JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2003, p. 1750–1752 Vol. 41, No. 4
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.4.1750–1752.2003

New PCR Test That Recognizes All Human Prototypes of Enterovirus: Application for Clinical Diagnosis

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Received 6 June 2002/Returned for modification 25 September 2002/Accepted 20 December 2002

We describe a new PCR test (Penter RT-PCR) that recognizes all 64 prototypes of enterovirus. Sixty clinical samples were analyzed in parallel with this Penter RT-PCR and previously described PCR tests: 34 and 32 samples tested positive, respectively. This assay is suitable for use in clinical diagnosis, and its ability to amplify all known serotypes makes it more useful than other consensus PCR tests.

Enteroviruses (EVs) are small single-stranded RNA viruses that comprise 64 serotypes recently redistributed into five species (22). They are responsible for a wide variety of clinical manifestations (eruptions and respiratory, ocular, cardiac, and neurological symptoms [8, 17]). The PCR test first described almost 10 years ago (3, 9, 18) has become the technique of choice for the diagnosis of these infections, particularly for cerebrospinal fluids (7). The rapidity of diagnosis by PCR has proved to be the determining factor in the management of patients, reducing the cost (by preventing unnecessary use of antibiotics) and duration of hospitalization (15, 16). None of the PCR techniques proposed to date, using primers located in the 5′ noncoding region, recognizes all 64 prototypes of EVs. The primers described by Zoll et al. do not amplify coxsackieviruses A11, A17, A24 or echovirus 16 (23). Furthermore, it is known that EVs exhibit a high degree of intratypic sequence heterogeneity (13). We describe a new PCR method (Penter RT-PCR) that detects all serotypes of EVs.

The Penter RT-PCR primers (Penter-1 and 2) were selected from within the 5′ noncoding region of the EV genome and are 85% identical to known enteroviral RNA sequences. A stair primer PCR system (5, 12) was used. A 420-bp fragment was amplified. This new reverse transcription (RT)-PCR test was performed with all prototypes of EV (obtained from tissue culture or suckling mouse brain), with serotyped field strains, and with frozen clinical samples. These samples were also analyzed by PCR (a technique hereafter referred to as in-house reference RT-PCR) with primers previously described by Zoll et al. (23) or by Rotbart et al. (19) and by means of the protocols described below.

RNA was extracted from prototypes of EV with guanidinium thiocyanate (4, 11). For specimens from patients, RNA was isolated from 140 μl of the sample, by using a viral RNA minikit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. RNA from a single extraction was used for both PCR tests, and an aliquot (10 μl) of RNA extract was used as a template for RT. The reaction was performed in a final volume of 20 μl, and we added 10 μl of cDNA to 40 μl of reaction mixture for all PCR tests. RNA extraction and RT, cDNA amplification, and amplicon detection were performed in three separate, nonadjacent rooms.

For the Penter RT-PCR test, the protocol was as follows: RT was performed with 0.5 μM Penter-2, 250 μM concentrations (each) of the four deoxynucleoside triphosphates (dNTPs), 50 U of Moloney murine leukemia virus reverse transcriptase (Stratagene, Ozyme, Montigny-le-Bretonneux, France). The cDNA obtained was then amplified in a volume of 50 μl containing 10 μl of cDNA. The PCR mixture, containing 1 μM concentrations of each primer (Penter-1 and Penter-2), 200 μM concentrations of each of the four dNTPs, and 2 U of Hot Star *Taq* polymerase (Qiagen) in the appropriate amplification buffer, was incubated in a Hybaid PCR Express thermal cycler (Life Science International, Cergy Pontoise, France) and, after *Taq* polymerase activation, was subjected to 5 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 60 s, and extension at 72°C for 50 s and 35 cycles at 94°C for 15 s, 54°C for 50 s, and 74°C for 50 s. The amplified product was detected in microtiter plates, with a specific 5′-biotinylated probe, by measuring absorbance. The primers (Penter-1 and Penter-2), amplification buffer, and specific probe were developed in collaboration with Argène Biosoft (Varilhes, France) and are commercially available as a kit, under the name "Enterovirus consensus." Each PCR experiment included negative controls prepared by replacing samples or infected cells with distilled water.

