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Identification of Enteroviruses in Clinical Specimens by Competitive PCR Followed by Genetic Typing Using Sequence Analysis

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A sensitive method based on competitive PCR was developed to detect and quantitate enteroviral RNA in clinical specimens, with special emphasis on controlling contamination and the presence of potential inhibitory factors in the specimens. Oligonucleotide primers from the conserved parts of the 5′ untranslated and VP2 capsid protein-coding regions were selected to differentiate between enteroviruses and rhinoviruses on the basis of the length of the cDNA amplicons. RNA transcribed from a truncated cDNA copy of the echovirus 11 genome was used as an internal control for the reverse transcription reaction and PCR. This allowed simple differentiation of the control and viral PCR products from each other by agarose gel electrophoresis and nonradioactive quantitation of the viral RNA in the clinical specimens. By direct sequencing of the PCR products and subsequent computer analysis of the data, potential laboratory contaminations could be monitored and enteroviruses from clinical samples could be grouped into four distinct clusters, thus enabling genetic typing of the viruses. The described method can be applied to the diagnosis and epidemiology of enteroviral infections.

Enteroviruses (polio-, coxsackie-, echo-, and enteroviruses 68 to 71) are a large group of human pathogens that cause a wide variety of clinical illnesses. The spectrum of diseases ranges from a mild febrile illness to myocarditis, meningoencephalitis, poliomyelitis, and neonatal multiorgan failure. Because infections caused by enteroviruses, particularly in children and neonates, cannot be clinically distinguished from diseases caused by other viruses or bacteria and because of possible therapeutic interventions by antipicornaviral agents in the near future, rapid and reliable diagnostic tests are needed.

Classical diagnostic methods, such as virus isolation followed by neutralization typing, are time-consuming and their results are often difficult to interpret. Furthermore, some enterovirus serotypes grow poorly in cell culture. Symptoms may also be postacute or chronic manifestations of enteroviral disease, and therefore, infectious virus may no longer be present. Serological diagnosis is complicated by the large number of serotypes and the high degree of prevalence of enterovirus antibodies in the general population. Nucleic acid hybridization assays have been developed, but they often lack sufficient sensitivity for the direct detection of viral RNA in clinical samples (17, 28).

Recent developments in molecular biology have enabled the detection of enterovirus genomes directly in clinical samples. Several research groups have developed assays, based on PCR, which can be used for the demonstration of virtually all the members of the enterovirus group by a single set of primers (5, 9, 11, 15, 23, 27, 33). These tests do not, however, allow typing of the virus strains, which would be highly important, e.g., for epidemiological purposes.

We describe here a competitive, nonradioactive PCR test followed by genetic typing of enteroviruses by DNA sequencing. By using a single set of primers, selected from the 5′ untranslated (5′-UTR) and VP2 capsid protein-coding regions of the picornavirus genome (9, 15, 23), human rhinoviruses can be differentiated from enteroviruses on the basis of the length of the PCR products in agarose gels (3, 23). By using a control RNA in competitive PCR, the known difficulties with PCR-based technologies, such as the influence of possible inhibitors in clinical specimens, the inefficiency of the reverse transcription (RT) reaction, and possible disturbing conditions in PCR (10, 32), can be controlled. Moreover, the amount of enteroviral RNA in clinical specimens can be determined. Finally, direct sequencing of cDNA amplicons allows genetic typing and epidemiological analysis of enteroviruses and, in addition, provides a means to monitor laboratory contaminations.

MATERIALS AND METHODS

Virus strains. Coxsackieviruses A9 (CAV9) and B4 (CBV4), poliovirus 3 (PV3), and human rhinovirus 2 prototype strains were originally obtained from the American Type Culture Collection (Rockville, Md.). They were propagated in LLC-Mk_2 or HeLa (human rhinovirus 2) cell cultures. CAV9 was purified as described earlier (2).

Clinical specimens. Clinical specimens were obtained from the specimen collection at the Department of Virology, University of Turku, where they had earlier been tested as part of the daily diagnostic routine. The samples consisted of 50 virus isolates and 20 original specimens. The virus isolates originated from stool (n = 28 samples), pharyngeal (n = 11), nasopharyngeal aspirate (n = 4), cerebrospinal fluid (n = 3), conjunctival secretion (n = 2), urine (n = 1), and vesicular fluid (n = 1) specimens. The 20 original samples (see Table 1) consisted of 7 fecal, 10 pharyngeal, 1 nasopharyngeal aspirate, and 2 conjunctival secretion specimens. Enteroviruses had originally been identified by isolation in appropriate cell cultures and then neutralization typing by using World Health Organization serum pools A to H and rhinoviruses by cell culture isolation and a subsequent acid lability test. The virus isolates had been stored for 5 to 10 years and the original specimens had been stored for 11 months to 4.5 years at −70°C prior to use in the present study.

