Jpn. J. Infect. Dis., 61, 497-499, 2008

Short Communication

Detection and Quantification of Enterovirus 71 Genome from Cerebrospinal Fluid of an Encephalitis Patient by PCR Applications

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(Received August 5, 2008. Accepted September 29, 2008)

SUMMARY: Enterovirus 71 (EV71) is one of the causative agents of hand, foot, and mouth disease (HFMD) and is known to cause encephalitis, but several reports have identified EV71 in cerebrospinal fluid (CSF). We detected EV71 in CSF from a 20-month-old infant. The patient was diagnosed with brainstem encephalitis associated with HFMD. The clinical features of the patient were high fever (39.1°C) and myoclonic jerks, and magnetic resonance imaging of the brain showed a bright signal area around the 4th ventricle. From a nasopharyngeal swab and rectal swab, EV71 was detected using reverse transcription (RT)-nested polymerase chain reaction (PCR). From CSF, the EV71 genome was identified using pan-enterovirus RT-nested PCR and sequencing. By real-time PCR, the nasopharyngeal swab, rectal swab, and CSF contained 1.8×10^4, 9.8×10^4, and 1.8×10 copies of the EV71 genome/μL, respectively. The enterovirus could only be isolated by cell culture from the rectal swab, and it was identified by a neutralization test using EV71-specific antiserum. RT-nested PCR and real-time PCR are considered to be sensitive tools for EV71 diagnosis in CSF.

Enterovirus 71 (EV71) is one of the significant causative agents of encephalitis. In Japan, the first outbreak of hand, foot, and mouth disease (HFMD) associated with central nervous system (CNS) disorders occurred in 1973; the causative agent of the outbreak was EV71 (1). Large outbreaks of EV71 infection associated with CNS diseases occurred in Malaysia in 1997 (2) and Taiwan in 1998 (3). In 2000, the EV71 outbreak accompanied by CNS diseases occurred in Hyogo Prefecture, Japan (4). Usually, the detection and identification of EV71 from the cerebrospinal fluid (CSF) of encephalitis patients are known to be difficult (5). In this study we report an acute encephalitis case in which EV71 was detected in CSF using reverse transcription (RT)-nested polymerase chain reaction (PCR) and sequencing.

A 20-month-old girl was admitted to the pediatric ward of a hospital in Kakogawa city in Hyogo Prefecture with a high fever of 39.1°C, rashes on her hands, general weakness and poor feeding for a 2-day duration on July 1st of 2003. Encephalitis associated with HFMD was suspected based on clinical findings including vesicular rashes on the hands and feet, meningeal signs, somnolence, and myoclonic jerks. A magnetic resonance imaging (MRI) showed a high signal area around the 4th ventricle. Laboratory tests were in the normal range except for a leukocyte count of 13,900/μL, lactate dehydrogenase (LDH) of 278 IU/μL, and blood sugar of 175 mg/dL. CSF showed pleocytosis: 2,608/3 μL of cells; mononuclear cells 400 and multinuclear leukocytes 2,208.

The clinical specimens including CSF, pharyngeal swabs, rectal swabs, and sera were taken on admission under informed consent of the parents and sent to Hyogo Prefectural Institute of Public Health and Environmental Sciences.

Immediately after the arrival of the samples, viral RNA was extracted from the clinical samples using the QIAamp Viral RNA Mini Kit (QIAGEN, Heidelberg, Germany). The DNA fragment was amplified by RT-PCR using SuperScript™ II One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen, Carlsbad, Calif., USA) and Ex-*Taq* (TaKaRa, Shiga, Japan). For the detection of the enterovirus RNA in CSF samples, RT-PCR was performed using primers E2 (5′-CCTCCGGCCCCTGAATG) and E1 (5′-CACCGG ATGGCCAATCCA). Consequently, semi-nested PCR was performed using E2 and entR1 (5′-ATTGTCACCATAAGC AGCCA) to amplify a 154-bp fragment (4). The PCR was carried out using 94°C for 10 s, 60°C for 10 s and 72°C for 20 s, and was continued for 45 cycles; in the first cycle, the denaturing step continued for 5 min at 94°C, and in the last cycle, the extension step continued for 1 min at 72°C. The same PCR conditions were used for the first and second PCR. An aliquot (4 μL) template was used for the PCR. For the semi-nested (or nested) PCR, PCR products diluted 100 times (in water) were used.

