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Detection of Enteroviruses and Rhinoviruses in Clinical Specimens by PCR and Liquid-Phase Hybridization

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A sensitive method based on PCR followed by liquid-phase hybridization for detection of enterovirus and rhinovirus RNAs in clinical specimens and cell culture supernatants is described. RNA was extracted from stool samples, throat swabs, nasopharyngeal aspirates, cerebrospinal fluid, urine, and plasma with a commercial phenol-guanidinium-chloroform reagent and purified on a polysulfone membrane, on which the reverse transcriptase reaction was also done. Two sets of oligonucleotide primers from the 5′ noncoding region of picornaviruses were selected for DNA amplification of 153-bp (enterovirus) and 120-bp (rhinovirus) regions. Double-stranded amplicons were digested into single strands with T7 gene 6 exonuclease and quantitated by an assay using a europium-labeled probe, streptavidin- and biotinylated probe-coated microtitration wells, and time-resolved fluorometry. The sensitivity of the assay was about one template molecule when purified coxsackievirus A9 RNA was used. All enterovirus prototype strains, except echoviruses 22 and 23, and clinical isolates grown in cell culture or suckling mice were strongly positive by the enterovirus PCR-hybridization, as were selected prototype strains and untyped isolates of rhinoviruses by the rhinovirus PCR-hybridization. In a series of 100 clinical specimens tested, the results for 92 agreed with virus culture results. The detection method described will be useful in etiopathogenic studies on enteroviruses and rhinoviruses.

Conventional laboratory diagnosis of enterovirus infections includes isolation of the virus in cell culture, less frequently in suckling mice, followed by neutralization typing. The diagnosis can be supported by serology, mainly on the basis of a titer increase between acute- and convalescent-phase serum specimens or detection of immunoglobulin M antibody. Rhinovirus diagnosis is done almost exclusively by culture of the virus followed by acid lability testing. Serology is complicated by more than 100 serotypes. Diagnosis can be delayed because many enteroviruses and rhinoviruses cause cytopathogenic changes in cell cultures only during prolonged incubation, particularly in the first passage. Increased information on enterovirus and rhinovirus sequences (26, 35) has made possible a new and more rapid approach to this diagnostic problem by detection of the viral genome directly in clinical specimens by the PCR. The first application of this technology was for detection of rhinovirus in nasal washes (9, 10). Later, methods were developed to amplify highly conserved sequences present in both viruses followed by a hybridization assay that differentiated between the groups, first with cell culture isolates (15) and then with clinical specimens (25). A number of groups have now reported the use of PCR for direct detection of enteroviruses (1, 14, 18, 24, 27, 31, 33, 38, 41, 43) and rhinoviruses (3, 17, 18, 39) in clinical specimens.

In the present report, we describe the development of a highly sensitive nonnested PCR assay followed by liquid-phase hybridization for detection of enteroviruses and rhinoviruses directly in clinical specimens. The selection of primers and probes was based on previously reported studies on the nucleotide sequences of human picornaviruses. The PCR format was a combination of those used for identification of influenza A virus (30) and poliovirus (41, 42) isolates. The basic concept of liquid-phase hybridization was adapted from a series of studies reported previously (5, 8, 23, 29, 32, 37) and used in our earlier study on PCR detection of adenoviruses (11), which includes quantitative adsorption of the hybrids of single-strand amplicons and Eu^+-labeled probes on microtiter wells precoated with streptavidin and a biotinylated probe. Novel developments described in the present report include combining a simple purification method for RNA extracts, reverse transcription of purified RNA on a membrane, enzymatic digestion of double-stranded amplicons into single strands (12) for hybridization, and further optimization of the liquid-phase hybridization procedure.