RNA from purified CAV9 and total nucleic acids from the prototype viral stocks, clinical isolates, and original specimens was extracted by proteinase K–sodium dodecyl sulfate treatment (at concentrations of 100 μg/ml and 0.5%, respectively) at 37°C for 1 h; this was followed by phenol-chloroform extraction and ethanol precipitation (17). The dried precipitate was dissolved in sterile distilled water and was stored at −70°C prior to use.

Oligonucleotides. The oligonucleotides used as primers were derived from the conserved parts of the 5′-UTR and the VP2 capsid protein-coding regions of the enterovirus genome (see Fig. 1) (9, 15, 23). By using the fact that the 5′-UTR in

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human rhinoviruses is about 120 nucleotides shorter than that in enteroviruses (13, 26), human rhinoviruses could be differentiated from enteroviruses by a smaller size of the PCR product in agarose gels (3, 23).

Preparation of control RNA. Control RNA was prepared for competitive PCR and for controlling the presence of RNase activity and the enzyme inhibitors possibly copurified with total nucleic acids from the sample. For the preparation of control RNA, a truncated (nucleotides 535 to 2199) cDNA clone of echovirus 11 (EV11) (6) was digested by using *Eco*NI and *Bsp*MI restriction enzymes, resulting in a 214-bp deletion in the EV11 cDNA. The ends of the cDNA fragments were trimmed with the Klenow fragment of *Escherichia coli* DNA polymerase I and were ligated together. The truncated construct was linearized with *Cla*I and was transcribed to the RNA with T7 RNA polymerase (Promega Corp., Madison, Wis.). RNA was purified by digestion with RNase-free DNase (Promega); this was followed by phenol-chloroform extraction and ethanol precipitation. The control RNA was dissolved in sterile distilled water, and the quantity of RNA was determined by measuring the optical density at 260 nm. Finally, the RNA was divided into aliquots in 10-fold dilutions and was stored at −70°C.

RT and PCR. cDNA synthesis was performed in a reaction mixture containing 5 μl of sample nucleic acids, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphates (dNTPs); (Pharmacia, Uppsala, Sweden), 20 U of rRNasin RNase inhibitor (Promega), 0.1 nmol of the 5(−) primer (Fig. 1), and 100 U of Moloney murine leukemia virus reverse transcriptase (Promega) in a total volume of 40 μl (15). The reaction mixture was incubated at 37°C for 1 h.

Five microliters of the cDNA reaction product was added to the PCR mixture (total volume, 100 μl) containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs, 0.1 nmol of the 5(−) and 3(+) primers or the 5(−) and 4(+) primers (Fig. 1), and 1 U of thermostable DNA polymerase (Dynazyme; Finnzymes, Espoo, Finland) (15). The final mixture was overlaid with mineral oil and was amplified in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). After an initial 5 min of heat denaturation at 95°C, 40 cycles of denaturation, annealing, and DNA synthesis for 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C, respectively, were performed; this was followed by a final extension period of 10 min at 72°C. Ten microliters of the final reaction products were analyzed in 2% agarose gels containing 1 μg of ethidium bromide per ml.

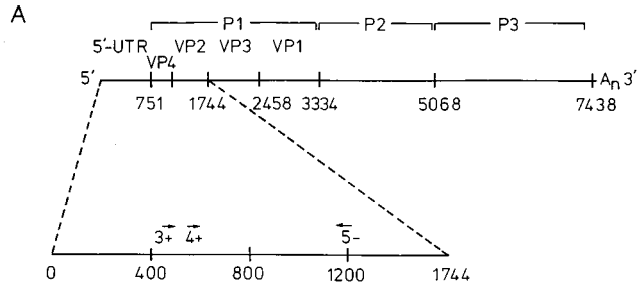
To avoid contamination, we used different rooms for handling specimens, reagents, and PCR products. RNA extractions were performed in a separate room and hood, as was pipetting for the RT reaction and PCR. All reagent mixtures were prepared in a clean room dedicated for that purpose only. The fourth room was used for agarose gel electrophoresis of the amplified material. UV light was regularly used to decontaminate the hoods and the clean room. We used separate pipettes, gloves, and coats during the various steps of the work.

