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Rapid Diagnosis of Enterovirus Infection by Magnetic Bead Extraction and Polymerase Chain Reaction Detection of Enterovirus RNA in Clinical Specimens

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We describe a rapid method for extraction and detection of enterovirus RNA in clinical samples. By using magnetic bead technology, enterovirus RNA was efficiently and rapidly extracted from cerebrospinal fluid, stool, saliva, blood, pericardial fluid, urine, and cryopreserved or formalin-fixed solid tissue. Enterovirus RNA was then detected by reverse transcription followed by polymerase chain reaction amplification with primers designed to allow detection of most enterovirus serotypes. For detection of enteroviruses in specimens from patients with acute enteroviral disease, the overall sensitivity of enzymatic RNA amplification was greater than that of cell culture isolation, especially in blood specimens and in stool specimens from patients with acute cardiac disease. Enterovirus RNA was also detected in cryopreserved and archival formalin-fixed myocardial tissue from patients with acute myocarditis and chronic dilated cardiomyopathy. The ability to study archival specimens is of particular value in conducting retrospective investigation. The RNA extraction procedure used was considerably faster than extraction methods using organic reagents, used less hazardous reagents, and was of similar sensitivity. This detection protocol may therefore be useful both for the diagnosis of enterovirus infection and in studying the pathogenesis of acute and chronic enterovirus-induced disease.

Enteroviruses are important human pathogens; at least 70 serotypes which infect humans have been identified, including the polioviruses, echoviruses, and group A and B coxsackieviruses (CVA and CVB). Acute enterovirus infection may be associated with a broad spectrum of clinical features (reviewed by Grist and Reid [12]), asymptomatic infection being the most common outcome. There is considerable evidence which suggests that enteroviruses may also cause persistent infection associated with such chronic diseases as dilated cardiomyopathy (DCM) and chronic relapsing pericarditis (reviewed by Muir [23]), the chronic fatigue syndrome (1, 11, 34), and more recently the postpolio syndrome (30).

The laboratory diagnosis of enterovirus infection may be achieved by isolation of virus from cerebrospinal fluid (CSF), blood, or heart or other affected organs. Isolation of virus from stool or throat also provides evidence of infection, but this is less significant, as asymptomatic excretion of enteroviruses is relatively common, particularly during the summer and autumn in temperate climates. However, attempts to isolate enteroviruses are frequently unsuccessful for a number of reasons. Some serotypes grow poorly in cell culture. In many cases infectious virus may no longer be excreted when patients present with symptoms, particularly in patients with cardiac symptoms, which are typically a postacute or chronic manifestation of enterovirus infection. Serological diagnosis of enterovirus infection is complicated by the large number of serotypes, the difficulty in demonstrating rising antibody titers in many patients, and the high prevalence of neutralizing antibody in the general population. The most useful serological marker is the presence of enterovirus-specific immunoglobulin M, which indicates a current or recent antigenic stimulus (7, 21, 22), and its persistence may be useful as a marker of chronic enterovirus-induced disease (25).

There is considerable nucleic acid sequence homology between the enterovirus serotypes, and hybridization probes generated from conserved regions of the enterovirus genome have been shown to be useful in detecting a wide range of enterovirus serotypes (3, 6, 14, 18, 29, 31). Enterovirus RNA has also been detected by hybridization in the absence of detectable infectious virus or viral antigens in cardiac muscle from patients with viral myocarditis or DCM (3, 19) and in skeletal muscle from patients with inflammatory muscle disease (2, 35). Polymerase chain reaction (PCR) may also be useful, and several primer pairs and amplification protocols for the detection of enterovirus RNA following cDNA synthesis have been described (4, 13, 28). By using this technology, enterovirus RNA has been demonstrated in cardiac muscle (17, 32), skeletal muscle (11), and stool and CSF (26). However, the application of PCR to the routine diagnosis of infectious disease has been limited, partly because of the time-consuming nature of sample preparation procedures. In particular, the successful detection of RNA viruses requires laborious RNA extraction procedures using toxic reagents in order to separate viral RNA from RNases and PCR inhibitors present in clinical samples.

