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Highly Sensitive Assay for Detection of Enterovirus in Clinical Specimens by Reverse Transcription-PCR with an Armored RNA Internal Control

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The objective of the present study was the development of a diagnostic reverse transcription (RT)-PCR for the specific detection of enterovirus (EV) RNA in clinical specimens controlled by an internal control (IC) RNA. The IC RNA contains the same primer binding sites as EV RNA but has a different probe region. The IC RNA was packaged into an MS2 phage core particle (armored) and was added to the clinical sample to allow monitoring of both extraction efficiency and RT-PCR efficiency. Serial dilutions of the IC RNA were made, and the detection limit of the RT-PCR was tested in a background of EV RNA-negative cerebrospinal fluid. The sensitivity and specificity of the RT-PCR assay were tested by using all 64 known EV serotypes, several non-EV serotypes, and two Quality Control for Molecular Diagnostics (QCMD) Program EV proficiency panels from 2001 and 2002. In total, 322 clinical specimens were tested by RT-PCR, and to establish the clinical utility of the RT-PCR, a comparison of the results of viral culture and RT-PCR was done with 87 clinical specimens. The lower limit of sensitivity was reached at about 150 copies of IC RNA/ml. All 64 EV serotypes were positive, while all non-EV serotypes were negative. All culture-positive samples of the 2001 QCMD proficiency panel (according to the 50% tissue culture infective doses per milliliter) were positive by RT-PCR. Invalid results, i.e., negativity for both EV RNA and IC RNA, due to inhibition of RT-PCR were observed for 33.3% of the members of the 2002 QCMD proficiency panel and 3.1% of the clinical specimens. Inhibition of RT-PCR could be relieved by the addition of 400 ng of bovine α-casein per μl to both the RT reaction mixture and the PCR mixture. With this optimized protocol, the results for all samples of the 2002 QCMD proficiency panel and all clinical specimens except one fecal sample (0.3%) were valid. Evaluation of the clinical samples demonstrated that EV infection could be detected in 12 of 87 samples (13.8%) by RT-PCR, while viral culture was negative. Our data show that the RT-PCR with armored IC RNA offers a very reliable and rapid diagnostic tool for the detection of EV in clinical specimens and that the addition of bovine α-casein relieved inhibition of the RT-PCR for 99.7% of clinical specimens.

The human enteroviruses (EVs) are members of the family *Picornaviridae*, are ubiquitous, and are mainly enterically transmitted. EVs have traditionally been identified by serotype-specific antisera in a virus-neutralizing test, and 66 EV types are known to infect humans (19). The 66 EV serotypes were initially recognized and divided into five major groups: polioviruses (PV; types 1 to 3), coxsackieviruses A (CVAs; types 1 to 22 and 24), coxsackieviruses B (CVBs; types 1 to 6), echoviruses (types 1 to 7, 9, 11 to 27, and 29 to 33), and EV types 68 to 71 (17). Recent molecular analyses have proved that echovirus types 22 and 23 are genetically distinct from the members of the genus *Enterovirus* and have been reclassified in a separate genus, *Parechovirus*, in the family *Picornaviridae* (13, 20, 23).

Infections with EVs cause a wide range of clinical outcomes, such as asymptomatic infections, aseptic meningitis (meningeal inflammation in the absence of a bacterial pathogen), encephalitis, paralytic poliomyelitis, and myocarditis. Although the majority of EV infections do not cause significant disease, infection can cause serious illness, especially in infants and immune-compromised patients. EV infections are the most common cause of aseptic meningitis and account for 80 to 90% of all cases of central nervous system infections for which a possible causative agent is identified (24). In the neonate, aseptic meningitis-induced complications and poor outcomes of EV infections generally occur within the first 2 days of life (1, 2). Aseptic meningitis in immune-competent adults is characterized by sudden onset of fever, but neurological abnormalities are rare, and both short-term and long-term outcomes are generally good. Encephalitis caused by EV infections is a less common but a more severe disease than aseptic meningitis (18, 29, 30). Immune-compromised children and adults who are infected with EV may develop chronic meningitis and encephalitis, which may last for years before becoming fatal (16).