Competitive RT-PCR. To study the feasibility of the competitive RT-PCR assay, serial 10-fold dilutions of CAV9 RNA were added to tubes containing a constant amount (10 fg) of the control RNA. CAV9 and the control RNA were reverse transcribed together to cDNA and were amplified by PCR through 40 cycles with the 5(−) and 4(+) primers. After agarose gel electrophoresis, the gel was photographed and the intensities of the bands corresponding to the control and CAV9 RNAs were measured by densitometric image analysis (MCID-M4, version 1.20; Imaging Research Inc., St. Catharines, Ontario, Canada).

To evaluate the relationship between input control RNA and calculated control RNA concentrations from the competitive PCR, 10-fold dilutions of the control were added to tubes containing a constant amount (10 fg) of CAV9 RNA. After competitive PCR and agarose gel electrophoresis, the intensities of the photographed bands were converted to relative areas by image analysis.

Finally, 10-fold dilutions (1 to 100 fg) of the control RNA were added to tubes containing unknown amounts of RNA from clinical specimens. After competitive PCR and agarose gel electrophoresis, the reactions in which the intensities of the sample bands were closest to those of the bands of the control were selected for quantitative analysis. The intensity ratio between the photographed control and specimen bands (expressed as relative areas after densitometric image analysis) was calculated, and subsequently, the amount of enteroviral RNA in the original specimens was determined by plotting the intensity ratio against the known concentrations of the control RNA.

Sequence analysis of amplified products. The cDNA amplicons from the clinical isolates and original specimens were purified by using the Qiaex DNA Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Nucleotide sequencing was carried out by using the dideoxynucleotide chain-termination method (29) with the 5(−) and 4(+) primers and modified T7 DNA polymerase (Sequenase version 2; United States Biochemical Corporation, Cleveland, Ohio) in the presence of ^{35}S-dATP precursor (Amersham, Buckinghamshire, United Kingdom). The sequence data were entered into a VAX computer manually by using the Genetics Computer Group (7) Seqed program. The Genetics Computer Group PileUp multiple sequence alignment program was used to generate dendrograms. Previously published enterovirus sequences used in the comparisons were obtained from the GenBank database.



B

Oligonucleotide	Sequence	Map position
primer 3+	5′- TCCTCCGGCCCCTGAATG-3′	449 - 466
primer 4+	5′- TACTTTGGGTGTCCGTGTTTC-3′	548 - 568
primer 5−	5′- GGCAACTTCCACCACCACCC-3′	1206-1187

FIG. 1. Oligonucleotide primers used in RT-PCR and nucleotide sequencing. (A) Schematic representation of positions and polarity of the primers in the 5′-UTR and VP2 capsid protein-coding regions of the enterovirus genome (6). The orientations of the primers are indicated by arrows. (B) Nucleotide sequences of the primers and their localization in the EV11 genome (6).

RESULTS

In order to develop an RT-PCR assay that could be used to detect enteroviruses directly from clinical material, we first wanted to exclude false-negative results possibly caused by inhibitory factors in the sample by using a control RNA, and, second, we wanted to monitor pre-PCR and crossover contaminations by sequencing of the PCR amplicons. There were two main criteria for the selection of the oligonucleotide primers used in the test: to distinguish between enteroviruses and rhinoviruses (3, 23) and to obtain accurate information for subsequent sequence analysis of the enteroviruses.

Sensitivity and specificity of RT-PCR. The sensitivity of the RT-PCR was first determined by using dilutions of CAV9 RNA and primer pair 5(−) and 4(+) and primer pair 5(−) and 3(+), representing sequences from the 5′-UTR and VP2 regions of the enterovirus genome (Fig. 1). By using the 5(−) and 3(+) primers, we obtained a longer (750-bp) amplicon which could have been more useful in subsequent sequence analysis. However, by using these primers, the sensitivity of the RT-PCR was 100 to 1,000 times lower (data not shown) than that obtained with the other primer pair. Therefore, the 5(−) and 3(+) primer pair was used only for the clinical specimens that were grown in cell culture and that were expected to contain sufficient amounts of viral RNA.

When the 5(−) and 4(+) primers and serial 10-fold dilutions of CAV9 RNA were used in the RT-PCR, a clearly visible band of the expected size (650 bp) was still seen in a dilution containing 1 fg of viral RNA, corresponding to approximately 240 viral genomes. All of the three picornavirus prototypes studied were successfully amplified with the primers: CBV4 and PV3 amplicons were visualized as single bands of 650 bp, whereas human rhinovirus 2 migrated as a readily smaller (530 bp) band in agarose gels (data not shown). Negative controls processed in each experiment through all of the steps from nucleic acid extraction to gel electrophoresis were negative.