MATERIALS AND METHODS

Viruses and clinical specimens. Working stocks of prototype enteroviruses were prepared in primary monkey kidney cells, LLC-MK_2 cells, or suckling mice, and those of rhinoviruses were prepared in HeLa (Ohio) cells (2). Enterovirus isolates were grown in LLC-MK_2 cells, and rhinovirus isolates were grown in HeLa cells. Enterovirus clinical specimens consisted of 10 stool samples, 11 throat swabs, 12 nasopharyngeal aspirates (NPA), three cerebrospinal fluid samples, two urine specimens, and one plasma sample (Respiratory and Enteric Viruses Branch, Centers for Disease Control and Prevention). Rhinovirus clinical specimens included five throat swabs from the Centers for Disease Control and Prevention and 58 NPA from the specimen library of the Department of Virology, University of Turku (kindly provided by Thedi Ziegler). NPA specimens were stored at −20°C; all other specimens were stored at −70°C.

Extraction of sample RNA. Viral RNA was extracted from cell cultures and clinical specimens with Ultraspec (Cinna/Biotecx Laboratories Inc., Houston, Tex.). After careful premixing of the reagent, 300 μl of Ultraspec, 80 μl of chloroform, and 100 μl of the sample to be tested were vortexed in a 1.5-ml Eppendorf tube on a rack shaker for 2 min, cooled in ice water for 5 min, and centrifuged at 13,000 × *g* for 15 min at 4°C by using a fixed-angle rotor (TMA-11) in a Tomy MTX-150 centrifuge (Tomy Tech USA, Inc., Palo Alto, Calif.). After centrifugation, 200 μl of the aqueous phase was collected and RNA was precipitated by adding an equal volume of cold isopropanol and kept at −20°C for 30 min. The precipitate was collected by centrifugation at 12,000 × *g* for 10 min at

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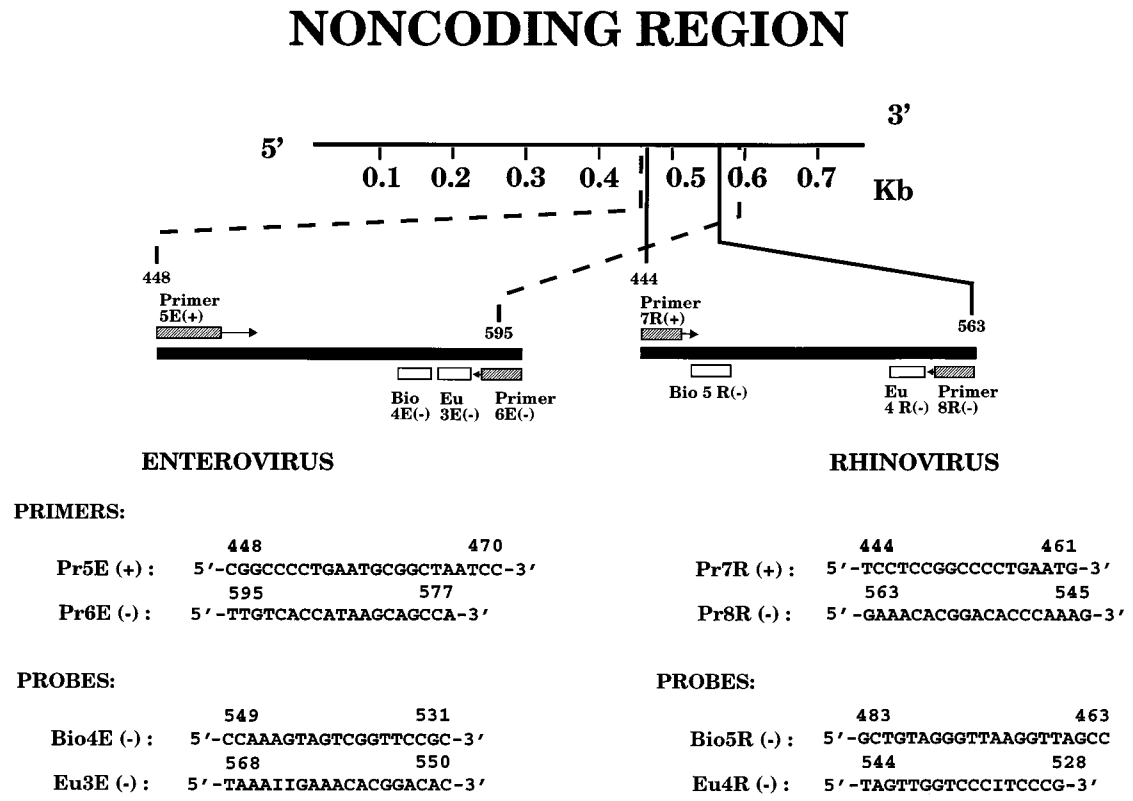

