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Detection and Rapid Differentiation of Human Enteroviruses following Genomic Amplification

MEI MEI KUAN*

National Institute of Preventive Medicine, Department of Health, Executive Yuan, Taipei, Taiwan, Republic of China

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By employing a nested PCR (n-PCR) with specific primers derived from the 5′ nontranslated and consensus region of the human enterovirus genome, we detected enterovirus RNA from 32 different serotypes of prototypic strains. A specific 297-bp fragment was amplified by this method from all of these strains. Not only was the method highly sensitive, detecting enterovirus RNA extracted from 0.01 50% tissue culture infective dose/50 μl (which is more sensitive than our current routine method of enterovirus diagnosis, based on the virus isolation and serotypic neutralization), but it was also relatively rapid. By using this technology, we also detected enterovirus RNA in uncultured specimens (including throat swabs and stools) from patients with respiratory illness and acute flaccid paralysis syndrome. This method enabled us to rapidly and directly distinguish enterovirus-infected specimens from nonenterovirus specimens in laboratory diagnosis. Furthermore, restriction fragment length polymorphism was assessed as an alternative means of differentiating various serotypes of prototypical enteroviruses. Fourteen of 16 human enterovirus-infected specimens exhibited restriction patterns identical to those of the corresponding prototypes.

Distinguishing enterovirus (EV)-related illnesses from those due to bacteria or nonenteroviruses is important for prognostic, therapeutic, and epidemiological purposes. The human EVs include the subgroups of polioviruses (PVs) (3 serotypes), coxsackieviruses A (CAs) (23 serotypes), coxsackieviruses B (CBs) (6 serotypes), echoviruses (Es) (32 serotypes), and 4 serotypes of newer EVs (EV68 to -71) (14). Classified within the family *Picornaviridae*, they contain the genome of a single-stranded RNA with positive polarity and are composed of approximately 7,500 nucleotides (15). Acute EV infections occur commonly and are associated with a broad spectrum of clinical features, including acute myopericarditis (2, 11), poliomyelitis (14, 24, 26), aseptic meningitis (1, 25), fatigue syndrome (27), respiratory and gastrointestinal infections, some asymptomatic presentations (14), and, less commonly, an encephalitis. The current diagnostic method for detection of EVs in clinical specimens relies on a combination of several cell cultures (6), which is hampered by a slow turnaround time and relatively low sensitivity, as well as by the serotypic diversity of EVs. Virus typing and neutralization with an intersecting viral antiserum pool are exceedingly tedious and expensive and therefore are unavailable in most diagnostic laboratories (12, 14, 20). Alignment studies of the sequenced EVs (3, 7, 9, 13, 15, 17, 19, 23, 24) have revealed a high degree of conservation within the 5′ nontranslated region of the EV genome. In addition, recent publications have revealed that nested PCR (n-PCR) is a more sensitive and time-saving technique (21, 22) than those employing PCR only or a combination of PCR and hybridization. This study employs a PCR-based method using a universal primer from the 5′ nontranslated region of the specifically EV genome in the reverse transcription (RT) step (4, 18, 28). However, the other three primers used in the subsequent step of n-PCR are derived from a fragment of homologous sequence known to be from the family *Picornaviridae* (8, 10, 16, 17). Such a combined design is a novel one and gives a DNA product with a size of 297 bp. In this study, n-PCR and restriction fragment length polymorphism (RFLP) were also used to directly examine the uncultured specimens.

* Mailing address: 161 Kun-Yang Street, Nan-Kang Taipei, Taiwan, R.O.C.

MATERIALS AND METHODS

Clinical samples. Throat swabs and stools were obtained from patients with a syndrome characterized by acute flaccid paralysis and upper respiratory symptoms. Stools and throat swabs were treated according to a previous described method of preparing the supernatant stocks of crude fluids (26). Part of these suspensions was also used for conventional virus isolations (20).

