

Enhanced Detection of Enteroviruses in Clinical Samples by Reverse Transcription-PCR Using Complementary Locked Primer Technology[∇]

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To increase detection sensitivity, we modified primers using complementary locked primer (CLP) technology. The sensitivity of the reverse transcription-PCR (RT-PCR) with CLP-modified primers was 10- to 100-fold higher than that of RT-PCR without these primers. CLP-modified primers can increase sensitivity, providing a widely accessible method for molecular diagnosis.

Human enteroviruses are very common and important viruses, and they are associated with several clinical manifestations, ranging from mild febrile illness to severe cases. Enteroviruses (EVs) are the most common causes of viral meningitis in both children and adults, accounting for 70% of all cases in which a causative agent is identified (4, 15). Although EVs are usually isolated from specimens obtained from cerebrospinal fluid (CSF), throat swab, or stool, the sensitivity of detection based on virus culture is lower than that of molecular diagnosis based on gene amplification due to several constraints using virus-replicable cell lines (1, 3, 13). In particular, for the clinical diagnosis of aseptic meningitis, the detection of EV in CSF is helpful to confirm the infection (10). Therefore, when diagnosing meningitis, it is widely accepted that culturing should be replaced by a nucleic acid amplification test, such as real-time PCR, due to the latter's lower detection limits and promptness in suggesting timely, practical treatment (5, 7, 8, 9, 12, 14, 17, 18).

The purpose of this experiment was to increase the sensitivity of EV detection using complementary locked primer (CLP) technology and to evaluate this method using clinical specimens combined with the conventional one-step reverse transcription-PCR (RT-PCR) assay. The key features of CLP technology (under the patent of iNtRON Bio, South Korea) are the unique sequence structure, modified by adding complementary sequences at the 5' end for primer-based hot-start PCR, and the regulation of annealing temperatures, which can minimize nonspecific amplification and provide better sensitivity. The target region located within the highly conserved 5' noncoding region (NCR), the primer sequences, and PCR conditions have been previously reported (16, 19, 20). The primers were modified by inserting several complementary sequences at the 5' end. The RT-PCR assay without CLP modification consisted of primers EntF (forward, position 160 to 180),

5'-CAAGCACTTCTGTTTCCCCGG-3', and EntR (reverse, position 580 to 599), 5'-ATTGTCACCATAAGCAGCCA-3' (20). The RT-PCR assay with CLP modification consisted of primers NCR-cF (forward), 5'-GCTTGAAGCACTTCTGTTTCCCCGG-3', and NCR-cR (reverse), 5'-ACAATATTGTCACCAATAGCAGCCA-3' (CLP-modified region underlined).

To evaluate RT-PCR sensitivity based on the CLP modification, the detection limit was tested using five reference strains that belong to distinct genogroups (enterovirus 71 [EV71], coxsackievirus B2 [CVB2], echovirus 30 [E30], coxsackievirus A24 [CVA24], and poliovirus type 1 [P1]) obtained from the American Type Culture Collection (ATCC). CLP-modified primers that recognized the various EV serotypes were confirmed using 25 different EV strains isolated from clinical specimens during 1997–2005 in South Korea. Twenty-five distinct serotypes used in this study consisted of 12 echovirus (E1, E3, E5, E6, E7, E9, E13, E14, E16, E18, E25, and E30), four coxsackievirus A (CVA10, CVA16, CVA22, and CVA24), six coxsackievirus B (CVB1 to CVB6), one poliovirus type 1 (P1), and two new enterovirus (EV71 and EV74) strains.

To check cross-reactivity with other enteric viruses detected in the human gastrointestinal tract, parechovirus strains 1 and 2 belonging to the reclassified genus *Parechovirus*, human norovirus, and rotavirus were used for gene amplification. Eighty-nine clinical specimens (70 from stools, 14 from throat swabs, 4 from CSF, and 1 from serum) from patients with suspected aseptic meningitis during the EV epidemics in South Korea from June to September 2008 were included in the protocol using CLP-modified primers.

For EV detection, viral RNA was extracted using silica-coated magnetic beads combined with an automatic liquid handling system (Tecan, Switzerland), adapting Boom's methods (2). Extracted RNA (5 μ l) was used as a template for gene amplification by one-step RT-PCR. RNA dilutions (10-fold) from 10^{-1} to 10^{-8} of the EV reference strains EV71, CVB2, E30, CVA24, and P1 were prepared to assess the sensitivity of the RT-PCR assay combined with CLP technology. The reference strains and negative control were tested in duplicate. The detection limit for RT-PCR with the CLP modification was 0.5

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TABLE 1. Comparison of CLP and non-CLP RT-PCR results by TCID₅₀

Virus	Serotype	TCID ₅₀ /RT-PCR ^a	
		CLP assay ^b	Non-CLP assay ^c
HEV-A	Enterovirus 71	0.5	50
HEV-B	Coxsackievirus B2	50	5,000
	Echovirus 30	5	50
HEV-C	Coxsackievirus A24	0.5	5
Poliovirus	Poliovirus 1	5	50

^a These assays were done in duplicate.

^b RT-PCR with complementary locked primer (CLP).

^c RT-PCR without complementary locked primer (CLP).

to 50 50% tissue culture infective doses (TCID₅₀)/RT-PCR depending on the EV serotype, which provides 10- to 100-fold improvement in sensitivity compared to the RT-PCR assay without CLP modification (Table 1). All 25 EV strains were detected using methods with or without CLP-modified RT-PCR, whereas non-EVs (parechovirus, human norovirus, and rotavirus) were undetected and lacked cross-reactivity (data not shown).

Of the 89 clinical samples obtained from stools, throat swabs, CSF, and serum that were tested, 49 (70%) and 20 (28.6%) out of 70 stool specimens were positive using CLP-modified and unmodified primers, respectively. Samples from four throat swabs, two CSF specimens, and one serum were positive only with the CLP-modified primers (Table 2). Although EV detection from clinical specimens with low viral titers, especially those from throat swab or CSF, depended on the status of the specimens, the CLP-modified primers used in this study were sensitive and dramatically enhanced the detection of EV compared to results of the RT-PCR without CLP technology.

Several studies have demonstrated the high sensitivity, specificity, and utility of molecular methods for the diagnosis of EV infection in comparison with conventional cell culture methods (6, 11). Various PCR assays have been developed, but most of them lack the simple applications used in diagnosis methods. The use of various primers, extraction methods, amplification conditions, and methods of detection has been reported in the literature. In our study, we modified a simple and easy method that enhances EV detection. The sensitivity of the existing

TABLE 2. Comparison of CLP and non-CLP RT-PCR results by sample type

Method	No. of positive results/total no. of samples from source:			
	Stool	Throat swab	CSF	Serum
CLP ^a	49/70	4/14	2/4	1/1
Non-CLP ^b	20/70	0/14	0/4	0/1
Total	70	14	4	1

^a RT-PCR with complementary locked primer (CLP).

^b RT-PCR without complementary locked primer (CLP).

PCR-based diagnostic methods can be improved simply by using different primers. Hence, nucleic acid amplification systems, such as RT-PCR, can be adapted to enhance sensitivity for many types of pathogens solely by using CLP technology to modify the primers without altering laboratory protocol.

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