To address this issue, we have developed a rapid and simple RNA extraction procedure using reagents of low toxicity and combined this with a reverse transcription and PCR amplification (RT/PCR) protocol using primers homologous to highly conserved regions of the 5′ noncoding region of the enterovirus genome. Using this procedure we have detected enterovirus RNA in (i) supernatants of cultures infected with enterovirus reference strains and clinical isolates of known serotype, (ii) specimens from patients with confirmed or suspected acute enterovirus infection, and (iii)

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TABLE 1. Results of RT/PCR detection of enterovirus RNA in clinical specimens from patient groups and controls

Type of specimen	Clinical diagnosis	No. of specimens	No. positive by RT/PCR
Virus isolation[a]	Suspected acute enterovirus infection	19[b]	16[c]
Stool filtrate	Healthy adults	15	0
CSF	Syndromes unrelated to enterovirus infection[d]	36	0
Endomyocardial biopsy (cryopreserved)	Viral myocarditis or DCM	18	2
Explanted myocardium			
Cryopreserved	End-stage DCM	12	3
	IHD	13	2
Formalin fixed	End-stage DCM	6	1
	IHD	6	1
	Other chronic heart disease	3	0

[a] See Table 2 for further details.
[b] From 11 patients.
[c] From 10 patients.
[d] See Materials and Methods for further details.

in myocardium of patients with chronic heart disease, using cryopreserved tissue and reporting for the first time, to our knowledge, PCR detection of enterovirus RNA in formalin-fixed myocardial tissue.

MATERIALS AND METHODS

Virus stocks. CVB types 1 to 6 (Whittaker Bioproducts) were propagated in Vero cells as described previously (24). The infectivity of virus-infected culture supernatant was determined by titration in microplate culture. Clinical isolates of other enterovirus serotypes were available in this laboratory. Virus-infected cell culture supernatant was used to prepare enteroviral RNA as described below.

Clinical samples. Samples for the isolation of enteroviruses in cell culture, including stool, CSF, throat swab, urine, heparinized blood, or serum samples, were obtained from 11 patients who presented at St. Thomas' Hospital with symptoms suggestive of acute enterovirus infection. Details of clinical symptoms and specimens obtained are given in Table 1. Stool samples were also obtained from 15 healthy adults without evidence of acute enterovirus infection. CSF samples were also obtained from 36 patients with diseases not associated with enterovirus infection, including multiple sclerosis (6 patients), Parkinson's disease (4 patients), Alzheimer's disease (6 patients), cerebrovascular diseases (6 patients), spinal cord compression (6 patients), muscular dystrophy (5 patients), and myasthenia gravis (3 patients). Detection of enterovirus in these samples was attempted by isolation in cell culture and by RT/PCR.

Cryopreserved endomyocardial bioptome biopsy samples were obtained from 18 patients undergoing cardiac catheterization at St. Thomas' Hospital for investigation of suspected acute viral myocarditis or DCM. The clinical diagnosis was confirmed by histological examination of endomyocardial biopsy tissue.

Cryopreserved and formalin-fixed donor myocardium samples were obtained from patients with end-stage DCM or other specific heart disease who received heart allografts at the Clinical Transplantation Unit, Papworth Hospital, Cambridge, United Kingdom. The clinical diagnosis was confirmed by histological examination of myocardial tissue. Cryopreserved specimens were received from 25 patients, including 12 with DCM and 13 with ischemic heart disease (IHD). Formalin-fixed specimens were received from 15 patients, including 6 with DCM and 9 with IHD or other specific heart disease.

If serum was available, detection of enterovirus-specific immunoglobulin M was employed (22, 24) to provide serological evidence of enterovirus infection.

Virus isolation. Isolation of enteroviruses from clinical specimens was attempted in Vero, human embryo lung, and rhesus monkey kidney cell cultures. Isolates were identified by cytopathic effect, and the serotype was determined by neutralization of infectivity with intersecting antiserum pools.

RNA extraction. To extract enteroviral RNA, specimens were pretreated to release viral RNA from virions or cells. Viral RNA was then extracted by hybridization with an enterovirus-specific oligonucleotide linked to magnetic beads. After the hybridization step, magnetic beads were washed to remove nonspecific components and the bead-bound viral RNA was used for RT/PCR.