The early clinical symptoms of meningitis caused by viruses, bacteria, and fungi are quite similar and are difficult to distinguish, but the diagnosis, therapy, and outcome of disease caused by these pathogens vary considerably. A reliable means of laboratory detection of EV is needed, since the specific and rapid diagnosis of EV meningitis has a significant impact on

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patient management (10). Although reverse transcription (RT)-PCR may become the method of choice for the diagnosis of EV infections of the central nervous system, in many laboratories the diagnosis of EV infections still relies on cell culture techniques. Stool specimens or rectal swab, throat swab, and cerebrospinal fluid (CSF) specimens are used for virus culture. Early in the acute phase of the illness, EV is frequently isolated from the throat, whereas isolation of the virus from CSF provides the most direct link to disease but is usually less successful. A cytopathic effect (CPE) is not recognizable within a few days, and some EVs do not grow in cell culture and therefore do not cause a CPE at all (15, 25). In recent years, several EV RT-PCR assays which are sensitive and rapid have been developed (8, 9, 14, 25, 26). In those previous studies, EV RNA was purified without an internal control (IC) RNA that monitored both RNA extraction efficiency and RT-PCR efficiency. Like the wild-type target, these IC RNAs should have the same length, contain the same primer binding sites, and have the same G+C content for identical extraction and amplification efficiency but should contain a different probe binding site for the differential detection of IC RNA. In molecular diagnostics, the use of an IC RNA is crucial for a reliable interpretation of results because it enables the verification of the sensitivity of the assay and avoids false-negative results. RNA transcribed in vitro can be used as IC RNA but is prone to degradation by RNases. Packaging of IC RNA into a phage protects the RNA from enzymatic degradation (22). These armored RNAs can be spiked into clinical specimens without degradation, thus enabling simultaneous monitoring of the complete nucleic acid extraction and amplification process for each specimen.

MATERIALS AND METHODS

Chemicals and enzymes. Lysis buffer, wash buffers, and silica suspensions were prepared as described previously (3). SuperScript II was obtained from Life Technologies (Gaithersburg, Md.). RNase inhibitor (RNAsin) was obtained from Promega (Madison, Wis.). Bovine serum albumin (BSA) was obtained from Roche Diagnostics (Almere, The Netherlands). Deoxynucleotides (dATP, dCTP, dGTP, dTTP, and dUTP), *Taq* DNA polymerase (Amplitaq Gold), and uracil-*N*-glycosylase (Amperase) were from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). Streptavidin-coated magnetic beads (Dynabeads M-280) were from Dynal (Hamburg, Germany). Tris, KCl, $MgCl_2$, calf thymus DNA, and bovine α-casein (lot no. 17H9551) were obtained from Sigma (Zwijndrecht, The Netherlands). Bovine α-casein was dissolved in 1× PCRII buffer (Applied Biosystems) at 20 mg/ml, and the mixture was stored at −20°C. Triton X-100 was obtained from Packard Instrument Corporation (Downers Grove, Ill.). Serum and plasma samples were obtained in Vacutainer tubes (Becton Dickinson Systems, Meylan, France).

Clinical specimens. In total, 322 clinical specimens, including 281 CSF samples, 18 fecal samples, 10 throat swab samples, 3 vesicle fluid samples, 3 pleural fluid samples, 2 broncheoalveolar lavage fluid samples, 2 amniotic fluid samples, 1 urine sample, and 2 brain biopsy specimens, were obtained from patients suspected to be infected with EV and were tested by the RT-PCR.

CPE for EV infection. Viral culture was done by cocultivation of CSF with human diploid fibroblasts, tertiary monkey kidney cells, or Vero cells. These viral cultures were examined twice weekly for the appearance of EV-specific CPEs. Preliminary identification of isolates was performed according to either the unstained CPE or the CPE obtained by incubation with a specific monoclonal antibody (DAKO-Enterovirus monoclonal antibody 5-D8/1; DAKO, Glostrup, Denmark).

Serotyping of isolates. Typing of EVs was performed at the National Institute of Public Health and the Environment (Bilthoven, The Netherlands) by neutralization tests with antiserum pools. These pools are prepared in combinations designed so that an isolate can be screened for identity by using eight pools of 42 antisera in a single test.

Viral strains. EV serotypes CVA (types 1 to 6, 18 to 22, 24), CVB (types 1 to 6), echovirus (types 1 to 9, 11 to 22, 24 to 27, 29 to 33), EV (types 68 to 71), PV vaccine types (types 1 to 3), and parechovirus types 1 and 2 were kindly provided by the National Institute of Public Health and the Environment. The rhinovirus serotypes (types 1A, 1B, 3, 8, 11, 13, 14, 15, 16, and 88) were kindly provided by the Department of Virology of the Utrecht Medical Center (Utrecht, The Netherlands). The hepatitis A virus (HAV) serotype (type HM175) was kindly provided by the Municipal Health Service, Amsterdam.