Competitive RT-PCR. Serial dilutions (from 10 pg to 1 ag) of CAV9 RNA were first amplified in the presence of a constant amount (10 fg) of the control RNA by competitive RT-PCR. After agarose gel electrophoresis, the CAV9 product was seen to migrate as the upper band of the anticipated size (650 bp)

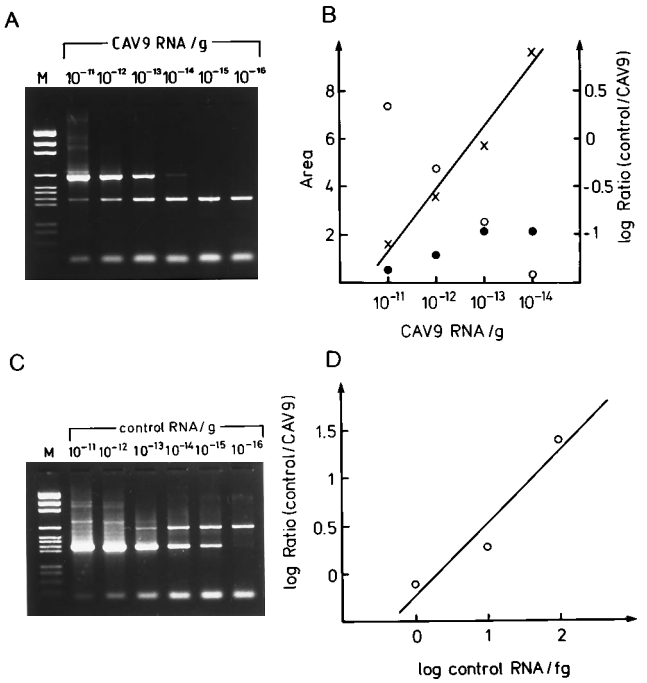


FIG. 2. Competitive PCR with purified RNA from the prototype CAV9 and control RNA transcripts prepared from a truncated cDNA clone of echovirus 11 (6). (A) Tenfold dilutions of CAV9 were added to tubes containing 10 fg of the control RNA. The amplification product of CAV9 (upper band) was clearly differentiated from that originating from the control RNA (lower band) in agarose gels. Lane M, DNA molecular weight marker. (B) Relative areas of the CAV9 (○), control (●), and the ratio of the control to CAV9 (×) after analyzing the intensities of the corresponding bands by densitometric image analysis. (C) Tenfold dilutions of the control RNA were added to tubes containing 10 fg of CAV9 RNA. Agarose gel electrophoresis of the amplified products is shown. Lane M, DNA molecular weight marker. (D) The ratio of the relative areas (control/CAV9), obtained after image analysis of the corresponding band intensities, plotted against the known concentrations of the control is shown on a log-log scale.

and was easily distinguished from the lower band (436 bp) representing the control (Fig. 2A). The 214-bp difference in the length of the cDNA amplicons most probably accounted for the more efficient amplification, and thus a more intensive band of the control in comparison with that of CAV9 when identical amounts were analyzed. As the amount of CAV9 RNA increased, so did the intensity of the upper band, while that of the lower band indicative of the control RNA simultaneously decreased slightly (Fig. 2A). When the intensities of the bands were subjected to densitometric image analysis and the areas thus obtained were plotted against the known concentrations of the CAV9 RNA, a linear relationship was seen between (i) the relative area of CAV9 and (ii) the ratio of the areas (control/CAV9) and the input concentrations of CAV9 RNA (Fig. 2B).

In order to evaluate quantitation of enteroviral RNA in clinical specimens by using the control RNA in the competitive PCR, dilutions of the control (from 10 pg to 1 ag) were amplified by competitive RT-PCR in the presence of a constant amount (10 fg) of CAV9 RNA (Fig. 2C). Again, there was a linear relationship between the ratio of the areas (control/CAV9) and the increasing concentrations of the control RNA, as shown on a log-log scale in Fig. 2D. However, more efficient amplification of the control was evident, indicating that relative rather than absolute values were obtained by the test.