Stool samples were resuspended in approximately 10 volumes of Eagle maintenance medium and clarified by centrifugation followed by successive filtration through syringe filters of decreasing pore size (Gelman Sciences; 5, 2, 0.45, and 0.2 μm). For pretreatment of stool filtrate, urine, throat swab medium, CSF, or virus culture supernatant, 90 μl of sample was mixed with 10 μl of 10× boiling buffer (100 mM Tris [pH 7.5], 100 mM vanadyl ribonucleoside complexes [VRC; GIBCO BRL], 1% sodium dodecyl sulfate [SDS]), heated in a boiling water bath for 2 min, and then chilled on ice. For pretreatment of cryopreserved tissue, up to 100 mg of tissue was ground to a fine powder in liquid nitrogen and then homogenized in 100 μl of ice-cold extraction buffer (100 mM Tris [pH 8.0], 500 mM LiCl, 10 mM EDTA, 5 mM dithiothreitol, 10 mM VRC). The homogenate was then clarified by brief centrifugation and chilled on ice. For pretreatment of formalin-fixed tissue, up to 20 30-μm-thick sections were deparaffinized by extracting twice with 10 ml of octane and then twice with 2 ml of absolute ethanol, in a scaled-up version of the method described by Wright and Manos (33). To approximately 10 mg of tissue was added 100 μl of proteinase K digestion buffer (50 mM Tris [pH 8.5], 1 mm EDTA, 0.5% Tween 20, and 10 mM VRC, with nuclease-free proteinase K [Sigma] added immediately before use to a final concentration of 200 μg/ml), and the mixture was incubated for 3 h at 55°C and then 8 min at 99°C to inactivate proteinase K. The sample was then clarified and chilled on ice. For pretreatment of proteinaceous body fluids such as serum, plasma, and pericardial effusion, 100 μl of sample was mixed with 100 μl of 2× proteinase K digestion buffer containing 400 μg of proteinase K per ml and incubated at 55°C for 1 h and then 99°C for 8 min. The sample was then clarified and chilled on ice. For pretreatment of leukocytes, approximately 10^6 washed peripheral blood lymphocytes or cells from 1 ml of whole heparinized blood were pelleted by centrifugation, suspended in 200 μl of cold hypotonic lysis buffer (10 mM Tris, pH 7.5; 140 mM NaCl; 5 mM KCl; 1% Nonidet P-40; 10 mM VRC), and incubated on ice for 1 min. Cell nuclei were pelleted by centrifugation, and 100 μl of the supernatant was used for RNA extraction.

Hybridization of enterovirus RNA. Streptavidin-linked magnetic beads (Dynal) coated with a biotinylated oligonucleotide which hybridizes to the 5′ noncoding region of many enterovirus RNA genomes (described below) were used for enterovirus RNA hybridization. To 100 μl of bead suspen-

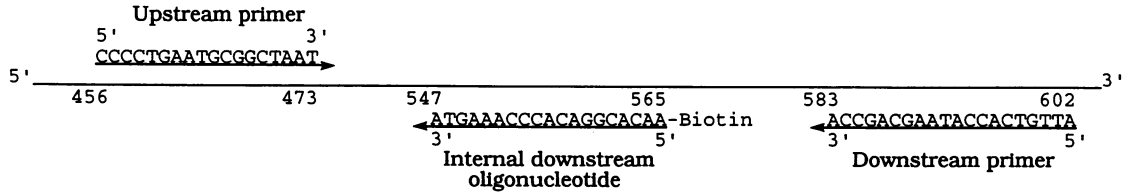

FIG. 1. Oligonucleotides used for RNA extraction, reverse transcription, PCR amplification, and Southern blot hybridization. The numbering refers to nucleotide positions based on those of CVB type 4 (16).

sion (containing 100 μg of beads) was added 300 pmol of biotinylated oligonucleotide. The suspension was mixed for 15 min at room temperature and then washed three times with 200 μl of freshly prepared 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) by using a magnetic bead concentrator to facilitate the removal of wash fluid. The prepared beads were finally resuspended in 1 ml of 12× SSPE and stored at 4°C.

For hybridization of enteroviral RNA to magnetic beads, 100 μl of pretreated sample was mixed with 100 μl of oligonucleotide-linked bead suspension for 5 min on ice. Magnetic beads were then washed three times with 200 μl of ice-cold 6× SSPE by using a magnetic concentrator and suspended in 20 μl of sterile water. The bead suspension was either used immediately for RT/PCR or stored at −70°C. Elution of bound RNA from the beads was found to be unnecessary and actually resulted in a reduction in the intensity of the PCR product band, since some RNA remained bound after elution. The presence of the beads did not result in any significant inhibition of reverse transcription or PCR amplification.

Oligonucleotides. Figure 1 shows the oligonucleotides used for RNA extraction, reverse transcription, PCR amplification, and Southern blot hybridization. Oligonucleotide sequences were based on those described by Rotbart (28). Optimal upstream and downstream primer sequences for PCR amplification were predicted by using OLIGO Primer analysis software (National Biosciences Inc.). These primers generate an approximately 148-bp PCR product (varying slightly in size, depending on the serotype). The internal downstream oligonucleotide was biotinylated at the 5′ end for use both as a capture oligonucleotide for enterovirus RNA extraction as described above and as a probe in a Southern blot hybridization as described below. Upstream and downstream primer sequences are conserved among enteroviruses serotypes, being present in all enterovirus genome sequences published to date (28). These sequences are not present in those rhinovirus sequences published to date. However, the internal downstream oligonucleotide sequence is conserved among both enteroviruses and rhinoviruses. All oligonucleotides were synthesized by British Biotechnology.