Primers. Random hexamers (Roche Diagnostics), which were diluted to 1.5 μg/μl in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), were used for RT. PCR primers were designed by computer-assisted analysis (OMIGA; Oxford Molecular, Oxford, England) of all available 5′ noncoding regions and the full genomes of the EV serotypes. High-pressure liquid chromatography-purified PCR primers were from Applied Biosystems and were diluted to 100 ng/μl in TE buffer. The sequences of the primers used for amplification of both wild-type EV RNA and IC RNA are located in the conserved 5′ noncoding region of the EV sequence. The primer pair used for amplification consisted of entero-1 (5′-CCC TGA ATG CGG CTA AT-3′; nucleotide positions [nt] 452 to 468) and Bio-entero-2 (5′-ATT GTC ACC ATA AGC AGC C-3′; 5′ biotinylated; nt 579 to 597). Nucleotide numbering was that for the Sabin PV type 2 strain, as described by Toyoda et al. (28).

Armored EV RNA control. The armored EV RNA control, which contained part of the 5′ noncoding region (nt 428 to 691), was obtained from RNA Diagnostics, Ambion, Inc. (Austin, Tex.), and was constructed by the armored RNA technique (22). The method is based on the packaging of recombinant RNA into MS2-like particles, which are produced in *Escherichia coli*. The particles are isolated through a series of conventional protein purification procedures. According to Ambion, the approximate factor used for the conversion of 1 mg of armored RNA to the numbers of RNA copies is about 2×10^{14}. The armored EV RNA stock solution used in the present study (lot no. 040D49012A) contained approximately 6.5×10^{14} copies of EV RNA/ml.

Construction of armored IC RNA control. We designed two oligonucleotides for the construction of IC RNA, and these were synthesized by Applied Biosystems: Ent-hyb-3 (5′-*CCC TGA ATG CGG CTA AT*C CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GTC TGT CGT AAC GCG CAA GTC TGT G**CT TGA GAC GTG**-3′) and Ent-hyb-4 (5′-*ATT GTC ACC ATA AGC AGC C*AT GAT AAA AAT AAC AGG AAA CAC GGA C**GG TTA CCA CGC ACG TCT CAA GCA** CAG ACT T-3′). These two oligonucleotides, which together represent the same part of the 5′ noncoding region (nt 428 to 691) present in the armored EV RNA control, overlapped over a stretch of 20 nt (underlined) and contained the same primer binding sites as the armored EV RNA control (italics), but with a different probe region (boldface). This IC probe region allowed discrimination between EV and IC RNA amplimers after hybridization and detection. The IC RNA control was constructed by hybridization and elongation of 1 ng of oligonucleotide Ent-hyb-3 and 1 ng of oligonucleotide Ent-hyb-4 in a mixture of 2.5 U of Amplitaq Gold; 5 μg of BSA; 1× PCRII buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl) dATP, dCTP, dGTP, and dTTP at a concentration of 200 μM each; and 3 mM $MgCl_2$. The mixture was incubated for 10 min at 95°C, 5 min at 55°C, and 10 min at 72°C. The resulting hybrid was subsequently amplified with primer pair entero-1 and non-Bio-entero-2 in the same mixture described above and was incubated for 10 min at 95°C, followed by 35 cycles, with each cycle consisting of 20 s at 95°C, 20 s at 55°C, and 1 min at 72°C, followed by 5 min at 72°C. The concentration of the resulting amplimer was estimated by measuring the UV absorption at 260 nm, and subsequently, 2 ng was cloned into a plasmid vector (PCRII; Promega), resulting in plasmid pEntIC 2. The IC RNA sequence was confirmed by dideoxynucleotide sequencing (Visible Genetics Inc., Toronto, Ontario, Canada). Packaging of the IC RNA control into MS2-like particles was performed with plasmid pEntIC 2 as the template and was custom made by Ambion. According to Ambion, the approximate factor for the conversion of 1 mg of armored RNA to the numbers of copies of RNA is about 2×10^{14}. The armored IC RNA stock solution used in the present study (lot no. 021D49013A) contained approximately 5.4×10^{14} copies of IC RNA/ml.