Analysis of clinical isolates. In the next part of the study, we investigated the 50 cell culture-grown picornavirus isolates described above. Forty-eight (96%) isolates were successfully amplified in the RT-PCR when the 5(−) and 3(+) primers were used. Of the two negative specimens, one was EV23 which, in fact, was not expected to be amplified because of sequence differences between it and other picornaviruses (31). The other specimen, earlier diagnosed as PV3, was negative in repeated tests. Reisolation of this sample was also negative, suggesting degradation of the RNA during the storage and several freezings-thawings of the specimen. Forty-six of the positive samples gave an amplification product of 750 bp corresponding to enteroviruses, while two rhinovirus isolates, earlier identified by their acid labilities, migrated faster (630 bp) in agarose gels.

In order to genetically type enteroviruses and control the specificity of the RT-PCR, we first sequenced the amplicons derived from 30 cell culture-grown clinical isolates and compared the sequences with those of the known prototype strains. Readable sequences were obtained from all 30 isolates when the 5′-UTR was studied, whereas accurate sequences were achieved from 24 (80%) isolates when the VP2 capsid protein-coding region of the genome was investigated. Because there is variability in the sequences among different enterovirus isolates in the part of the 5′-UTR analyzed, these sequences could be used to monitor contamination and to confirm the specificity of the amplification. However, no useful grouping for genetic typing was obtained in this region (data not shown). On the other hand, sequences from the VP2 region of the prototype strains have been shown to group into four clusters (6, 14, 24, 25) (Fig. 3): cluster A contains CAV2 and CAV16; cluster B contains CAV9, CBV1, CBV3, CBV4, CBV5, EV11, and EV12; cluster C contains PV1, PV2, and PV3 together with CAV21 and CAV24; and cluster D contains enterovirus 70. In the present study, all of the isolates whose sequences (89 nucleotides) were determined were relatively closely related to the earlier sequenced prototypes and were shown to group into the four clusters (Fig. 3). All of the polioviruses grouped into cluster C; CAV16 grouped readily into cluster A; CAV9, all of the coxsackieviruses B, and all of the echoviruses were rather close relatives of the corresponding prototype strains and grouped in cluster B. There was no clear correlation between the sequence homology and the moment of collection of the specimens in this material, although no definite conclusions could be made because of the limited number of isolates whose sequences were determined.

Direct detection and typing of enteroviruses in clinical specimens. For direct amplification of viral RNA from clinical specimens, the 5(−) and 4(+) primers were used. Seventeen (85%) of the 20 specimens (Table 1) were successfully amplified and gave a product of 650 bp corresponding to enteroviruses in agarose gels. In repeated cell culture isolations, a cytopathic effect could be seen in 15 of the 20 samples. Two of the four cell culture-negative specimens were also negative in the PCR; two of the samples did not contain sufficient material for reisolation (Table 1).

For quantitation of enteroviral RNA in clinical specimens, total nucleic acids and empirically chosen two to three 10-fold dilutions (1 to 100 fg) of the control RNA were reverse transcribed and coamplified in the same test tube with the same primers. After agarose gel electrophoresis, the reactions in which the intensities of the bands were closest to each other were selected for quantitative analysis. The intensities of the bands were converted to relative areas by densitometric scanning followed by image analysis. The ratio of the areas (control/virus) was then calculated and used for quantitation of viral RNA in eight specimens. Accordingly, the amount of viral RNA ranged from 4.5 to 650 pg/ml in the original samples,