For quantitative analyses, viral RNAs were extracted from a total of 140 μL of samples and eluted by 60 μL of the elution buffer. For cDNA synthesis, 15 μL of the 60 μL elutants were used. Thus, the original samples were calculated to include 1.71 times the number of copies of viral genome obtained by using real-time PCR. Therefore, the copy numbers in the samples for detection limit (copies/μL) experiments were multiplied by 1.71. The RT procedure prior to the PCR was performed using 45°C for 45 min and 94°C for 2 min. The temperature conditions were established using the DNA thermal cycler Dice (TaKaRa).

For amplification of the 5′-untranslated (5′-UTR)/VP4/VP2 region of enterovirus in the clinical samples, RT-PCR (6) was performed using P-2 (5′- CCTCCGGCCCCTGAATGCGGC

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TAAT) and E33 (5′- TCCGGGAATTTCCAGTACCA) as the primers. Nested PCR was performed using the primer set EVP-4 (5′-CTACTTTGGGTGTCCGTGTT) and OL68-71R (5′-GGGAACTTCCAGTACCAYCC) (2). The PCR was carried out at 95°C for 10 s, 53°C for 45 s and 72°C for 45 s, and was continued for 40 cycles; in the first cycle, the denaturing step continued for 5 min at 95°C, and in the last cycle, the extension step continued for 4 min at 72°C.

Quantification of enterovirus was also performed by real-time RT-PCR (7,8). The primers and probes used were a forward primer (5′-TCCTCCGGCCCCTGA), reverse primer (5′-GATTGTCACCATAAGCAGCCA) and TaqMan Probe (5′-CGGAACCGACTACTTTGGGTGTCCGT). The machine used for the real-time PCR was an ABI PRISM 7700 (Applied Biosystems, Foster City, Calif., USA). In order to prepare a positive control standard for EV71, the RT-PCR product of an EV71 isolate (GenBank accession no. AB286954; position 1-190) was cloned into a plasmid using a TOPO TA cloning kit (Invitrogen). Plasmid DNA was purified using a HiSpeed Plasmid Midi kit (QIAGEN). The concentration of the plasmid DNA was measured by spectrophotometer at 260 nm, and the copy number/μL of inserted plasmid was calculated. Copies of EV71 genomes/μL were determined in clinical samples and serially diluted EV 71 genomes.

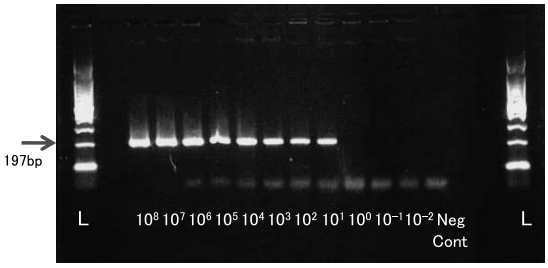

Fig. 1. Detection limit of PCR by primers E1 and E2. PCR result by primer E1 and E2 were shown. EV71 genome (1.0×10^8 copies/μL) were serially diluted to 10^8 - 10^{-3} copies/μL. Amplifications of PCR products (197 bp) were visible by an electrophoresis 10^8 - 10^1. The detection limit in original sample was calculated 1.7×10 copies/μL, because the (clinical) samples were 1.7 times diluted during genome extraction and cDNA synthesis procedure. L, 100-bp DNA ladder.

Table 1. Detection limits of PCR applications

PCR application	Calculated detection limit (EV71 genome copies/μL) in samples[1] (n = 3)
5′-UTR PCR	
primers E1+E2 (primer set 1)	1.7×10 - 1.7×10^2
primers entR1+E2 (primer set 2)	1.7×10^2 - 1.7×10^3
5′-UTR nested PCR[1] (primer set 1+2)	1.7 - 1.7×10
Real-time PCR	1.7 - 3.4×10^2

[1]: Calculated from serially diluted cDNA of EV71 in this study.