Dilution buffer for armored RNA controls. The armored RNA controls were diluted in TSM dilution buffer, which contained 20 ng of calf thymus DNA per μl, 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, and 0.1% gelatin (catalog no. G-9382; Sigma), and stored at −20°C. The final solution of armored EV RNA contained 500 copies/μl, and the final solution of armored IC RNA contained 100 copies/μl.

RNA purification. EV RNA was purified from clinical specimens as described earlier (4), with the following modifications: 20 μl (50 μl for fecal samples [7]) of size-fractionated silica particles was used in combination with 900 μl of lysis buffer L6. The clinical specimen and 500 armored IC RNA copies were added to this silica-lysis buffer mixture. RNA was eluted in 100 μl of TE buffer.

TABLE 1. PCR sensitivity for armored EV RNA and IC RNA control phages

RNA type and no. of copies/200 μl of CSF by extraction	No. of RNA copies in the following mixture[a]:		Proportion positive[b]
	RT (2/5)	PCR (1/5)	
EV RNA			
900	360	180	10/10 (100)
450	180	90	9/10 (90)
225	90	45	8/10 (80)
112	45	23	8/10 (80)
84	34	17	5/10 (50)
56	23	11	6/20 (30)
28	11	6	5/16 (31)
14	6	3	1/10 (10)
7	3	1	1/10 (10)
0	0	0	0/10 (0)
IC RNA			
225	90	45	10/10 (100)
112	45	23	9/10 (90)
56	23	11	8/10 (80)
28	11	6	6/10 (60)
0	0	0	0/10 (0)

[a] The following assumptions were made for the copy numbers presented: (i) the armored RNA stocks contain the specified number of phage particles, and all contain EV RNA or IC RNA; and (ii) the efficiencies of extraction, RT, and PCR were 100% each.

[b] The data represent number of samples positive/number of samples tested (percent).

RT-PCR. Forty microliters of the 100 μl (2/5) of RNA eluate was used for RT. The final RT mixture (50 μl) contained 1,500 ng of hexamers, 1× CMB1 buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.1% Triton X-100), 0.4 U of SuperScript II per μl, 120 μM (each) deoxynucleoside triphosphate, 0.08 U of RNAsin per μl, and 5 mM $MgCl_2$. The mixture was incubated for 30 min at 42°C, and 25 μl (corresponding to 40 μl [1/5] of CSF) was subsequently used as input in the PCR. The PCR was performed in a 50-μl volume containing 200 ng of primer entero-1 (5′-CCC TGA ATG CGG CTA AT-3′; nt 452 to 468) and 200 ng of primer Bio-entero-2 (5′-ATT GTC ACC ATA AGC AGC C-3′; 5′ biotinylated; nt 579 to 597); 0.05 U of Amplitaq Gold DNA polymerase per μl; 0.01 U of Amperase per μl; 0.1 μg of BSA per μl; 1× PCRII buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl); 400 ng of bovine α-casein per μl; dATP, dCTP, and dGTP at a concentration of 200 μM each; and 400 μM dUTP. The final $MgCl_2$ concentration in the PCR mixture was 2.5 mM. PCRs were performed in an Applied Biosystems 9600 thermocycler, as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles each consisting of 20 s at 95°C, 20 s at 55°C, and 1 min at 72°C, followed by 5 min at 72°C.

Optimized RT-PCR. Bovine α-casein (400 ng/μl) was added to the RT mixture, and the mixture was processed as described above.

Hybridization and measurement by ECL. After RT-PCR, excess primers were removed as described earlier (5), and amplicons were hybridized with Tris (2,2′-bipyridine) ruthenium [II] chelate (TBR)-labeled probes specific for EV RNA and IC RNA, as described recently (5). The probes (nt 531 to 551) were labeled with TBR at the 5′ end and were as follows: TBR-entero-1 (EV-specific probe; 5′-GCG GAA CCG ACT ACT TTG GGT-3′) and TBR-entero-2 (IC-specific probe; 5′-CTT GAG ACG TGC GTG GTA ACC-3′). Hybrids were captured with streptavidin-coated magnetic beads; and the electrochemiluminescence (ECL) signal, expressed in luminosity units (LU), was measured with the M8 system (IGEN, Oxford, England). A 96-well plate is used with this device, and unhybridized TBR-labeled probes are automatically removed by washing. The amount of labeled hybrids is determined after excitation by applying an electric field.