Reverse transcription. Optimal conditions for reverse transcription and PCR amplification were determined empirically. Enterovirus cDNA was generated with Moloney murine leukemia virus reverse transcriptase (BRL Life Technologies) with the reverse transcription buffer supplied with the enzyme. The reaction was carried out in 20-μl volumes containing 200 U of reverse transcriptase, 1.33 U of Inhibit-ACE (RNase inhibitor; 5 Prime to 3 Prime Inc., Boulder, Colo.), 0.75 μM downstream primer, deoxyribonucleoside triphosphates (dNTPs; Cambio) at a concentration of 1 mM each, and 2 μl of magnetic bead-bound RNA suspension. The reaction mix was overlaid with mineral oil, incubated in a thermal cycler using a thermocouple which monitors the internal tube temperature (Hybaid Thermal Reactor; Hybaid Ltd, Teddington, Middlesex, United Kingdom) at 37°C for 90 min, and then heated at 99°C for 5 min to inactivate reverse transcriptase. Reverse transcription was found to proceed more efficiently in reverse transcription buffer than in PCR buffer. Inhibit-ACE was found to give greater RNA protection than placental RNase inhibitor; in some titration experiments an approximately 100-fold increase in the sensitivity of enterovirus RNA detection was observed. The activity of Inhibit-ACE is not dependent on the presence of dithiothreitol.

PCR amplification. Following reverse transcription, 80 μl of PCR mix was added directly to each reverse transcription reaction. PCR mix consisted of single-strength PCR buffer (Cetus), 1.5 mM $MgCl_2$, 0.1875 μM upstream primer, and 2.5 U of *Taq* polymerase (AmpliTaq; Cetus). The final PCR thus contained 1.8 mM $MgCl_2$, each dNTP at 200 μM, and each primer at 0.15 μM. The addition of PCR mix to the reverse transcription mix following cDNA synthesis provided salt and pH conditions similar to those of standard single-strength PCR buffer and did not affect PCR product yield. The reaction mix was incubated in a thermal cycler to give the following temperature cycling protocol: 94°C for 5 min, 50°C for 15 s, and 72°C for 15 s (1 cycle); then 94°C for 15 s, 50°C for 15 s, and 72°C for 15 s (50 cycles); and then 72°C for 5 min (1 cycle). The reaction mix was then analyzed by agarose gel electrophoresis (AGE) in a 4% agarose (3:1 NuSieve/SeaKem; FMC Bioproducts) gel for the presence of an approximately 148-bp PCR product. An amplification protocol using 15-s plateau times resulted in greater PCR product yield than one using 2-min plateau times. Furthermore, at short plateau times, the accumulation of PCR product continued at high cycle numbers: significantly more PCR product was observed after 50 amplification cycles than after 40 cycles, particularly at low input RNA template concentrations.

Southern blot hybridization. To confirm the identity of enterovirus cDNA PCR products, Southern blot hybridization using a biotinylated oligonucleotide which hybridizes to the specific PCR product (Fig. 1) was used. Only where PCR products of the correct size reacted with this oligonucleotide probe by Southern blot hybridization was a specimen considered enterovirus positive. After AGE, PCR products were transferred to positively charged nylon membranes (Hybond N^+; Amersham) according to the manufacturer's protocol. Nylon membranes were prehybridized in 5× SSPE–5× Denhardt's solution containing 0.5% SDS and 0.1 mg of sonicated denatured herring sperm DNA per ml at 42°C for 1 h. Biotinylated internal downstream oligonucleotide was then added to a final concentration of 20 ng/ml, and hybridization was continued for a further 3 h at 42°C. Membranes were then washed successively with 6×, 1×, and 0.15× SSPE containing 0.1% SDS, each time at 42°C for

15 min. Nylon membranes were then reacted with streptavidin-peroxidase conjugate to detect bound biotinylated oligonucleotide. Membranes were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 3% skim milk powder for 30 min at room temperature. Streptavidin-peroxidase conjugate (Sigma) was then added to a final concentration of 2 μg/ml. Membranes were incubated for a further 1 h and then washed with PBS containing 0.1% Tween 20, once for 15 min and then four times for 5 min each. Membranes were reacted with enhanced chemiluminescent substrate (Amersham) according to manufacturer's protocol and exposed to Kodak XAR5 photographic film for 1 to 4 min. Photographic film was developed with a Kodak RP X-Omat Processor.