FIG. 1. Locations and sequences of enterovirus and rhinovirus primers and probes in the picornavirus 5′ noncoding region of the genome. The nucleotide positions in the enterovirus sequences correspond to those of poliovirus type 1 strain Mahoney (28), and those in the rhinovirus sequences correspond to those of rhinovirus 1B (13). Kb, kilobases.

4°C. The isopropanol was discarded; the RNA pellet, with about 30 μl of residual isopropanol, was then dissolved in 100 μl of distilled water at 56°C for 15 min.

Removal of inhibitors from RNA extracts. The dissolved RNA was diluted with 170 μl of Tris-EDTA buffer (10 mM Tris-HCl, 50 μM EDTA, pH 7.2). One half was stored at −70°C for later use, and the other half was filtered through an Ultrafree-MC 10,000 NMWL filter unit with polysulfone type PTGC membrane (UFC3-TGC-000; Millipore Corp., Bedford, Mass.). After centrifugation at 13,000 × *g* for 10 min at 4°C, the RNA retained on top of the filter (11) was washed once with 200 μl of Tris-EDTA buffer and centrifuged as described above. Immediately after this washing, the reverse transcriptase reaction was done in these filter units as described below.

Primers and probes. Primers and probes were designed from the sequences in the 5′ noncoding region of picornaviruses (15). Primers and probes were selected from the consensus regions of enteroviruses and rhinoviruses and shared some homology between these two virus groups. The locations and sequences of the enterovirus (153-bp) and rhinovirus (120-bp) regions and primers are shown in Fig. 1.

We labeled two oligonucleotide probes (Bio4E and Bio5R) with biotin by incorporating 1-dimethoxytrityloxy-Biotin-C6-PA (Cambridge Research Biochemicals Inc., Wilmington, Del.) at the 3′ end during standard synthesis on a 380B ABI DNA synthesizer (Applied Biotechnology, Inc., Foster City, Calif.). The 1-dimethoxytrityloxy group was retained on the biotinylated oligonucleotide to facilitate purification by reverse-phase high-performance liquid chromatography (4). Probes Eu3E and Eu4R contained 20 modified deoxycytidines at the 5′ end (36) and were labeled with europium as described previously (8). All probes were stored at −70°C, and diluted stocks (2 μg/ml in Tris-EDTA buffer, pH 7.2) were kept at 4°C for several weeks.

Reverse transcriptase and PCR. The reverse transcriptase reaction and PCR were done essentially as previously described (30). For the former reaction, RNA retained on the filter was resuspended in 25 μl of 4× PCR buffer (Gene Amp Kit, Perkin-Elmer/Cetus Corp., Norwalk, Conn.), 800 μM deoxynucleoside triphosphates, 25 U of RNase inhibitor (Boehringer GmbH, Mannheim, Germany), 50 pM of a negative-strand primer (Pr6E or Pr8R), and 10 U of the avian myeloblastosis virus transcriptase enzyme (Boehringer GmbH). After incubation at 42°C for 60 min, the reaction mixture (cDNA) was diluted to 100 μl with sterile distilled water containing 50 pmol of a positive-strand primer and 5 U of *Taq* polymerase (Perkin-Elmer/Cetus) and transferred to a PCR tube. Two drops of mineral oil were added, and the tubes were incubated in a DNA Thermal Cycler (Perkin-Elmer/Cetus), first for 3 min at 94°C, then through 40 cycles of programmed amplification (denaturation, 94°C, 30 s; annealing, 53°C, 45 s; extension, 72°C, 1 min), and finally for 7 min at 72°C (42). Each PCR experiment included a positive control and several negative controls, intercalated throughout the samples examined. The PCR amplicons, in 10-μl volumes, were analyzed on 1.8% agarose gels at 120 V for 1 h with 0.5 μg of ethidium bromide per ml in the electrophoresis running buffer and by the hybridization assay described below.