Virus stock. Stocks of EV strains were obtained from our institutes as well as others. These different serotypes of EV stocks were recovered in Vero, Hep-2, or rhabdomyosarcoma cells for viral cultures. After having demonstrated a complete cytopathic effect, the cultures were frozen and thawed three times. Debris were removed by centrifugation at 1,000 × *g* for 15 min at room temperature, and the virus suspensions were stored at −70°C.

RNA extraction. Nucleic acids were purified by successive extractions with phenol, phenol-chloroform-isoamyl alcohol (24:24:1), and chloroform-isoamyl

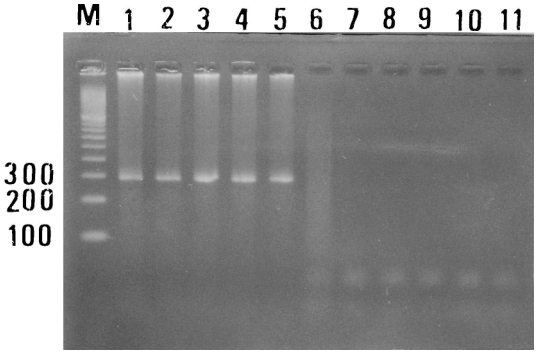

FIG. 1. Detection of EV RNA extracted from CB5-infected cell culture supernatant. After single RT-PCR and n-PCR, respectively, 8 μl of those products was visualized by agarose gel electrophoresis. Lanes 1 to 6 show products derived from the amplification of RNA extraction of 10-fold serial dilution of CB5 in 50 μl of cell culture medium, corresponding to 100 $TCID_{50}$ (lane 1) to 0.001 $TCID_{50}$ (lane 6). The product obtained from n-PCR (297 bp) was detected at 0.01 $TCID_{50}$ (lane 5). Lanes 7 to 11, negative controls, including rhinovirus 1B, adenovirus, measles virus, Hep-2 cells, and water; lane M, molecular size marker (100-bp ladder).

TABLE 1. Detection of EV prototypes propagated by n-PCR with a specific set of primers

EV prototype	Cell type used for propagation	n-PCR result[a]
PV1	Vero	+
PV2	Vero	+
PV3	Vero	+
CA1	RD	+
CA2	RD	+
CA3	RD	+
CA4	RD	+
CA5	RD	+
CA6	RD	+
CA7	RD	+
CA8	RD	+
CA9	RD	+
CA24	Stock	−
CB1	Vero	+
CB2	Vero	+
CB3	Vero	+
CB4	Vero	+
CB5	Vero	+
CB6	Vero	+
E4	Vero	+
E6	Vero	+
E7	Vero	+
E9	Vero	+
E11	Vero	+
E12	Vero	+
E14	Vero	+
E19	Vero	+
E20	Vero	+
E25	Vero	+
EV68	Vero	+
EV69	Vero	+
EV70	Vero	+
EV71	Vero	+
Adenovirus	Stock	−
Measles virus	Vero	−
Rhinovirus 1B	ATCC[b] stock	−

[a] +, positive by both sets of primers; −, negative by both sets of primers (see text for details).
[b] ATCC, American Type Culture Collection, Rockville, Md.

alcohol followed by ethanol precipitation (5). An aliquot of 50 μl of each EV supernatant was used for the RNA preparation or the quantitative test of RT-PCR. However, an aliquot of 100 to 200 μl of each suspension of crude stool and throat swab was prepared with the RNA for a direct RT-PCR test. Purified RNA was collected in 20 μl of distilled diethylpyrocarbonated water.

