral RNA which occurs during nucleic acid purification. In order to address the issue of reproducibility, we have quantitated 6,000 $TCID_{50}$ of CVB3 from two different preparations of RNA and found less than a twofold difference in quantity (results not shown). However, it is conceivable that the yield of RNA can vary with each preparation, particularly as reagents are replaced, or can depend on the method of RNA purification used. To control for this, one positive sample should be compared with a standardized graph.

Another factor to consider is that the standard quantitates at the 5′ end of the viral genome; however, there may be variability in the representation of this section, particularly in samples from tissues in which there is viral persistence. In such cases, variability may be caused by the specific degradation of RNA or by an abundance of incomplete or defective viral particles. The relative persistence of different regions of the enteroviral genome in infected tissues remains to be established.

Third, the engineered restriction site in the standard RNA is unique compared with known enteroviral sequences. However, since the RNA sequence of most enteroviruses remains unknown, one confirmational control in which the enterovirus is amplified in the absence of standard should always be run at the same time and subjected to enzyme digestion.

Quantitation of enteroviral RNA by competitive PCR can be routinely performed. The standard fragment has been cloned into *E. coli*; consequently, it is relatively easy to store, and can be grown as required in large quantities in most microbiology laboratories. We have also found that the transcribed standard RNA stored in aliquots at −70°C in 5 M ammonium acetate and 70% ethanol is very stable. However, the use of radioactivity for routine applications does warrant further consideration. We chose to do a radiolabel incorporation with [α-^{32}P]dCTP followed by electrophoresis, band excision, and direct scintillation counting because we felt that this method was the most accurate and the least time-consuming. There are alternative techniques for product analysis, though, particularly if scanners are available. Autoradiography followed by densitometry is one alternative, which also affords the option of performing the assay in the absence of radioactivity if a biotinylated primer is used in the PCR. Direct scanning of the EtBr-stained gels also circumvents the use of radioactivity, particularly when the gel bands show high levels of intensity and distinction.

The standard enteroviral template in routine clinical application serves as a valuable control during reverse transcription and PCR amplification of enteroviral RNA. Since the template is added directly to the test sample, then samples prepared in bad reagents or samples containing inhibitors of the amplification process (potential false-negative results) can be identified by their inability to amplify the standard template.

When used in a competitive manner, the standard template can quantitate RNA from a broad range of enteroviruses. Since the standard is based on genetic sequence, the vast antigenic variance among enteroviruses is no longer a hindrance. The standard RNA contains the sequences of the highly conserved 5′ NTR of the enteroviral genome. Known enteroviral sequences (CVB1, -3, and -4, and polioviruses 1, 2, and 3) (12, 13, 16–18, 29) and clones of several more serotypes (CVA16 and -21 and EV 25 and 34) (3) contain sites for primers A and D, and they exhibit considerable homology to the standard template. Combining these results with recent reports of PCR amplification from the 5′ NTR of many additional enteroviruses (3, 22, 35), it seems likely that as many as 60 serotypes, spanning the poliovirus, CVA, CVB, EV, and numbered enterovirus groups, may be quantitated by this technique.

This wide range of applicability might prove particularly useful for studying the pathogenesis of enteroviral infections. For example, the quantitative PCR assay could determine whether the amount of viral RNA is a clinical determining factor by correlation to either disease severity or patient outcome. This application would be most useful for determining the role of enteroviral RNA is chronic infections in which the viral genome cannot be quantitated by more conventional methods such as cell culture.

The standard template may also be used to evaluate the efficacy of new drug treatments. To date, there are very few treatments for enteroviral infection and for those available, such as gamma globulin, pretreatment is often required (19). Clearly, new therapeutic approaches towards enteroviral infection are needed. In a recent study, one promising agent, the calcium channel blocker verapamil, appeared capable of ameliorating viral murine myocarditis (6). Competitive PCR could prove useful in further examining the mechanism of action of this drug by monitoring viral RNA levels from very small samples over the course of treatment.

We conclude that quantitative PCR detection of enteroviruses by using panenteroviral primers and a mutated standard is feasible, accurate, and sensitive. This technique may have important application in the clinical diagnosis and prognosis of enteroviral infections.

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REFERENCES

1. **Becker-André, M., and K. Hahlbrock.** 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res. **17**:9437–9446.
2. **Bowles, N. E., C. A. Sewry, V. Dubowitz, and L. C. Archard.** 1987. Dermatomyositis, polymyositis, and coxsackie B virus infection. Lancet **i**:1004–1007.
3. **Chapman, N. M., S. Tracy, C. J. Gauntt, and U. Fortmueller.** 1990. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. J. Clin. Microbiol. **28**:843–850.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
5. **Cronin, M. E., L. A. Love, F. W. Miller, P. R. McClintock, and P. H. Plotz.** 1988. The natural history of EMC virus-induced myositis and myocarditis in mice: virus persistence demonstrated by *in situ* hybridization. J. Exp. Med. **168**:1639–1648.
6. **Dong, R., P. Liu, L. Wee, J. Butany, and M. Sole.** 1992. Verapamil ameliorates the clinical and pathological course of murine myocarditis. J. Clin. Invest. **90**:2022–2030.
7. **Gilland, G., S. Perrin, K. Blanchard, and H. F. Bunn.** 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA **87**:2725–2729.
8. **Higuchi, R., B. Krummel, and R. K. Saiki.** 1988. A general

method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16:**7351–7367.