To avoid contamination of the samples, three rooms and two hoods were used in the PCR work. All reagent mixtures were prepared in a separate room in a clean hood. All RNA extractions and pipetting with positive-replacement pipettors were done in another room and hood, and the third room was used for agarose gel work. Contamination of the negative controls was found only twice, each time after purified, highly concentrated coxsackievirus A9 RNA was pipetted.

Biotinylation of bovine serum albumin (BSA), coating of microtitration strips, and saturation with streptavidin and biotinylated probes. BSA (GIBCO/BRL, Gaithersburg, Md.) was purified, biotinylated (EBP-406; Enzo Biochemical, Inc., New York, N.Y.), and used for coating of microtitration strips (1 × 12 well Microstrip PS; catalog no. 9502107; Labsystems, Helsinki, Finland). The coated strips were saturated with streptavidin (Boehringer GmbH) as previously described (11). The strips, previously coated with streptavidin, were saturated with a biotinylated probe (Bio4E or Bio5R), diluted to 15 to 60 ng/ml in DELFIA Assay Buffer 1244-106 (Tris-HCl-buffered NaCl solution [pH 7.8] containing 0.05% NaN_3, BSA, bovine gamma globulins, Tween 40, diethylenetriaminepentaacetic acid, and an inert red dye; Wallac Oy, Turku, Finland). A diluted probe (200 μl per well) was incubated for 1 h at ambient temperature. Finally, the strips were washed six times with a commercial wash solution (DELFIA 1244-144; Wallac Oy) in an automated Platewash apparatus (model 1296-024; Wallac Oy) and stored in sealed plastic bags with moist paper for several weeks at 4°C.

Digestion of PCR amplicons with exonuclease. The double-stranded PCR amplicons were digested into single strands with T7 gene 6 exonuclease (United States Biochemical, Cleveland, Ohio). The positive strand was protected from digestion by phosphorothioate analogs incorporated in the primer, and only the complementary strand, which competes with the probes, was digested (12). The enzyme was first diluted in 2× buffer (80 mM Tris-HCl [pH 7.5], 40 mM $MgCl_2$, 100 mM NaCl, 2 mM dithiothreitol) to contain 1.5 U/μl; 5 μl of this enzyme dilution was then mixed with an equal volume of the PCR amplicon. The mixture

was incubated for 10 min at 37°C and then for 10 min at 75°C to inactivate the enzyme.

Hybridization assay. The design of the hybridization reaction is a slight modification of that described previously (11), which uses biotinylated BSA and streptavidin-precoated microtiter wells. Two oligonucleotides are used for hybridization, one labeled with biotin to absorb the hybrids to the solid phase and a second labeled with Eu^{+}-chelate for detection. For hybridization, the digested amplicons were diluted to 100 μl with 2× hybridization solution (DELFIA Assay Buffer 1244-106 with an additional 2.92 g of NaCl per 50-ml bottle) containing 3 to 10 ng of the Eu-labeled probe per ml. The diluted amplicon-probe mixture was added to the biotinylated probe-coated wells and then incubated for 1 h at ambient temperature. The washing steps, addition of the enhancement solution, and measurement of europium fluorescence were done as previously described (11). Results were expressed as counts per second. Each hybridization test included a positive control, consisting of a pool of PCR products from selected prototype strains of enteroviruses or rhinoviruses, and a negative control, consisting of a similar pool of negative specimens. The cutoff value for a positive specimen was 10 times the background value obtained with diluent controls.