Criteria for diagnostic RT-PCR. A signal of more than 500 LU (2.5 times the mean background signal for either probe) was considered a positive result. A clinical specimen was considered positive for EV RNA if more than 500 LU was measured with the EV-specific probe, regardless of the result obtained with the IC-specific probe. A clinical specimen was considered negative for EV RNA if less than 500 LU was measured with the EV-specific probe and IC RNA was detected at more than 500 LU. In the diagnostic RT-PCR, four controls, two positive controls and two negative controls, were included in the RNA extraction procedure. The high-positive control contained 12,500 armored EV RNA copies/extraction, and the low-positive control contained 2,500 armored EV RNA copies/extraction, both together with 500 armored IC RNA copies. The first negative control contained 500 armored IC RNA copies and served as a control for the entire procedure and should be negative for the EV-specific probe but positive for the IC-specific probe. The second negative control contained no armored EV RNA copies or armored IC RNA copies and should be negative for both probes with a mean of 200 LU.

Parechovirus-specific RT-PCR. The conditions of the RT-PCR for the detection of parechoviruses were similar to those for the protocol described above, except for the primers used in the PCR. For the specific detection of parechoviruses, we used the primer pair described by Oberste et al. (21).

RESULTS

Determination of the lower limit of detection of the EV RT-PCR assay. To determine the lower limit of detection of the EV RT-PCR assay, we spiked decreasing amounts of both armored EV RNA and armored IC RNA diluted in TSM buffer into 200 μl of EV-negative CSF before RNA extraction by the method of Boom et al. (4). RNA was eluted in 100 μl, and 40 μl of extracted RNA (2/5 of the extracted RNA) was

TABLE 2. Specificity of EV RT-PCR for prototype strains of EVs and controls

Virus (virus serotype or titer)	Source	Result	Mean (range) LU of EV RNA
CVA (2–4, 6–18, 20, 21, 24[a])	Cell culture	Positive	87,000 (57,000–117,000)
CVA (1, 5, 19, 22)	Suckling mice	Positive	87,000 (73,000–100,000)
CVB (1–6)	Cell culture	Positive	91,000 (75,000–107,000)
Echovirus (1–9, 11–16, 17–21, 24–27, 29–33[b])	Cell culture	Positive	98,000 (86,000–111,000)
Echovirus (22, 23[c])	Cell culture	Negative	218 (209–227)
Enterovirus (68–71)	Cell culture	Positive	77,000 (68,000–86,000)
Poliovirus (1, 2, 3)	Cell culture	Positive	104,000 (79,000–129,000)
Rhinovirus (1A, 1B, 3, 8, 11, 13, 14, 15, 16, 88)	Cell culture	Negative	228 (205–277)
HAV	Cell culture	Negative	237
Negative control		Negative	226 (217–235)
HPC[d] (25,000 EV RNA copies/ml)		Positive	85,000 (62,000–106,000)
LPC[e] (5,000 EV RNA copies/ml)		Positive	58,000 (46,000–70,000)
IC (2,500 IC RNA copies/ml)		Positive	51,000 (40,000–63,000)

[a] Echovirus type 34 is a variant of CVA type 24.

[b] Echovirus type 8 is a variant of echovirus type 1, CVA type 23 is a variant of echovirus type 9, echovirus type 10 was reclassified as reovirus type 1, and echovirus type 28 was reclassified as human rhinovirus type 1A.

[c] Echovirus types 22 and 23 were reclassified as parechovirus types 1 and 2, respectively.

[d] HPC, high-positive control.

[e] LPC, low-positive control.

TABLE 3. Composition of and results for the 2001 QCMD proficiency panel

Code	Serotype	Virus titer[a]	LU		Result
			EV RNA	IC RNA	
EV-C01	CVA 9	0.036	31,104	65,482	Positive
EV-C02	CVA 9	0.36	87,325	81,793	Positive
EV-C03	CVA 9	3.6	90,570	71,435	Positive
EV-C04	No virus		214	89,860	Negative
EV-C06	Echovirus 11	25.2	96,460	85,396	Positive
EV-C11	Echovirus 11	252	99,930	43,751	Positive
EV-C09	Echovirus 11	25,200	87,380	860	Positive
EV-C07	CVB 5	317	83,867	32,092	Positive
EV-C08	EV 71	56.4	98,508	31,411	Positive
EV-C10	No virus		226	74,234	Negative
EV-C05	Echovirus 6	20,000	82,529	27,549	Positive