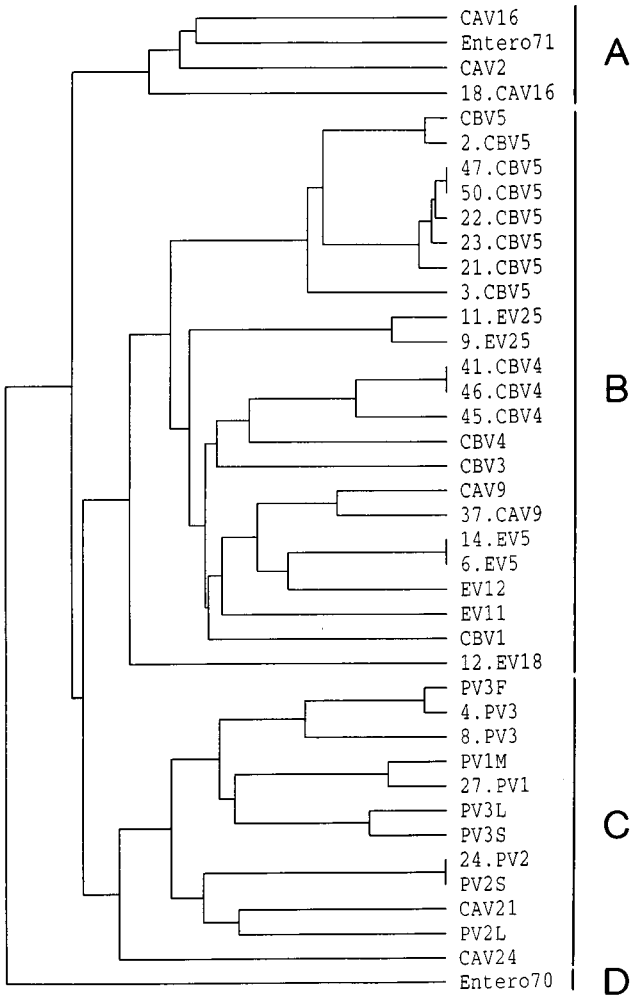


FIG. 3. Dendrogram generated with nucleotide sequences from the VP2 capsid protein-coding region of the enterovirus genome from clinical isolates. The sequences obtained in the present study are indicated by the sample number preceding the virus strain. Different genetic clusters (A, B, C, and D) are indicated. PV1M, PV1/Mahoney; PV2L, PV2/Lansing; PV2S, poliovirus 2/Sabin; PV3F, PV3/Finland; PV3L, PV3/Leon; PV3S, PV3/Saukett.

corresponding to 1×10^6 to 150×10^6 viruses per ml. When the PCR-negative original specimens (Table 1) were amplified in the presence of 10 fg of control RNA, they all gave a correct amplification signal excluding inhibition of the RT reaction and the PCR by factors in the sample, at least at the level of sensitivity studied. The result suggests degradation of viral RNA in these specimens during the storage and/or preparation of the samples. However, sequence variation between different virus strains at the primer recognition site as a cause of failure to amplify RNA in these specimens cannot be ruled out completely.

All of the cDNA amplicons from the original specimens were sequenced in both directions. When the 5-UTR and VP2 regions were studied, readable sequence was obtained from 11 and 13 of the 17 PCR-positive specimens, respectively. The sequences (99 nucleotides) clustered similarly to those representing the clinical isolates (Fig. 4). The two CAV16 specimens grouped into cluster A together with the prototype CAV2 and CAV16 strains and were more closely related to each other than to the CAV16 prototype. CAV9 and coxsackieviruses B were close relatives of the corresponding prototype strains and grouped together with all of the sequenced echoviruses into cluster B. All of the sequenced EV18 strains were seen close to each other in the dendrogram, while EV30 was relatively distantly related to other members of cluster B. CAV15 grouped into cluster C together with polioviruses, CAV21, and CAV24 (data not shown).

DISCUSSION

During the past few years, PCR-based technologies have proved very promising in the diagnosis of enteroviral infections, and they have the potential to gradually replace the conventional methods because of their superior sensitivity and rapidity. Furthermore, serotypes that grow poorly in cell cultures as well as replication-defective virus genomes can be detected by PCR. However, several problems need to be resolved before the assay system can be successfully used in the daily diagnostic routine. Special organization of laboratory space is necessarily needed in order to avoid contamination by material from other specimens and, in particular, previously amplified samples. Moreover, clinical specimens may contain RNases and factors that act as inhibitors in RT reactions and PCRs, giving rise to false-negative results. Finally, further information on the identities of the detected enteroviruses is often needed. In the present study, we have made an effort to solve these problems by developing a competitive PCR which is followed by sequence analysis of the cDNA amplicons.

Most PCR assays described for enteroviruses to date use primers from the conserved 5′-UTR of the picornavirus genome (15, 27). This allows for the detection of both enteroviruses and rhinoviruses by a single set of reagents. These primers, however, do not allow differentiation between the members of these two virus groups by agarose gel electrophoresis. To circumvent this problem, we used previously described primers from the conserved 5′-UTR and VP2 capsid protein-coding regions of the picornavirus genome (9, 15, 23) that distinguish between enteroviruses and rhinoviruses on the basis of the lengths of their cDNA amplicons (3, 23). Furthermore, use of the VP2 primer allows for sequencing of a part of the capsid protein-coding region that can be used for genetic typing of enteroviruses. The amplicons obtained were long enough for sequence analysis, and the level of sensitivity (1 fg of enteroviral RNA was clearly detectable in agarose gels) of the test was comparable to that obtained in earlier studies (9, 23). The sensitivity could be further increased by different hybridization methods (11, 18, 21) or by using nested PCR methodologies (19, 30). Since nested PCR is particularly prone to contaminations and labeled probes are needed for the hybridization assays, we wanted to avoid these additional procedures because the level of sensitivity obtained in the present study was considered sufficient for studying clinical material, at least that from patients with acute infections.