RESULTS

Selection of primers and probes. Although it was possible to identify primers which are able to produce detectable amplicons from both enteroviruses and rhinoviruses when visualized by gel electrophoresis and staining (15), attempts to find primers with consensus sequences for both enteroviruses and rhinoviruses which functioned with group-specific probes for identification of the amplicons with the hybridization detection system described here were unsuccessful. For example, amplicons were made with primers Pr2(+);162-182 and Pr3(−);443-462 but no reactivity was detected in liquid-phase hybridization with these 313-bp amplicons when a biotinylated probe (5′-CATTCAGGGGCCGGAGGA-3′) and an Eu-labeled probe (5′-GGCCGCCAACGCAGCC-3′) were used. It was concluded that these amplicons were too long for the liquid-phase hybridization assay and possibly had formed secondary structures that prevented hybrid formation with the probes. Therefore, separate sets of primers and probes were made for enteroviruses and rhinoviruses. Although the new rhinovirus primer pair contained consensus sequences to amplify both groups of viruses, separate PCR primer sets were chosen to include group-specific probes for identification of enterovirus and rhinovirus amplicons. High counts (more than 100,000 counts per second) were immediately obtained when these new 153- and 120-bp amplicons were used with new probes. The locations and sequences of the primers and probes are shown in Fig. 1.

Testing of prototype strains and isolates. All available enterovirus prototypes (65 serotypes) and selected rhinovirus prototypes and isolates were tested with enterovirus primers and probes (Table 1). All enterovirus prototypes were strongly reactive, except for echoviruses 22 and 23, and the positive/negative ratios (calculated from the negative-control counts) were often 100 or higher. The nonreactivity of echoviruses 22 and 23 was expected, since these viruses are only distantly related to enteroviruses and evidently represent an independent picornavirus group (6, 16). In addition, 23 isolates of enteroviruses from the years 1967 to 1992 were tested by the enterovirus PCR-hybridization assay; all were strongly positive (data not shown), indicating that no major changes occurred in this conserved region of the enterovirus genome during this period. Some rhinoviruses, particularly types 38 and 48, were also reactive in the enterovirus assay, but the counts were lower than with enteroviruses.

Non-picornaviruses, such as respiratory syncytial virus; parainfluenza virus types 1, 2, 3, and 4; herpes simplex virus type 1; and adenovirus (untyped), were all negative in enterovirus and rhinovirus PCR assays when analyzed by gel electrophoresis and hybridization (Table 1). Hepatitis A virus was also nonreactive by enterovirus PCR-hybridization.

TABLE 1. Specificity of enterovirus PCR-hybridization tests determined with prototype strains of enteroviruses, selected rhinoviruses, and some non-picornaviruses

Virus (serotype[s])	Specimen source	Cps[a] range
Poliovirus (1–3)	Cell culture	117,925–303,190
Echovirus (1–6, 8, 9, 11–21, 24–27, 29, 31–33)	Cell culture	107,602–643,621
Echovirus (22)	Cell culture	2,628
Echovirus (23)	Cell culture	8,646
Coxsackievirus A (2, 4–6, 8–10, 16, 21, 24)	Cell culture	154,288–480,930
Coxsackievirus A (1, 3, 7, 11–15, 17, 19, 20, 22)	Suckling mice	200,924–462,079
Coxsackievirus B (1–6)	Cell culture	280,988–595,122
Enterovirus (68–71)	Cell culture	141,782–308,184
Rhinovirus (1B, 29, 36, 38, 48)	Cell culture	1,023–96,899
Hepatitis A virus	Cell culture	1,376
Adenovirus-coronavirus	Cell culture	404–2,119
Rous sarcoma virus-parainfluenza virus (1–4)	Cell culture	1,079–12,622
Herpesvirus type I	Cell culture	38,380
Negative control		1,215–10,461
Positive control		401,709–602,909

[a] Cps, counts per second.

The selected rhinovirus prototypes tested by rhinovirus PCR-hybridization were also strongly positive, and there was no reactivity with the five enteroviruses tested (Table 2). However, the rhinovirus PCR primers, as expected, also amplified the enteroviruses tested (Fig. 2), but these amplicons did not hybridize to the rhinovirus-specific probes, and therefore the resulting signal was negative.