[a] The titers of the original virus stocks before inactivation and freeze-drying were as follows: CVA type 9, 3.6×10^6 50% tissue culture infective doses ($TCID_{50}$s) per ml; echovirus type 6, 2.0×10^8 $TCID_{50}$s/ml; echovirus type 11, 2.5×10^7 $TCID_{50}$s/ml; CVB type 5, 3.2×10^7 $TCID_{50}$s/ml; EV type 71, 5.6×10^6 $TCID_{50}$s/ml.

used for RT; subsequently, 50% of the cDNA was used for PCR (1/5 of the extracted RNA). Limiting dilutions of the armored EV RNA revealed a detection limit of 84 EV RNA copies in the extraction procedure with a 50% hit rate (5 of 10 runs), resulting in a detection limit of 17 copies of EV DNA by PCR (1/5). Limiting dilutions of the armored IC RNA revealed a detection limit of 28 IC RNA copies in the extraction procedure with a 60% hit rate (6 of 10 runs), resulting in a detection limit of 6 copies of IC DNA by PCR (1/5) (Table 1). Identical results were found after the direct release of EV RNA and IC RNA from its phage (without CSF as a background) after incubation at 70°C for 5 min (results not shown).

Testing of EV serotypes. All known EV serotypes ($n = 64$) were tested by the EV RT-PCR described here. The extraction of every single EV serotype and non-EV serotype was done alternately with negative extraction controls that contained IC RNA. All EV serotypes were positive, whereas the non-EVs, including echovirus types 22 and 23, rhinoviruses, HAV, and all negative controls, were negative. The negative results were truly negative, since the ECL signals for coextracted armored IC RNA were all positive (Table 2).

Testing of 2001 and 2002 QCMD proficiency panels. The Quality Control for Molecular Diagnostics (QCMD) Program EV proficiency panels from 2001 and 2002 consisted of coded freeze-dried samples, including an echovirus type 11 and CVA type 9 dilution series, other EV serotypes representing different genetic clusters of human EVs, and negative controls. The freeze-dried samples were reconstituted with 1 ml of sterile water by the QCMD Program protocol, and 200 μl of the reconstituted sample was used for extraction and amplification, as described in Materials and Methods. All samples of the 2001 QCMD proficiency panel were positive, regardless of the serotype, and all negative controls included tested truly negative, since the ECL signals for coextracted armored IC RNA were all positive (Table 3). However, when we tested the 2002 QCMD proficiency panel, four invalid results (33%), i.e., no result for either EV or IC, were found, which meant that no conclusive result could be reported (Table 4). These four invalid results were probably caused by inhibition of the RT reaction, and we therefore decided to add 400 ng of bovine α-casein per μl to the RT reaction mixture as well as to the PCR mixture. After the addition of 400 ng of bovine α-casein per μl to the RT reaction mixture, the results for all panel members were valid. All samples expected to be positive were found to be positive except for one sample with CVA type 9 with the lowest virus titer, which was negative. All negative results were truly negative, since the ECL signals for coextracted armored IC RNA were all positive (Table 4).

Testing of clinical specimens. A total of 322 clinical specimens were obtained from patients suspected to be infected with EV and were tested by the RT-PCR. Forty-five positive results (14%), 267 negative results (82.9%), and 10 invalid results (3.1%) were found. These invalid results were found for 7 of 281 CSF samples (2.5%), 2 of 18 fecal samples (11.1%), and 1 of 10 throat swab samples (10%). Repeat tests by the optimized RT-PCR with these 10 samples with invalid results revealed that all invalid results apart from those for one fecal

TABLE 4. Composition of and results for the 2002 QCMD proficiency panel with and without addition of bovine α-casein to the RT reaction mixture[a]

Code	Serotype	Virus titer[b]	Without bovine α-casein		Result	With bovine α-case in LU		Result
			EV RNA	IC RNA		EV RNA	IC RNA	
EV-D01	Echovirus 11	25	32,782	20,526	Positive	53,764	12,046	Positive
EV-D02	Echovirus 11	250	45,173	13,993	Positive	59,508	4,922	Positive
EV-D03	CVA 9	36	64,338	221	Positive	50,855	3,836	Positive
EV-D04	No virus		214	11,495	Negative	219	18,970	Negative
EV-D05	**CVA 9**	**0.36**	**224**	**347**	**Invalid**	20,137	12,794	Positive
EV-D06	Echovirus 11	25,000	55,558	1,410	Positive	57,322	798	Positive
EV-D07	CVA 16	0.25	12,636	14,211	Positive	38,583	14,211	Positive
EV-D08	CVA 9	0.36	1,413	12,754	Positive	15,560	33,891	Positive
EV-D09	**CVA 9**	**0.036**	**216**	**202**	**Invalid**	216	7,318	Negative
EV-D10	**No virus**		**247**	**246**	**Invalid**	225	19,841	Negative
EV-D11	**CVB 5**	**32**	**225**	**237**	**Invalid**	32,701	8,544	Positive
EV-D12	CVA 9	3.6	197	8,156	Positive	38,271	15,007	Positive