Quantitation of RT/PCR sensitivity. The sensitivity of the RT/PCR method was determined by using enteroviral RNA prepared from virus culture supernatant of known titer. Enterovirus RNA was extracted from 10-fold dilutions of a CVB type 4-infected culture supernatant and amplified by RT/PCR. PCR products were analyzed by AGE with ethidium bromide staining and by Southern blot hybridization. For comparison, enterovirus RNA was extracted from the same dilutions of culture supernatant by using RNAzol B (Biotecx Laboratories), a guanidinium thiocyanate- and phenol-containing reagent, according to manufacturer's protocol, which is based on that of Chomczynski and Sacchi (5). Briefly, 90 μl of sample was mixed with 400 μl of RNAzol B and 80 μl of chloroform, incubated on ice for 5 min, and then centrifuged in a microcentrifuge for 15 min. RNA was twice precipitated from the aqueous phase in isopropanol with 10 μg of linear polyacrylamide as a carrier (9), washed in 70% ethanol, air dried, and dissolved in 20 μl of sterile water. RNA prepared in this way was amplified and analyzed in parallel with RNA prepared with magnetic beads.

PCR detection of enterovirus RNA from reference enterovirus strains, clinical enterovirus isolates, and other viruses. Viral RNA was extracted from the supernatants of cell cultures infected with the following enterovirus serotypes: poliovirus types 1 to 3, CVB types 1 to 6, CVA types 9 and 16, and echovirus types 4, 6, 7, 11, 18, and 30. By using RNAzol B, total RNA was extracted from cultured human embryo fibroblasts and from fibroblast or Vero cell cultures infected with cytomegalovirus, herpes simplex virus type 1, adenovirus, mumps virus, and rubella virus. RNA was then tested by RT/PCR.

Prevention of PCR contamination. Because of the high potential sensitivity of PCR, precautions must be observed to exclude the possibility of contamination of a reaction tube with previously amplified PCR product or target RNA from another specimen. In this study, a number of procedures were adopted to eliminate this risk. The processing of clinical samples and preparation of reagents were performed in a room different from that used for PCR amplification and analysis of amplification products. For extraction of RNA from virus-infected tissue culture samples used as positive control, a class I safety cabinet in a third room was used for the dispensing of culture fluid prior to heat treatment and magnetic bead extraction. Dedicated reagents, micropipettes, sterile reagent tubes, and filtered pipette tips were used for RNA extraction and RT/PCR assembly. The RNA extraction procedure described in this paper requires minimal sample manipulation, thus minimizing the risk of carryover. Negative control blank extractions in which no clinical material or positive control virus or RNA was added were processed alongside patient specimens and positive control samples on each occasion, and equal numbers of test samples and reagent blanks were included in each RT/PCR assay. Positive PCR results in these negative controls were not observed in experiments reported in this paper. Tissue grinders and homogenizers were rendered free of contaminating nucleic acid and nucleases after use by immersion in dilute Decon 90 for at least 1 h, followed by rinsing and immersion in approximately 2 M HCl for at least 1 h and then by rinsing and baking overnight at 180°C.

RESULTS

Quantitation of RT/PCR sensitivity. Figure 2 shows the results of two titration experiments using CVB type 4 RNA. RNA was extracted with magnetic beads (Fig. 2A and B) and with RNAzol (Fig. 2C and D). RT/PCR was capable of detecting viral RNA extracted from 10 50% tissue culture infective doses (Fig. 2A) by using magnetic beads after AGE. Following Southern blot hybridization, the sensitivity was increased to 0.1 50% tissue culture infective dose (Fig. 2B). With RNA prepared with RNAzol B, the sensitivity of RT/PCR was comparable or slightly lower (Fig. 2C and D).

PCR detection of enterovirus RNA from reference enterovirus strains, clinical enterovirus isolates, and other viruses. Following RT/PCR amplification of enterovirus RNA in all cases, a band of approximately 148 bp was clearly visible in ethidium bromide-stained gels. In addition, all PCR products derived from these serotypes reacted with the biotinylated detection probe in a Southern blot hybridization assay. In contrast, no specific PCR product was detected by AGE or Southern blot hybridization following PCR amplification of RNA extracts of human fibroblasts or of cell cultures infected with cytomegalovirus, herpes simplex virus type 1, adenovirus, mumps virus, or rubella virus.