Quantitation of amplified products, and thus determination of the amount of viral RNA or DNA in clinical specimens, could have important applications in virology. In future, quantitation will be essential for monitoring the efficacy of antiviral therapy. It could also provide new information about the pathogenesis of infections, e.g., whether infection of secondary target organs depends on the degree of viremia and if the amount of viral RNA or DNA correlates with the severity or prognosis of the disease. In recent years, several quantitative PCR methods have been developed. A major problem in their use has been the restriction of the PCR analysis to the exponential phase of amplification (32). In addition, in assays with unrelated or noncompetitive control RNAs, unequal amplification of the templates because of possible differences in their melting temperatures, optimal magnesium concentration,

TABLE 1. Results of cell culture isolation and neutralization typing, reisolation, and PCR for original specimens

No.	Specimen[a]	Enterovirus serotype originally identified	Reisolation	PCR
1	F	CAV16	+	+
2	F	CAV9	+	+
4	PS	CBV3	+	+
5	PS	CAV16	+	+
7	F	EV9	+	+
8	PS	EV18	+	+
9	F	EV30	NT[b]	+
10	F	CAV15	+	+
12	PS	CBV3	−	+
13	F	CBV5	+	+
15	PS	CBV5	+	+
16	PS	CAV9	+	+
22	C	EV15	−	−
24	PS	EV30	+	+
32	PS	EV18	+	+
42	C	CBV4	−	−
52	NPA	CBV5	+	+
72	PS	EV9	NT	−
82	F	EV30	+	+
92	PS	EV18	+	+

[a] F, fecal specimen; PS, pharyngeal specimen; NPA, nasopharyngeal aspirate; C, conjunctival specimens.
[b] NT, not tested.

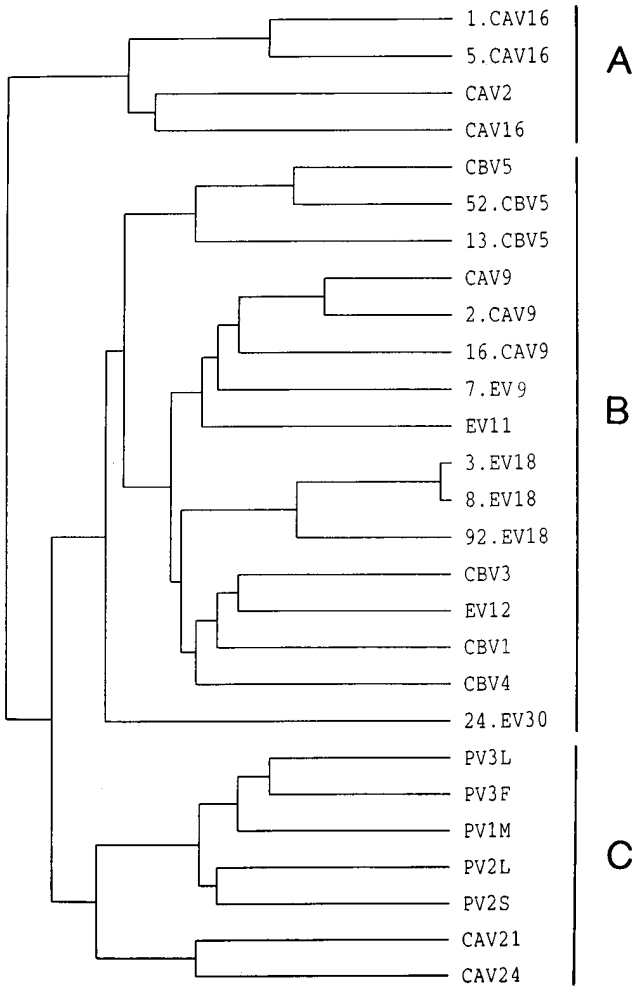


FIG. 4. Dendrogram generated with nucleotide sequences from the VP2 protein-coding region of the enterovirus genome from original specimens. The sequences obtained in the present study are indicated by the sample number preceding the virus strain. A, B, and C indicate different genetic clusters. PV1M, PV1/Mahoney; PV2L, PV2/Lansing; PV2S, PV2/Sabin; PV3F, PV3/Finland; PV3L, PV3/Leon.