[a] Bovine α-casein was used at a concentration of 400 ng/μl. Invalid results are in boldface.
[b] The titers of the original virus stocks before inactivation and freeze-drying were as follows: echovirus type 11, 2.5×10^7 50% tissue culture infective doses ($TCID_{50}$s) per ml; CVA type 9, 3.6×10^6 $TCID_{50}$s/ml; CVA type 16, 2.5×10^5 $TCID_{50}$s/ml; CVB type 5, 3.2×10^7 $TCID_{50}$s/ml.

TABLE 5. Summary of results after testing of clinical specimens

Specimen	No. (%) of specimens with the following results:				
	Total	Positive	Negative	Invalid	
				Without bovine α-casein	With bovine α-casein at 400 ng/μl
CSF	281	36	238	7 (2.5)	0
Feces	18	6	10	2 (11.1)	1 (50)
Throat swab	10	0	9	1 (10)	0
Other	13	3	10	0	0
Total	322	45 (14)	267 (82.9)	10 (3.1)	1 (0.3)

sample were valid and negative for EV and could be reported to the clinician (Table 5).

Comparison of virus culture with RT-PCR. Eighty-seven clinical specimens suspected to be infected with EV were available to be tested by virus culture and RT-PCR. Agreement of the results between virus culture and RT-PCR was found for 72 of 87 clinical specimens (82.8%). A negative result by both virus culture and RT-PCR was found for 54 clinical specimens, whereas positive results by virus culture and RT-PCR were found for 18 clinical specimens. Discordant results were found for 15 clinical specimens. For three of these the result of virus culture was positive for EV RNA, whereas the result of RT-PCR was truly negative for EV RNA, since the results for IC RNA were positive. Two of these RT-PCR-negative samples were subjected to a parechovirus-specific RT-PCR and were found to be positive (results not shown). For the other RT-PCR-negative sample, a CPE was found only after 4 weeks. The remaining samples with discordant results consisted of 12 clinical specimens that were RT-PCR positive and virus culture negative (Table 6).

DISCUSSION

Isolation of EV in cell culture is still regarded as the diagnostic "gold standard." There are, however, many disadvantages to culture, such as its labor-intensiveness, the delay of days to weeks to obtain a positive result, and its false negativity rate of approximately 25 to 35% because of failures of antibody neutralization and the inability of certain CVA serotypes to grow in cell culture (15, 25).

Many of the disadvantages of cell culture can be overcome by RT-PCR. The primer and probe sets most frequently used for the detection of EV are those described by Chapman et al. (9) and Rotbart et al. (25). These primers and probes have been proved to be reactive with all known EVs and fail to amplify echovirus types 22 and 23, which have been reported to be members of a genetically distinct genus in the family *Picornaviridae* (13, 20, 23). We designed a primer and probe set with minor modifications in comparison to those first described by Chapman et al. (9) and Rotbart et al. (25) not only to ensure that all known EV serotypes would be detected but also to avoid cross-reactivity between EV serotypes and rhinoviruses. This cross-reactivity with rhinoviruses could have an important impact if nasal or pharyngeal swab specimens are used for testing.

We have described an RT-PCR assay in a nonnested format for the detection of EV RNA in clinical specimens in which 2,500 copies of armored IC RNA mimicking the EV target per ml were included in the RNA extraction step and all subsequent steps of the procedure. This armored IC RNA can be spiked directly into clinical specimens without degradation of the RNA and enables monitoring of the complete nucleic acid extraction and amplification processes for each specimen.