9. **Ho, N. S., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
10. **Hyypia, T., P. Auvinen, and M. Maaronen.** 1989. Polymerase chain reaction for human picornaviruses. J. Gen. Virol. **70:** 3261–3268.
11. **Hyypia, T., C. Horsnell, M. Maaronen, M. Khan, N. Kalkkinen, P. Auzinen, L. Kinnunen, and G. Stanway.** 1992. A distinct picornavirus group identified by sequence analysis. Proc. Natl. Acad. Sci. USA **89:**8847–8851.
12. **Iizuka, N., S. Kuge, and A. Nomoto.** 1987. Complete nucleotide sequence of the genome of coxsackievirus B1. Virology **156:**64–73.
13. **Jenkins, O., J. D. Booth, P. D. Minor, and J. W. Almond.** 1987. The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the picornaviridae. J. Gen. Virol. **68:**1835–1848.
14. **Jin, O., M. J. Sole, J. W. Butany, W.-K. Chia, P. R. McLaughlin, P. Liu, and C.-C. Liew.** 1990. Detection of enterovirus RNA in myocardial biopsies from patients with myocarditis and cardiomyopathy using gene amplification by polymerase chain reaction. Circulation **82:**8–16.
15. **Kandolf, R., D. Amies, P. Kirschner, A. Canu, and P. H. Hofschneider.** 1987. *In situ* detection of enteroviral genomes in myocardial cells by nucleic acid hybridization: an approach to the diagnosis of viral heart disease. Proc. Natl. Acad. Sci. USA **84:**6272–6276.
16. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J.J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291:**547–553.
17. **La Monica, N., C. Meriam, and V. R. Racaniello.** 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. J. Virol. **57:**515–525.
18. **Lindberg, A. M., P. O. K. Stalhandske, and U. Petterson.** 1987. Genome of coxsackievirus B3. Virology **156:**50–63.
19. **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, Ltd., New York.
20. **Mullis, K. B., and F. A. Faloona.** 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. **155:**335–350.
21. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
22. **Rotbart, H. A.** 1990. Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol. **28:**438–442.
23. **Rotbart, H. A., M. J. Abzug, and M. J. Levin.** 1988. Development and application of RNA probes for the study of picornaviruses. Mol. Cell. Probes **2:**65–73.
24. **Rotbart, H. A., P. S. Eastman, J. L. Ruth, K. K. Hirata, and M. J. Levin.** 1988. Nonisotopic oligomeric probes for the human enteroviruses. J. Clin. Microbiol. **26:**2669–2671.
25. **Rotbart, H. A., M. J. Levin, and L. P. Villarreal.** 1984. Use of subgenomic poliovirus DNA hybridization probes to detect the major subgroups of enteroviruses. J. Clin. Microbiol. **20:**1105–1108.
26. **Rotbart, H. A., M. J. Levin, L. P. Villarreal, S. M. Tracy, B. L. Semler, and E. Wimmer.** 1985. Factors affecting the detection of enteroviruses in cerebrospinal fluid with coxsackievirus B3 and poliovirus 1 cDNA probes. J. Clin. Microbiol. **22:**220–224.
27. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
28. **Schmidt, N. J.** 1989. Cell culture procedures for diagnostic virology, p. 51–99. *In* N. J. Schmidt and R. W. Emmons (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 6th ed. American Public Health Association, Inc., Washington, D.C.
29. **Stanway, K. G., P. J. Hughes, R. C. Mountford, P. Reeve, P. D. Minor, G. C. Schild, and J. W. Almond.** 1984. Comparison of the complete nucleotide sequences of the genomes of the neurovirulent poliovirus P3/Leon/37 and its attenuated Sabin vaccine derivative P3/Leon 12a,b. Proc. Natl. Acad. Sci. USA **81:**1539–1543.
30. **Wang, A. M., M. V. Doyle, and D. F. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86:**9717–9721.
31. **Wee, L., P. Liu, L. Penn, J. W. Butany, P. R. McLaughlin, M. J. Sole, and C. C. Liew.** 1992. Persistence of viral genome into late stages of murine myocarditis detected by polymerase chain reaction. Circulation **86:**1605–1614.
32. **Wilfert, C. M., and J. Zeller.** 1985. Enterovirus diagnosis, p. 85–107. *In* L. M. de la Maza and E. M. Peterson (ed.), Medical virology IV: proceedings of the 1984 International Symposium on Medical Virology. Lawrence Erlbaum Associates, London.
33. **Yousef, G. E., E. J. Bell, G. F. Mann, V. Murugesan, D. G. Smith, R. A. McCartney, and J. F. Mowbray.** 1988. Chronic enterovirus infection in patients with postviral fatigue syndrome. Lancet **i:**146–149.
34. **Zhang, H. Y., G. E. Yousef, N. E. Bowles, L. C. Archard, G. F. Mann, and J. F. Mowbray.** 1988. Detection of enterovirus RNA in experimentally infected mice by molecular hybridisation: specificity of subgenomic probes in quantitative slot blot and *in situ* hybridisation. J. Med. Virol. **26:**375–386.
35. **Zoll, G. J., W. J. G. Melchers, H. Kopecka, G. Jambroes, H. J. A. van der Poel, and J. M. D. Galama.** 1992. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J. Clin. Microbiol. **30:**160–165.