RT-PCR. cDNA was synthesized and amplified as described above. A 20-μl reaction mixture contained 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM dTT, 0.2 mM (each) deoxynucleoside triphosphates, 50 pmol of primer 1 (5′-ATTGTCACCATAAGCAGCCA-3′ [positions 577 to 596]), 5 U of avian myeloblastosis virus reverse transcriptase, and RNA isolated from the supernatant of 50 to 100 μl of virus-infected cells. After incubation at 37°C for 60 min, the PCR mixture was added. The PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.9), 3.6 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 50 pmol of primer 1 and primer 2 (5′-ACCTTTGTACGCCTGTT-3′ [positions 67 to 83]), and 1 U of *Taq* DNA polymerase. RNA-cDNA hybrids were denatured at 94°C for 5 min. The amplification was performed in 35 cycles consisting of denaturation for 1 min at 94°C, primer annealing for 40 s at 40°C, and elongation for 2 min at 72°C. For the n-PCR, 5 to 8 μl of the first PCR mixture was added to 95 to 92 μl of the second PCR mixture, which was the same as first mixture, but with different primers, primer 3 (5′-AAGCACTTCTGTTTCCC-3′ [positions 166 to 182]) and primer 4 (5′-ATTCAGGGGCCGGAGGA-3′ [positions 447 to 463]). After another set of 35 cycles under the conditions described above, aliquots of 5- to 8-μl products of RT-PCR or n-PCR were separated by electrophoresis in 3% agarose gels and stained with ethidium bromide (0.5 mg/ml).

Restriction analysis. Aliquots of 15 μl of n-PCR products were incubated with 2 to 5 U of restriction enzyme (RE) in a 20- to 25-μl reaction volume with the buffer recommended by the manufacturers. Samples were incubated at 37°C for 4 h and were then analyzed by polyacrylamide (7%) gel electrophoresis. REs were obtained from Promega and Stratagene.

RESULTS

Quantitation of RT–n-PCR. Quantificative experiments with n-PCR used RNA extracted from a 10-fold dilution serial of cell culture supernatants infected by CB 5. At the same time, virus isolation was performed for 6 days and resulted in a barely positive result: a cytopathic effect at a virus dose of less than 1 50% tissue culture infective dose ($TCID_{50}$). However, the n-PCR successfully reduced the time deemed necessary to achieve a significantly positive result within 1 to 2 days at 0.01 $TCID_{50}$/50 μl. Figure 1 shows the results from agarose gel analysis of the RT-PCR of the virus culture's stock by a 10-fold serial dilution. The bands in the expected size region of about 500 bp in the case of the first-round PCR were barely visible (data not shown), but those around 297 bp in the case of n-PCR were relatively definite and clear.

Detection of EV RNA by RT–n-PCR and identification of various serotypes by RFLP. To verify whether the n-PCR method used in this study would allow the detection of EV strains of all serotypes, 32 different EV stocks of prototype strains were applied to the n-PCR. Both sets of primers gave successful amplifications of all of these viruses, including PV1, PV2, PV3, CA1 to 9, CB1 to 6, E4, E6, E7, E9, E11, E12, E14, E19, E20, E25, and EV68 to -71, resulting in DNA bands of the expected size (about 297 bp after the final n-PCR) with a visible band detected on agarose gel analysis. Otherwise, CA24, measles virus, adenovirus, and rhinovirus 1B were negative (Table 1). Restriction patterns of various prototypes were further evaluated, although so far there has been a lack of sequence data for most of them. The restriction patterns of CA2, CA3, and E4 are identical, as are those of CA5 and CA7, by *Sty*I, *Bgl*I, and *Xmn*I digestion (Table 2 and Fig. 2). Some of the lanes show additional weak submolecular bands that were attributable to incomplete cleavage or the presence of the first-round PCR product or products derived from one nested primer and one outer primer.