The sensitivity of the RT-PCR was evaluated with limiting dilutions of both armored EV RNA and armored IC RNA. We found a detection limit of 6 copies of IC DNA in 60% of the samples by PCR. Poisson statistics predict that 63% of the reactions will be positive by PCR with a single copy of DNA (12). Thus, if RNA is extracted and reverse transcribed with efficiencies of 100%, the 60% detection rate by PCR should represent 1 copy of IC DNA. Since we found comparable results after the direct release of RNA from the armored RNA by incubation at 70°C for 5 min and after extraction of the RNA from the armored RNA by the method of Boom et al. (4), our results might suggest a less efficient RT step or the presence of a certain percentage of empty armored RNA phages. In addition, the approximately threefold difference in detection between EV RNA and IC RNA that we observed might be explained by inaccuracies in the determination of the concentration and the steps used for dilution of the stock solutions. The lower limit of sensitivity of the RT-PCR was based on the lower detection rate of IC RNA and was about 150 copies of IC RNA/ml. The presence of 500 copies of IC RNA during extraction from 200 μl of clinical specimens allowed us to draw the conclusion that a specimen found to be negative for EV RNA but positive for IC RNA would contain less than 2,500 copies of EV RNA/ml.

The use of the armored IC RNA was critical in the detection of false-negative reactions or invalid results. Recently, it was shown (6) that an inhibitor(s) of DNA-processing enzymes could be introduced by the extraction method of Boom et al. (4) itself and after DNA extraction from clinical specimens, such as CSF and urine. This inhibition could be relieved by a novel buffer containing bovine α-casein. Similar results were found in a recent study by Boom et al. (7), in which inhibitors were relieved after extraction of DNA from fecal specimens by the addition of bovine α-casein directly to the PCR mixture. Despite the presence of bovine α-casein in the PCR mixture, strong inhibition of IC RNA was still found for 4 of 12 (33.3%) samples of the 2002 QCMD proficiency panel and 10 of 322 (3.1%) clinical specimens. Thus, the invalid results were

TABLE 6. Comparison of results of RT-PCR and virus culture with different clinical specimens from patients with clinically suspected EV infections

Clinical specimen	No. (%) of specimens with the following results[a]:				
	Total	RT-PCR +, culture +	RT-PCR +, culture −	RT-PCR −, culture −	RT-PCR −, culture +
CSF	68	15	10	40	3
Feces	13	3	1	9	0
Throat swap	4	0	0	4	0
Urine	1	0	0	1	0
Biopsy	1	0	1	0	0
Total	87	18 (20.7)	12 (13.8)	54 (62.1)	3 (3.4)[b]

[a] +, positive result; −, negative result.
[b] Two isolates were identified by culture as parechovirus and not EV, and one isolate was positive by culture only after 4 weeks, indicating a very low virus titer.

caused not only by inhibition of the PCR but also by inhibition of the RT reaction. The optimized RT-PCR protocol with the addition of bovine α-casein in the RT reaction relieved the inhibition of the RT-PCR, resulting in valid results for all panel members of the 2002 QCMD proficiency panel and for all clinical specimens except one fecal specimen. We found similar results by the extraction of MS2 RNA in a background of fecal specimens and a subsequent RT reaction as a model system (unpublished data). The mechanism of relief of inhibition is not known, but it has been speculated (6) that stretches of phosphorylated serine residues present in bovine α-casein may be involved. These phosphorylated regions give bovine α-casein a high chelating force toward positively charged metal ions (3, 11, 27). This chelating power may be involved in the enhancing properties of bovine α-casein by capturing coextracted metal ions which might otherwise act as inhibitors of both RT and PCR.

At present, viral culture is still the gold standard for detection of EV infections. Validation of an RT-PCR assay for the diagnosis of EV infections is difficult because cases are defined clinically and viral culture has a poor sensitivity (approximately 70%), partly due to the inability of certain CVA serotypes to grow in cell culture (15, 25). We found an overall good agreement of 82.8% between the results of virus culture and those of RT-PCR. CSF provides the most direct link to disease but is usually less successful in virus culture. We found high rates of discordant results (17.2%) between virus culture and RT-PCR, mainly with CSF samples. Delayed processing and improper handling of the clinical specimens may have contributed to the false-negative results of virus culture, and our results showed the added value of the RT-PCR assay described here for the diagnosis of EV infections.

In conclusion, viral culture for EV will still be of use for the clinical diagnosis of EV infections. However, the EV RT-PCR assay described here will be of benefit as a sensitive, highly specific, and faster assay for the detection of EV in clinical specimens. The use of armored IC RNA strongly reduces the rates of false-negative results, and the addition of bovine α-casein relieves inhibition of the RT-PCR in 99.7% of cases.

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