In-house reference PCR was carried out according to two different in-house protocols. At one center (Saint-Etienne Medicine Faculty), the primers 1, 2, and 3 used were those described by Zoll et al. (23). The following modified procedure (2) was applied. The cDNA was produced with 1 μM primer, 3,250 μM concentrations of each of the four dNTPs, and 20 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Cergy Pontoise, France). After a first round of 30 cycles (94°C for 30 s, 42°C for 60 s, and 72°C for 2 min) in a Thermocis thermocycler (Cisbio International, Gif sur Yvette, France) with primers 1 through 3, 2.5 U of *Taq* polymerase (GibcoBRL) and 250 μM concentrations of each of the four

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TABLE 1. Enterovirus serotypes that tested positive by Penter RT-PCR

Species	Serotype[a]	Prototype
Poliovirus	PV-1[b]	Mahoney
	PV-2[b]	Lansing
	PV-3[b]	Leon
Human enterovirus A	CV-A2	Fleetwood
	CV-A3	Olson
	CV-A5	Swartz
	CV-A7	Parker
	CV-A8	Donovan
	CV-A10	Kowalik
	CV-A12[c]	Texas-12
	CV-A14[c]	G-14
	CV-A16	G-10
	EV-71	BrCr
Human enterovirus B	CV-B1	Conn-5
	CV-B2	Ohio-1
	CV-B3[c]	Nancy
	CV-B4[c]	JVB
	CV-B5	Faulkner
	CV-B6	Schmitt
	CV-A9[c]	Bozek
	E-1[c]	Farouk
	E-2	Cornelis
	E-3[c]	Morrisey
	E-4	Pesascek
	E-5[c]	Noyce
	E-6[c]	D'Amori
	E-7	Wallace
	E-9	Hill
	E-11[c]	Gregory
	E-12	Travis
	E-13[c]	Del Carmen
	E-14	Tow
	E-15	Ch 96-51
	E-16	Harrington
	E-17	CHHE-29
	E-18	Metcalf
	E-19	Burke
	E-20[c]	JV-1
	E-21[c]	Farina
	E-24	DeCamp
	E-25	JV-4
	E-26	Coronel
	E-27	Bacon
	E-29[c]	JV-10
	E-30[c]	Bastianni
	E-31	Caldwell
	E-32	PR-10
	E-33	Toluca-3
	EV-69	Toluca-1
Human enterovirus C	CV-A1	Tompkins
	CV-A11[c]	Belgium-1
	CV-A13[c]	Flores
	CV-A15	G-9
	CV-A17	G-12
	CV-A18	G-13
	CV-A19	Dohi
	CV-A20	IH-35
	CV-A21	Coe
	CV-A22	Chulman
	CV-A24	Joseph
Human enterovirus D	EV-68	Fermon
	EV-70	J 670/71
Unclassified	CV-A4[c]	High Point
	CV-A6	Gdula

[a] PV, poliovirus; CV, coxsackievirus; E, echovirus; EV, enterovirus.
[b] Poliovirus Sabin strains also tested positive.
[c] Serotyped field strains also tested positive.

TABLE 2. Results of Penter RT-PCR and in-house reference RT-PCR for clinical specimens

Clinical specimen	No. of specimens (S + R)[a]	No. with positive result by:	
		Penter RT-PCR	In-house reference RT-PCR
Stool	17 (4[b] + 13)	9	10
Throat	7 (0 + 7)	2	1
Nasopharynx	5 (0 + 5)	4	4
CSF[c]	31 (15 + 16)	19	17
Total	60 (19 + 41)	34	32

[a] Number of specimens studied at the Saint-Etienne Faculty of Medicine (S) and at the Rennes Faculty of Medicine (R).
[b] Tested positive by tissue culture.
[c] CSF, cerebrospinal fluids.

dNTPs, 2 μl of the PCR product was used for a second round of 20 cycles (94°C for 15 s, 49°C for 60 s, and 72°C for 2 min) with primers 1 and 2. The amplified products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide, and the fragments, 154 bp in length, were observed under UV light. At another center (Rennes Faculty of Medicine), the primers and probe were those described by Rotbart et al. (19). RT was performed as for Penter RT-PCR, except that we used primer EV2. The PCR mixture, containing 1 μM concentrations of each primer (EV1 and EV2), 200 μM concentrations of each of the four dNTPs, 10 μM digoxigenin-11-dUTP (Roche Diagnostics Systems, Neuilly-sur-Seine, France), and 2 U of *Taq* polymerase (Promega, Charbonnières, France), was incubated in a Hybaid PCR Express thermal cycler (Life Science International) over 40 cycles (92°C for 15 s, 54°C for 75 s, and 72°C for 75 s). Amplicons were detected in microtiter plates (PCR ELISA DIG detection, Roche) by using a specific 5′-biotinylated probe, EV3.