By using serially diluted EV71 genome extracted from EV71 isolate in this study, the detection limits of the PCR applications were compared as shown in Fig. 1. The detection limit was calculated by using serially diluted cDNAs. Because the clinical sample size in this study was small, we deduced the detection limit of EV71 using repeated PCR diagnoses of these samples.

The detection limits of single PCR (primers E1 + E2) and semi-nested PCR (primers entE1 + E2) were 1.7×10 - 1.7×10^2 and 1.7 - 1.7×10 copies/μL, respectively. Single-PCR (primers entR1 + E2) had 1.7×10^2 - 1.7×10^3 copies/μL of detection limit for single-PCR use (Table 1). These results show that 5′-UTR nested RT-PCR was the most sensitive method. The real-time PCR method (detection limit, 1.7 - 3.4×10^2 copies/μL) was less sensitive than the 5′-UTR nested RT-PCR. The 5′-UTR nested RT-PCR was 10 - 10^3 times more sensitive to single 5′-UTR PCR applications. 5′-UTR/VP4/VP2 nested PCR could not detect EV71 in CSF and appeared to be less sensitive than other PCR applications in this report.

Enterovirus gene was detected from CSF, pharyngeal swabs, and rectal swabs using PCR. Serum samples were negative for enterovirus using all types of PCR applications in this study. From the real-time PCR, CSF, pharyngeal swabs, and rectal swabs contained enterovirus genome in 1.8×10, 1.8×10^4, and 9.8×10^4 copies/μL in clinical samples, respectively. The copy number 1.8×10 copies/μL obtained for CSF was within the detection limit of the real-time PCR application used in this study.

Virus isolation using GL37 cells (4) was positive only with rectal swabs, and EV71 was identified by a neutralization test using type-specific antiserum. No virus could be isolated from any specimens using RD cells. The results of PCR and virus isolation are summarized in Table 2.

EV71 was detected from CSF using RT-nested PCR and real-time PCR. The amount of EV71 virus in CSF was minute, approximately $1/10^4$ of the amount detected from rectal swabs. Using RT-nested PCR, cerebrospinal specimens were positive only in PCR for the 5′-UTR. Its sequence (AB183003) matched 100% with the appropriate region of the sequence of the EV71 strain isolated from rectal swabs (AB238695). These results indicated that it is difficult to detect EV71 from CSF because the amount of virus is very small. However, we detected EV71 using highly sensitive RT-nested PCR a day after hospital admission and were able to have the diagnosis results fed back to the clinical site. By using real-time RT-PCR, quantitative detection of the EV71 genome was possible. RT-nested PCR and real-time PCR are considered to be sensitive tools for EV71 diagnosis in CSF.

Detecting EV71 in CSF is significant because, as Koch's

Table 2. PCR and isolation results of EV71 from an encephalitis patient

Specimen	5′-UTR/VP4/VP2 nested PCR	5′-UTR nested RT-PCR	Cell culture using GL37 cells	real-time PCR (copies/μL)
Cerebrospinal fluid	−	+[1]	−[2]	1.8×10
Pharyngeal swabs	+	ND[3]	−	1.8×10^4
Rectal swabs	+	ND	+	9.8×10^4
Serum	−	−	ND	ND

[1]: EV71 positive.
[2]: negative.
[3]: not done.

postulates state, to detect a virus from a diseased organism is important; especially for enteroviruses, which are often isolated from healthy children. Detection of the EV71 genome from CNS samples can provide strong evidence of EV71 encephalitis. Yen et al. (9) reported that the detection limit of coxsakievirus B type 3 was 20 copies/μL. Their result was similar to our real-time PCR result.

The viral RNA extraction kit which was used in this study recommended concentrating samples of up to 3.5 mL to a final volume of 140 μL. This procedure was not included in this study. Further study of the effect of CSF concentration methods on CSF diagnosis is thought to be necessary.

Although the clinical sample number in this study is small, our results show that the PCR applications in this study are very sensitive and appear to be applicable to the diagnosis of EV71 in CSF.

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