Testing of clinical specimens. In limited preliminary tests with clinical specimens, all culture-positive enterovirus specimens were positive by enterovirus PCR-hybridization and all culture-negative specimens were negative (data not shown). The positive/negative ratios were as large as those obtained from prototype strains grown in cell culture, indicating that the source of the specimen had no effect on the test results. However, this is not direct evidence of total removal of inhibitors in clinical specimens since the amount of viral RNA in the specimens was not known. All culture-positive rhinovirus specimens were positive by rhinovirus PCR-hybridization, but one of three culture-negative specimens was strongly positive and remained positive in repeated tests.

Comparisons of PCR-hybridization results with the original isolation tissue culture results for an extended panel of clinical

TABLE 2. Specificity of rhinovirus PCR-hybridization tests of selected prototype strains of rhinoviruses and enteroviruses

Virus (serotypes)	Specimen source	Cps[a] range
Rhinovirus (1A, 1B, 2, 3, 9, 11, 12, 14, 22, 29, 36, 38, 39, 48, 89)	Cell culture	23,836–284,337
Enterovirus (echovirus 5, 18, 30; enterovirus 70, 71)	Cell culture	140–944
Negative control		209–958
Positive control		77,347–194,902

[a] Cps, counts per second.

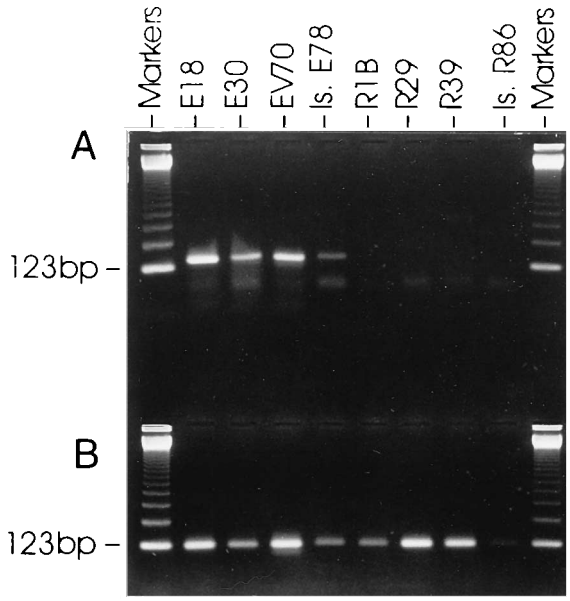

FIG. 2. Cross-reaction of the enterovirus and rhinovirus PCR. The 1% agarose gel shows 1/10 of the PCR amplification products of some enterovirus and rhinovirus specimens obtained with the enterovirus primer pairs (A) and the rhinovirus primer pairs (B). DNA molecular size markers are a 123-bp ladder (5613 SA; GIBCO/BRL). The enteroviruses included are the prototype strains for echovirus 18 (E18), echovirus 30 (E30), enterovirus 70 (EV70), and a clinical isolate of echovirus 5 from 1978 (Is. E78). The rhinoviruses included are the prototype strains for rhinovirus 1B (R1B), rhinovirus 29 (R29), rhinovirus 39 (R39), and a clinical isolate of an untyped rhinovirus from 1986 (Is. R86).

specimens are summarized in Tables 3 and 4. In enterovirus PCR-hybridization assays, 20 of 22 culture-positive specimens were positive and all 15 culture-negative specimens were PCR negative. In rhinovirus PCR-hybridizations, 36 of 37 culture positive specimens were positive and 5 of 26 culture-negative specimens were PCR positive.