With the Penter primers, all 64 prototypes gave a positive signal on microtiter plates (Table 1). The three Sabin poliovirus strains and 18 serotyped field strains were also recognized. The sensitivity of the test was determined by using a culture of coxsackievirus B4 and poliovirus strain Sabin 2 and was 0.1 50% tissue culture infective dose. DNA viruses (Epstein-Barr virus, cytomegalovirus, herpes simplex virus type 1) and RNA viruses (respiratory syncytial virus, hepatitis C virus) gave no amplification products. A field rhinovirus strain gave no amplification product, but the rhinovirus 3 serotype was detected. Parechoviruses 1 and 2 (formerly echoviruses 22 and 23) were not detected (20).

We tested 60 samples (Table 2). The specimens of the Saint-Etienne Medicine Faculty were taken from patients who had already tested positive by PCR (Amplicor Enterovirus, Hoffmann-Laroche, Basel, Switzerland) or by tissue culture. Specimens from the Rennes Faculty of Medicine were taken from patients for whom investigation for EV had been prescribed. Thirty-four samples tested positive with the Penter primers, and 32 tested positive with the Rotbart or Zoll primers. In four cases, the results of the two types of test were discordant: positive detection was achieved in three cases with the Penter system only and in one case with the Rotbart primers only. All the samples giving discordant results were retested with the Penter system and by in-house reference PCR. A total of 35

samples tested positive with one or the other PCR test. All samples known to be positive from the Saint-Etienne Medicine Faculty retested positive by in-house PCR or Penter PCR.

Between 1970 and 1983, Strikas et al. (21) identified the 15 serotypes of EV most frequently diagnosed in the United States. The serotypes coxsackievirus A11, A17, A24 and echovirus 16 (coxsackievirus A15 was not tested) not recognized by the primers described by Zoll et al. (23) were not among these serotypes, but neither were dozens of other serotypes. The aforementioned epidemiological study, involving isolation of the virus, showed that the unrecognized serotypes are not those most frequently detected in epidemics to date. However, the recognition of all serotypes, as observed with the test we propose, may nonetheless be advantageous in the face of epidemics due to serotypes of enteroviruses that circulate more rarely. It has been shown, for example, that echovirus 16 (1) may cause epidemics and that coxsackievirus A24 and its variants are a major cause of acute hemorrhagic conjunctivitis (10). These two serotypes are not recognized by the EV primer currently used and described in published studies. Halonen et al. (6) described a consensus PCR test, but they did not test coxsackievirus A18 or echovirus E7. Pozo et al. (14) reported that the Rotbart primers used in the Amplicor Enterovirus test do not always recognize echoviruses 1 and 5. In short, by using the principle of stair primers, the Penter RT-PCR test is able to detect all serotypes of EVs, including both prototype and field strains.

In the highly conserved 5′ noncoding region of enteroviruses used to generate the Penter primers, the probe was less specific than expected (recognition of rhinovirus 3). Hyypiä et al. (9) also reported this abnormality. The binding energy of these probes was found to be higher than that deduced from linear sequence analysis. We think that this is due to the particular three-dimensional structures of these regions.

This new consensus RT-PCR test for enteroviruses, which seems as sensitive as techniques previously described for use with clinical samples, should increase the reliability of EV infection diagnosis because it recognizes all known serotypes, without exception. Clinical studies on epidemics and diverse samples should confirm the ability of this new test to diagnose enterovirus infections reliably.

We are indebted to Jean Larroque, Martine Joannès, Philippe Bourgeois, and Come Barranger from Argène-Biosoft for their constant support. We are grateful to Danielle Thouvenot and to Jean-Jacques Chomel from Rockefeller University (Lyon I) for providing coxsackievirus strains B1 to B6.

Isabelle Josselin is funded by Argène-Biosoft.

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