Direct detection of uncultured specimens from patients with EV infection by PCR-RFLP. Eleven throat swabs from patients with upper respiratory syndromes underwent virus isolation and were identified as CB1 and CB4 infections by serum neutralization. These originally uncultured samples then were re-evaluated by n-PCR. All showed positive results by n-PCR, at

TABLE 2. Fragments resulting from digestion by REs of 297-bp-amplified EVs

RE	DNA fragment size (bp)	Prototype EV(s)
*Sty*I	297	E4, E9, E11, E20, PV3, CA2, CA3, CA5, CA7, CB4
	226 + 71	E6, E19, PV1, CA6, CB2, CB3
	197 + 100	E7, PV2, CB1
	212 + 75 + 10	CB2, CB3, CB6
	112 + 102 + 83	CB5
*Bgl*I	297	E7, E9, E11, E20, PV2, PV3, CB1, CB3, CA1, CA5, CA6, CA7,
	217 + 80	E4, E6, E14, E19, PV1, CA2, CA3, CB2, CB4, CB5
	21 + 80 + 196	CB6
*Xmn*I	297	E7, E11, PV2, PV3, CA1, CB3
	236 + 61	E4, E6, E9, E14, E20, PV1, CA2, CA3, CA5, CA6, CA7, CB1, CB2

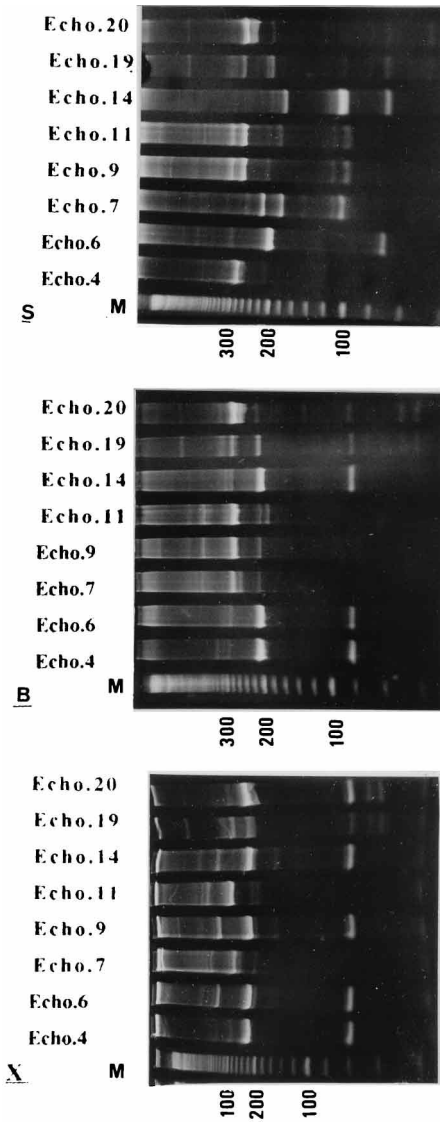

FIG. 2. Separation of *Sty*I-, *Bgl*I-, or *Xmn*I-digested 297-bp product amplified by n-PCR for EV RNA from different prototypes by polyacrylamide gel electrophoresis (7.0% polyacrylamide). Echo., echovirus; S, *Sty*I; B, *Bgl*I; X, *Xmn*I; M, molecular size marker (20-bp ladder).

least in terms of proving the existence of EV RNA in the 11 throat swabs, and 7 of the 11 specimens also showed an RFLP pattern identical to that of the prototype, CB1 (Table 3). Four were not defined, because the additional bands appeared to come from severely incomplete cleavage or mutation sequences in one of the three RE cleavage sites or from one nested primer and one outer primer (Fig. 3). We also collected the uncultured crude suspension of stools from some patients with acute flaccid paralysis syndrome. These patients had been diagnosed as being infected with the Sabin strain or other strains according to the serum neutralization method. Again, after PCR-RFLP, we could firmly verify the existence of the EV genome (Table 3).