TABLE 3. Comparison of the enterovirus PCR-hybridization assay with virus culture for detection of enteroviruses in clinical specimens

Specimen type[a]	Reference test (culture) result[a]	No. of specimens	No. positive	Cps[b] range
CSF[c]	+	3	3	156,564–406,251
CSF	−	0		
Stool	+	5	4[d]	293,913–561,183
Stool	−	3	0	2,265–13,152
NPA	+	1	1	637,137
NPA	−	11	0	404–8,441
Throat swab	+	10	10	180,432–686,351
Throat swab	−	1	0	32,772
Urine	+	2	1[e]	204,524
Urine	−	0		
Plasma	+	1	1	315,875
Plasma	−	0		

[a] +, growth in tissue culture; −, no growth.
[b] Cps, counts per second.
[c] CSF, cerebrospinal fluid.
[d] One stool positive for echovirus 30 was not PCR amplified.
[e] One urine specimen positive for CB3 was not PCR amplified.

TABLE 4. Comparison of the rhinovirus PCR-hybridization assay with virus culture for detection of rhinoviruses in clinical specimens

Specimen type	Reference test (culture) result[a]	No. of specimens	No. positive	Cps[b] range
NPA	+	33	32	32,721–223,082
NPA	−	25	5	0–1,184
Throat swab	+	4	4	34,402–375,770
Throat swab	−	1	0	5,742

[a] +, positive; −, negative.
[b] Cps, counts per second.

Sensitivity of PCR-hybridization assays. When purified coxsackievirus A9 RNA was diluted to contain 100, 10, 1, and 0.1 molecule in the PCR volume, approximately 1 molecule was detectable in repeated experiments. The sensitivity was also compared with the infectivity titer. The echovirus 25 prototype strain (titer, $10^{8.5}$ 50% tissue culture-infective doses per ml) and the echovirus 33 prototype strain (titer, $10^{3.0}$ 50% tissue culture-infective doses per ml) were serially diluted in LLC-MK_2 cell suspensions. After extraction and purification of RNA from each dilution, PCR and hybridization were done. A positive signal was obtained with echovirus 25 up to a dilution of $10^{-8.0}$ ($10^{0.5}$ 50% tissue culture-infective doses per ml) and with echovirus 33 up to $10^{-6.0}$ ($10^{-3.0}$ 50% tissue culture-infective doses per ml).

DISCUSSION

The results of this study indicate that enteroviruses and rhinoviruses can be detected with a high sensitivity directly in clinical specimens by nonnested PCR followed by liquid-phase hybridization. The improvements compared with the previously reported methods (1, 3, 9, 10, 17, 18, 25, 27, 31, 33, 38, 43) include a simple and efficient RNA extraction and purification procedure, reverse transcriptase reaction in filter units using a total RNA extract, additional specificity obtained by liquid-phase hybridization, and printout results which are obtained within 2 h after PCR. A possibility for bulk testing is an additional advantage. Even with these improvements, further developments are required before RNA detection of enteroviruses and rhinoviruses can be used in daily diagnosis except in highly specialized virus-diagnostic laboratories.

Removal of PCR inhibitors from RNA extracts is essential for a sensitive PCR test of clinical specimens (11). Previous methods have been laborious, requiring a long time and many steps. It is possible that some of the negative results on PCR detection of enteroviruses in heart biopsy or autopsy specimens from patients with idiopathic dilated cardiomyopathy and inflammatory myopathies (19–22, 34) may be explained by insufficient removal of these inhibitors. In fact, an internal control for an inhibitory activity in PCR of each clinical specimen may be required, at least during the development of the tests. The method reported here for the extraction and purification of specimen RNA is relatively simple; e.g., several washings with 70% ethanol are not required. Up to 18 specimens, including a negative control, were treated in 2 to 3 h. Another advantage is that all of the sample RNA can be used in the reverse transcription reaction without the requirement for precipitation to concentrate the RNA. Recently, another method was reported for extraction of enterovirus RNA from pretreated clinical specimens by hybridization with an enterovirus-specific biotinylated oligonucleotide linked to streptavi-

din-coated magnetic beads (24). In another recent report, guanidinium isothiocyanate extraction of rhinovirus RNA from clinical specimens, as used in the present study, provided greater sensitivity in PCR detection than proteinase K plus phenol-chloroform extractions (3).