primer length, specificity, and sequence has made accurate quantitation impossible (12, 22). These problems can be avoided by using competitive PCR with control RNA that is similar to the RNA of interest (4, 10). In competitive PCR, the ratio of the unknown product to the competitor product has been shown to remain constant through the amplification process and is not dependent on cycle number or changes in any of the variables mentioned above (4, 10). The first study to quantitate enteroviral RNA by competitive PCR was reported by Martino et al. (20). It was based on the construction of a panenteroviral standard, which was differentiated from the viral amplicon by enzymatic digestion, resulting in a smaller fragment in agarose gels and subsequent counting of the radioactivity of the bands excised from the gel. By that approach, 15 50% tissue culture infective doses could be detected and a fourfold difference in viral titers could be clearly distinguished (20).

The competitive PCR assay described here provides a relatively simple and rapid method for the nonradioactive quantitation of enteroviral RNA in clinical specimens. By RT and amplification of the viral template and the control in the same test tube, variable effects due to differences in reaction conditions are controlled and affect similarly the yields of the two PCR products. Inhibitors in the specimen can be identified by the inability of the test to amplify the control. Furthermore, the control RNA could serve as a useful indicator of potential RNA loss during storage and nucleic acid extraction of the specimen. Failure to amplify RNA from clinical specimens could, of course, also be due to sequence variation between different virus strains at the primer recognition site. This alternative explanation, however, is less evident since we have shown that virtually all the echovirus prototype strains (except EV22 and EV23) (14) and 23 of the 25 coxsackievirus A prototype strains (25) can be amplified with the same primers as used in the present study. In the present study, the amount of enteroviral RNA in clinical specimens, determined by the intensity ratio between the bands of the amplified control and viral DNAs, varied from approximately 1×10^6 to 150×10^6 genomes per ml of specimen. The test was not absolutely quantitative, evidently because of the length difference (214 bp) between the control and viral templates. However, it provides an idea of the viral concentrations in clinical specimens and could readily be used for determination of the differences in the amount of viral RNA in serial samples, e.g., during viral chemotherapy.

Today, the complete genomic sequences of more than a dozen different enterovirus serotypes are known. This makes it possible to analyze whether the present subgrouping and serotyping based on phenotypic and antigenic characteristics could be replaced by genetic typing. The latter would be desirable since it would provide exact criteria for the identification of virus isolates and because diagnostic procedures are increasingly based on PCR. It has earlier been shown that human enteroviruses fall into four genetic clusters when the VP2 protein-coding region is analyzed: cluster A contains CAV16 and related coxsackieviruses A; cluster B contains CAV9, coxsackieviruses B, and the majority of echoviruses; cluster C contains polioviruses and certain coxsackieviruses A (including serotypes 21 and 24); and cluster D contains entero-

virus 70 (6, 24). In addition, recent studies have shown by partial sequencing of the VP2 region of virtually all of the enterovirus prototype strains that this clustering could be used as the basis of molecular classification of the whole group (14, 25), Moreover, EV22 and EV23 have been shown to be genetically distant from enteroviruses, and, at least in molecular terms, they form a distinct picornavirus group (16, 31). The cluster information will be sufficient for most clinical applications; detailed sequence information can be used for exact epidemiological studies. The different steps of the assay protocol have the potential for automation, including interpretation of the results, which is one of the future trends in PCR-based diagnostic methodologies. One practical problem, however, concerns the appearance of certain coxsackieviruses A in the same cluster as polioviruses. Although accumulating data on the sequences of these coxsackieviruses A may reveal specific sequences that could be used to differentiate between these two virus groups, additional PCR or hybridization procedures are currently needed for the specific identification of polioviruses (1, 8, 23).

We sequenced a number of enterovirus strains from cell culture-grown and original clinical specimens. Readable sequence was obtained from all of the clinical isolates and from the majority of original specimens. However, some of the sequences could not be accurately interpreted, suggesting that optimization of sample preparation in various steps preceding sequencing is still needed. All of the enterovirus serotypes sequenced in the present study were relatively closely related to the earlier sequenced prototype strains. The 5′-UTR sequences could be used to confirm the enterovirus specificity of the assay and to exclude contaminations due to the sequence variation in picornavirus genomes. However, they were not otherwise useful, because no such clustering that could be used for genetic typing of enteroviruses was seen. On the other hand, the sequence analysis of the VP2 protein-coding region revealed four genetic clusters into which all of the sequences studied could clearly be divided. These results indicate that genotypic classification of enteroviruses could be used in the clinical diagnosis of enteroviral infections.

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