PCR detection of enterovirus RNA in clinical samples. The results of RT/PCR of RNA extracted from different types of clinical specimen from patients with clinical syndromes of suspected or confirmed enterovirus etiology and controls are summarized in Table 1. No PCR products were detected in stool samples from healthy individuals or CSF samples from patients without evidence of acute enterovirus infection. In addition, no PCR products were observed in extraction or reagent blanks. The results of RT/PCR of RNA extracted from clinical specimens from patients with suspected or confirmed acute enterovirus infection, together with results of attempts to isolate enteroviruses from the same specimens in cell culture, are shown in Table 2. The results of AGE and Southern blot hybridization for representative clinical specimens and clinical isolates are shown in Fig. 3. It can be seen that the use of Southern blot hybridization improves the rate of PCR detection and allows the specific PCR product to be distinguished from nonspecific amplification products. Because some nonspecific amplification products were of sizes similar to that of the specific PCR product, samples were considered positive only when a PCR product of the correct size reacted with the detection probe following Southern blot hybridization.

Overall, the results of RT/PCR compare favorably with those of virus isolation. Enterovirus RNA was detected by RT/PCR in six stool or throat swab specimens in which an enterovirus could not be cultured (patients 3 to 8). In three of these cases there was independent confirmation of the diagnosis of enterovirus infection from examination of other specimens from the same patient: in two cases, an enterovirus was cultured from another specimen from the same patient—from pericardial fluid from patient 4 and from urine and stool from patient 7. In the third case (patient 5) rising

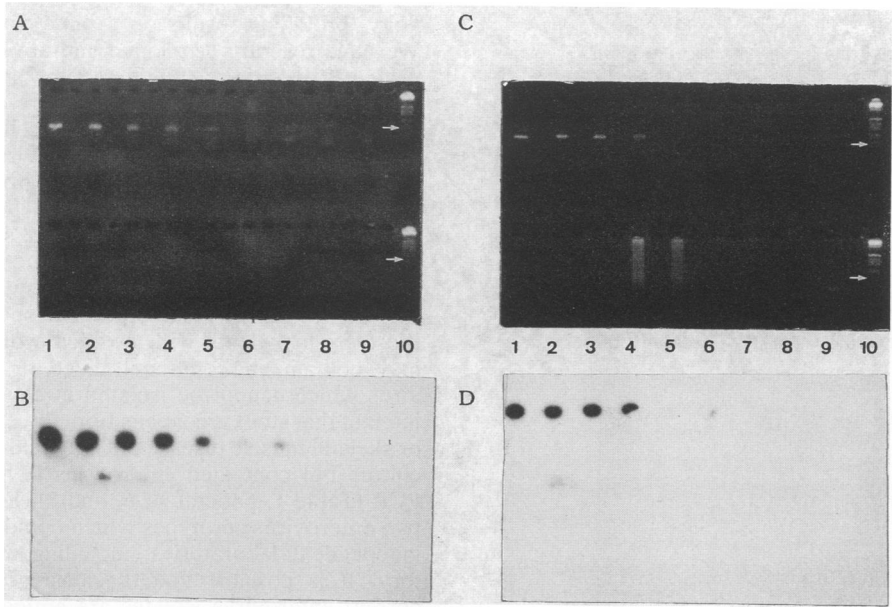

FIG. 2. Titration of RNA extracted from CVB type 4-infected cell culture supernatant with magnetic beads (A and B) and RNAzol (C and D). PCR products were visualized by AGE (A and C) and Southern blot hybridization (B and D) as described in Materials and Methods. Upper track: lanes 1 to 9, PCR products derived from amplification of 10-fold dilutions of viral RNA corresponding to 10^5 (lane 1) to 10^{-3} (lane 9) 50% tissue culture infective doses; lanes 10, molecular weight markers (kilobase ladder; Bethesda Research Laboratories). Lower track: lanes 1 to 9, PCR reagent blanks; lanes 10, molecular weight markers. The arrows indicate the position of a 142-bp–154-bp doublet band.

enterovirus-specific immunoglobulin M levels were detected in sera collected during convalescence. Although enterovirus RNA could also be detected by RT/PCR in blood components (patient 3), it was not possible to isolate virus, as these specimens were found to be cytotoxic in cell culture. There were, however, three specimens in which an enterovirus was detected by culture but not by RT/PCR.