DISCUSSION

Primer 1, used in the RT step described herein, is a universal primer intended to detect all EV genome sequences published to date (including those of PVs) but is not designed to amplify adenovirus, rhinovirus 1B, measles virus, influenza virus A (partly shown in Fig. 1), or the viruses detected by the previous assays, including cytomegalovirus, herpes simplex virus 1, parainfluenza virus 1, mumps virus, or rubella virus (4, 18, 28). However, primers 2, 3, and 4, available for the first-round PCR and n-PCR, were highly homologous with sequences of both EVs and rhinoviruses (8, 10, 16, 17). This is a different approach from those used in other published studies. According to our analysis, the n-PCR used in this study is specific for EV detection and is able to obtain a band with a size of 297 bp for further restriction analysis. This n-PCR achieved a degree of sensitivity and specificity comparable to those in other recent reports (21, 22) and was more convenient than the additional hybridization methods used by most other investigators to intensify the RNA signal from RT-PCR. Furthermore, the EV prototypes detected by our method outnumbered those of other reports. Therefore, we can assume that this n-PCR is appropriate for routine diagnosis of nearly all groups of human EVs. It can also eliminate a variety of clinical problems caused by contamination during processing of the cell culture or that occur in the current EV neutralization step of diagnosis (limited by slow turnaround time, relative insensitivity, and serotypic diversity in viral typing). From the clinical samples detected, our results successfully show that n-PCR can be very useful for rapid, direct investigation of a full spectrum of clinical specimens from a diverse population of patients with acute or persistent EV infections. That is, this method can play a critical role in the rapid screening of acute and potential EV infection, not only in our present surveillance, aimed at the

TABLE 3. Detection of EVs in uncultured clinical samples by n-PCR

Patient no.	Clinical characteristic(s)	Sample	n-PCR result	RE pattern	Virus isolation result
1	Upper respiratory symptoms	Throat swab	+	CB4	+ (CB4)
2–11	Upper respiratory symptoms	Throat swab	+	CB1 (7 patients) and unclear (3 patients)	+ (CB1)
12	Acute flaccid paralysis	Stool	+	PV2	+ (Sabin 2)
13	Acute flaccid paralysis	Stool	+	E4, CA2, or CA3	+ (untypeable)
14	Acute flaccid paralysis	Stool	+	PV1	+ (Sabin 1)
15	Acute flaccid paralysis	Stool	+	PV2	+ (Sabin 2)
16	Acute flaccid paralysis	Stool	+	PV3	+ (untypeable)
17	Acute flaccid paralysis	Stool	−	ND[a]	+ (Sabin 3)
18	Acute nosocomial infection	Throat swab	+	E11	−
19	Acute nosocomial infection	Throat swab	+	E11	−

[a] ND, not detected.

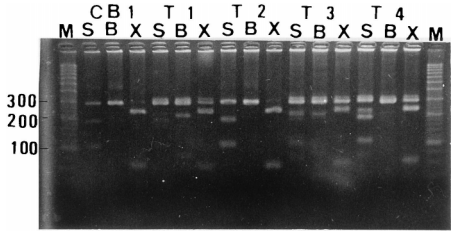

FIG. 3. Three percent agarose gel electrophoresis analysis of *Sty*I-, *Bgl*I-, or *Xmn*I-digested 297-bp product directly amplified by n-PCR for EV RNA in throat swabs from patients with upper respiratory infection with prototype CB1. T1 to T4, throat swab samples 1 to 4; S, *Sty*I; B, *Bgl*I; X, *Xmn*I; M, molecular size marker (20-bp ladder).

eradication of polio, but also in a broader sense of increasing the speed of public health measures taken against other diseases. Although the 5′ region is the most conserved region among EVs, utilization of differential restriction patterns to distinguish various serotypes may yield the following inconclusive results. (i) Some of the prototypes possess recognition sequences identical to those of the current restriction endonucleases, so that additional kinds of REs should be adopted to achieve further classification. (ii) Some wild-type strains may carry mutations or recombination sequences, so more specimens of each serotype should be examined in the future. (iii) Some patients with clinical cases of infection may occasionally have been coinfected with more than one virus strain. Nevertheless, by employing three REs (*Sty*I, *Bgl*I, and *Xmn*I each have their own specific six-base recognition site), we obtained a digestion pattern that was not too complicated and thus was easily visible. Therefore, this method of restriction analysis may provide one of the most efficient aids currently available for rapid tracking and further clarification of EV-infected specimens for epidemiological surveillance.

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