Another improvement in our technology is the use of microtitration strips in the assay of amplicons (7). The procedure is technically similar to antigen and antibody assays used widely in diagnostic laboratories. We used Eu-labeled oligonucleotide probes in the hybridization, which requires time-resolved fluorometry. However, practically the same results can be obtained by digoxigenin-labeled probes, which can be assayed by colorimetric or chemiluminescent reactions (12). Further developments may include additional simplification of DNA amplification techniques, such as isothermal amplification, which is carried out at a single temperature (40).

Our failure to detect 313-bp amplicons in liquid-phase hybridization may be explained by formation of secondary structures of single strands after boiling and cooling followed by hybridization, conditions which are also optimal for self-annealing and loop formation. Another possibility is that the competing strand with higher binding capacity will displace the probes, which are only 19 to 21 nucleotides long. The position of biotinylated and Eu-labeled probes may have some effect on the reaction, since similar problems were not found in the liquid-phase hybridization of human parvovirus B19 with 284-bp-long amplicons (12). Until more information is available, we suggest that shorter-than-200-bp, preferably 100- to 150-bp, amplicons be used in liquid-phase hybridizations.

Our PCR protocol, which was adapted from influenza A virus and poliovirus PCRs (30, 41), had nearly optimal sensitivity since about one molecule of highly purified coxsackievirus A9 RNA was detected in repeated experiments. However, this is not a direct indication of the number of template molecules that can be detected in clinical specimens. Since purified RNA could not be mixed with a negative specimen, which would then be extracted and purified, another approach used to evaluate the sensitivity of our assays was comparison of PCR-hybridization with infectivity. In these experiments, cell culture-grown viruses were diluted in cell extract. RNA was extracted and purified from each dilution in the same way as from clinical specimens. The PCR-hybridization and infectivity titers were almost the same with a virus with a high infectious titer and an apparently low number of virions per infectious unit (echovirus 25). With a low-infectivity virus (echovirus 33), the PCR-hybridization titer was at least 1,000 times higher.

The clinical sensitivities and specificities could be calculated only from the culture results, an approach which has inherent limitations. In addition, culturing was done with fresh specimens, whereas PCR was done with stored material. The sensitivity of the enterovirus PCR-hybridization was 90.9% (20 of 22), and the specificity was 100% (15 of 15). The viruses from the culture-positive and PCR-negative stool and urine specimens were identified as echovirus 30 and coxsackievirus B3, respectively. We believe that these samples were not amplified because of loss of infectivity during storage and repeated freeze-thaw cycles before use in the PCR assays. Reisolation from the original material was not attempted.

The sensitivity of the rhinovirus PCR-hybridization was 97.2% (35 of 36) and the specificity was 80.8% (21 of 26). The five culture-negative but PCR-positive specimens were probably true positives since they were repeatedly positive and the negative controls were repeatedly negative. Three previous studies (10, 17, 18) have suggested that PCR is more sensitive than isolation for detection of rhinoviruses. It would not be surprising were PCR-hybridization also to prove to be a more sensitive diagnostic method than virus culture with many enteroviruses, which do not grow well in cell culture.

The partial cross-reactions of rhinoviruses in the enterovirus PCR-hybridization were expected because of sequence homology between these two picornavirus groups. We studied these cross-reactions with only five rhinovirus prototype strains. These experiments must be extended to include all rhinovirus prototypes. Similar findings have been described in an earlier report, in which discrimination of rhinoviruses from enteroviruses by restriction enzyme (*Ava*II) digestion of the amplified products was suggested (1). Our cross-reactions were not identical, since rhinovirus 1B did not react in our enterovirus PCR-hybridization tests but reacted in their enterovirus PCR. In the future, if rhinovirus prototypes that are more reactive than types 38 and 48 in the enterovirus PCR-hybridization are found, several possibilities can be explored to decrease these cross-reactions, including more stringent conditions in hybridization, such as higher temperature, a higher annealing temperature in PCR, or modifications in probes and primers. Another interesting possibility for a modification in our test design is to use the rhinovirus primers as a universal primer set followed by specific hybridization for enteroviruses and rhinoviruses.

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