Enterovirus RNA was detected by RT/PCR in endomyo-

TABLE 2. Results of RT/PCR and cell culture detection of enteroviruses in clinical specimens from patients with suspected or confirmed acute enterovirus infection

Patient no. (age [yr])	Clinical details	Sample	PCR result		Virus isolation result
			AGE	Southern blot	
1 (37)	Aseptic meningitis	Throat swab	+	+	Echovirus type 30
		Rectal swab	−	−	Echovirus type 30
2 (8)	Fever, headache, abdominal pain	Stool	−	−	CVB type 5
3 (59)	Respiratory illness, malaise	Stool	+	+	Negative
		PBL[a]	+	+	Toxic
		Whole blood cells	+	+	Toxic
		Serum	+	+	Toxic
		Plasma	+	+	Toxic
4 (56)	Acute pericarditis	Stool	+	+	Negative
		Pericardial fluid	+	+	Enterovirus[b]
5 (37)	Bornholm disease	Stool	+	+	Negative[c]
6 (73)	Left and right ventricular failure	Stool	+	+	Negative
7 (25)	Aseptic meningitis	Stool	−		Echovirus type 11[c]
		Urine	+	+	Echovirus type 11
		Throat swab	−	+	Negative
		CSF	NT[d]	NT	Echovirus type 11
8 (38)	Acute polymyositis	Stool	+	+	Negative
9 (33)	Meningism	Stool	+	+	Echovirus type 11
10 (21)	Meningism, pyrexia, sore throat	Throat swab	−	+	Enterovirus[b]
11 (62)	Aseptic meningitis	CSF	+	+	NT

[a] PBL, peripheral blood mononuclear lymphocytes.
[b] Enterovirus isolated: slow growth in culture did not allow serotypic identification by virus neutralization.
[c] Rising enterovirus-specific immunoglobulin M levels were detected in sequential convalescent-phase sera.
[d] NT, not attempted (insufficient sample).

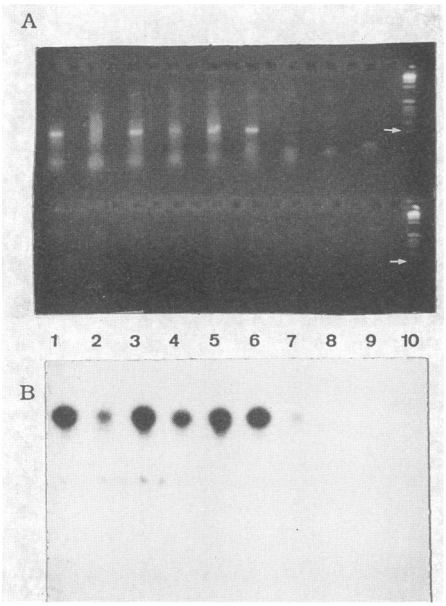

FIG. 3. PCR products following amplification of RNA derived from clinical samples and cell cultures infected with representative enterovirus serotypes, visualized by AGE (A) and Southern blot hybridization (B). Patient numbers refer to Table 1. Upper track: lanes 1, endomyocardial biopsy sample from a patient with healed myocarditis; lanes 2, endomyocardial biopsy sample from a patient with acute myocarditis; lanes 3, patient 6 stool; lanes 4, patient 8 stool; lanes 5, patient 4 pericardial fluid; lanes 6, patient 3 peripheral blood lymphocytes; lanes 7, patient 7 throat swab; lanes 8 and 9, stool from healthy adults; lanes 10, molecular weight markers (arrows indicate a 142-bp–154-bp doublet band). Lower track: lanes 1, healthy adult stool; lanes 2 to 9, PCR reagent blanks; lanes 10, molecular weight markers.

cardial biopsy samples from 2 of 18 patients: 1 with healed myocarditis and 1 with acute myocarditis. In the latter case, a PCR product was detected only after Southern blot hybridization. Enterovirus-specific RNA was detected following Southern blot hybridization in cryopreserved myocardial tissue from 3 of 12 patients with DCM and 2 of 13 with IHD. Using formalin-fixed myocardium removed at transplant, enterovirus RNA was detected in one of six patients with DCM, one of six patients with IHD, and none of three with other chronic heart diseases.

DISCUSSION

We have described procedures for the rapid detection of enterovirus RNA in clinical samples. PCR detection of RNA sequences requires a degree of RNA purity (8) which is usually achieved by extraction with phenol-chloroform or guanidinium isothiocyanate. The method described here is simpler, safer, faster, and of comparable sensitivity. As RNA extraction requires only 10 to 15 min per specimen, it is possible to complete extraction, amplification, and electrophoretic analysis in 1 day. Although Southern blot hybridization extends this time by a further 24 h, a preliminary identification is possible for many samples after AGE. However, nonspecific amplification products or nucleic acid smears are observed with some clinical specimens. Southern blot hybridization is required to identify specific PCR products in such cases. A second nested PCR may provide a faster means of increasing sensitivity and confirming specificity, providing results on a same-day or overnight basis. We have recently developed and are currently assessing a nested PCR assay for the detection of enterovirus RNA.

In this study, more diagnoses were achieved by RT/PCR than by enterovirus isolation in cell culture. There are several possible reasons for this. Firstly, it is likely that many enterovirus serotypes which grow poorly in cell culture will be detected by RT/PCR. Secondly, enterovirus RNA may be detected by PCR in specimens which are frequently toxic in cell culture, such as serum, plasma, peripheral blood lymphocytes, and whole blood (Table 1, patient 3). Thirdly, some clinical specimens, particularly those from patients with postacute or chronic symptoms, may contain antibody-neutralized or replication-defective virus which cannot be isolated in cell culture (34). It is of interest that stool specimens from three patients with cardiac or skeletal muscle disease did not yield an enterovirus in cell culture but contained enterovirus RNA detectable by RT/PCR (Table 1, patients 4, 6, and 8). RT/PCR also detected two enterovirus serotypes which could not be typed by two independent laboratories, including one reference laboratory. It is possible that, because of genetic drift, many current enterovirus isolates are antigenically distinct from standard reference strains which were used to generate serotyping antisera. RT/PCR may therefore help to confirm the identity of an enterovirus isolate in such cases. Since 10 of the 11 patients with suspected acute enterovirus infection were adults (Table 2), it is unlikely that asymptomatic carriage in the gastrointestinal tract or recent poliovirus vaccination accounted for enteroviruses detected in stool or throat swabs.

To our knowledge, there is only one other report of PCR detection of enterovirus in pericardial fluid (26), and the examination of pericardial fluid by PCR may be useful in establishing a diagnosis of enteroviral heart disease, as well as in studying the pathogenesis of acute and chronic relapsing pericarditis (25).

There were only three specimens in which an enterovirus was detected by culture but not by RT/PCR (Table 2, patients 1, 2, and 7), and in only one patient did RT/PCR fail to detect an enterovirus in any specimen (Table 2, patient 2). The presence of high levels of RNase activity or of enzymatic inhibitors which copurify with viral RNA in some clinical specimens may limit the sensitivity of RT/PCR, leading to false-negative results. Since many specimens were tested retrospectively in this study, it is also possible that virus and viral RNA was degraded during storage and freeze-thawing. All three culture-positive specimens where RT/PCR failed to detect enterovirus RNA were stool specimens, and it may be that some stool specimens are particularly inhibitory for RT/PCR.

Cryopreserved solid tissue can also be processed and analyzed rapidly for the presence of enterovirus RNA. By total RNA extraction methods, the size of tissue sample that can be successfully analyzed is limited, because high RNA concentrations inhibit PCR amplification. However, the use of a selective extraction method as described here, in which most nonspecific RNA is removed, allows larger tissue samples to be analyzed. We have successfully amplified enterovirus RNA from RNA extracts prepared from up to 100 mg of tissue. This may reduce sampling error involved in the detection of focally distributed RNA sequences. However, we have also shown that these methods are sufficiently sensitive to detect enterovirus RNA in endomyocardial bioptome biopsy samples (approximately 1 mg of tissue). PCR detection of enterovirus RNA in cryopreserved myo-

cardial tissue from a small number of patients has been reported previously (17, 32). However, some recent studies employing PCR to detect enterovirus RNA in biopsy samples from patients with DCM (27) or skeletal muscle biopsy samples from patients with inflammatory muscle disease (20) have failed to demonstrate the association with the presence of enterovirus RNA reported in nucleic acid hybridization studies. This may be due to differences in the relative sensitivity and specificity of hybridization and PCR assays or differences in the patients studied. Clearly, parallel studies in which the same patients are studied by both PCR and hybridization methods will be required to resolve these apparent contradictions.

The ability to study formalin-fixed tissue by PCR for the presence or viral RNA is of major significance, as this allows archival tissue to be examined, considerably increasing the possible scope of investigation. The amplification of measles virus RNA from formalin-fixed tissue has been reported (15). We now report the successful amplification of enterovirus RNA from formalin-fixed tissue by a considerably shorter extraction procedure.

The speed and sensitivity of this protocol and its ability to detect enterovirus RNA in tissue and other specimens from which virus cannot be cultured must be offset against the high cost of PCR reagents. The risk of false-positive results due to PCR contamination must also be rigorously excluded. Positive results were not observed in the extraction or reagent blanks included in these experiments. We are continuing our studies in order to accumulate a larger body of data. This will give a clearer indication of the feasibility of using this protocol for the laboratory diagnosis of enterovirus infection.

As the sequence of the capture oligonucleotide used here is conserved among enteroviruses and rhinoviruses, this extraction procedure may also be useful for the detection of rhinovirus RNA in conjunction with appropriate PCR primers (10, 13, 26). The principle of selective RNA hybridization should also be useful for the isolation and detection of other viral or cellular RNA species.

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