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Optimization of RT-PCR methods for enterovirus detection in groundwater

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ABSTRACT

Enteroviruses (EVs), which belong to the Picornaviridae family, infect individuals asymptomatically or cause mild symptoms (fever, runny nose, cough, skin rash, sneezing, mouth blister). Severe cases can cause various diseases, such as acute hemorrhagic conjunctivitis, aseptic meningitis, or myocarditis, especially in infants. These viruses can be transmitted via the fecal-oral route via contaminated water. In this study, we established a polymerase chain reaction (PCR) method for detecting EVs in water sample using Coxsackievirus B5 (CV-B5) and Echovirus 30 (E-30), which belong to species B of the four species of EVs (EV-A to D). Several methods have been investigated and compared for the detection of EVs, including real-time reverse transcription (RT) polymerase chain reaction and conventional RT-PCR. The most sensitive primer sets were selected, and the PCR conditions were modified to increase sensitivity. We also quantified the detection limits of real-time and conventional RT-PCR. The detection limits of conventional RT-PCR were detected in 10^5–10^6 copy/mL for CV-B5 and 10^6–10^7 copy/mL for E-30, respectively. This optimized method for detecting EVs is expected to contribute substantially to the investigation of EV outbreaks in water samples.

1. Introduction

Enterovirus is a single-stranded positive-sense RNA virus that belongs to virus group iv, *Picornaviridae* family, and the *Enterovirus* genus. According to the International Committee on Taxonomy of Viruses (ICTV), enteroviruses (EVs) are phylogenetically categorized into 15 species. EVs A–D are known to cause infections in humans and primates [1–3]. EVs have a non-enveloped structure and a total nucleic acid length of approximately 7.5 kb in length [4]. The capsid that surrounds RNA is composed of four polypeptides: VP1, VP2, VP3, and VP4 [5]. Of these, VP1, VP2, and VP3 are externally exposed, whereas VP4 is completely internalized and not exposed to the outside world. The P1 region encodes structural proteins (VP1, VP2, VP3, and VP4), whereas the P2 and P3 regions encode seven nonstructural proteins (2A-2C and 3A-3D) [6,7].

EVs are waterborne viruses that are primarily transmitted via fecal-oral contamination [8]. It is transmitted by the ingestion of contaminated food and drinking water or through person-to-person transmission. It is found in water, including rivers, oceans, and groundwater, but wastewater treatment does not completely eliminate EVs; therefore, continuous contamination monitoring is

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required [9,10].

EVs infect infants and children and clinical manifestations range from asymptomatic to hand, foot, and mouth disease; herpetic stomatitis; aseptic meningitis; and encephalitis [5,11,12]. Enteroviral outbreaks occur periodically in the Asia-Pacific region [13]. In 2018, Vietnam reported an outbreak of more than 53,000 cases [14]. In addition, large outbreaks caused by EVs occur every to 2–3 years in Japan and Taiwan [15]. In Korea, there was a case of a 12-month-old infant dying from enterovirus in 2009 [16]. In a study of 63 pediatric patients in the Department of Pediatrics at Severance Hospital, cerebrospinal fluid or fecal samples were collected, and 38 were positive for enteroviral infection [17]. According to the Korea Disease Control and Prevention Policy, the number of reported cases of enterovirus infection has increased from 2187 in 2017 to 4589 in 2019. Seventy-five percent of enterovirus infections occur in people aged 15 years or younger, with an infection rate that is particularly high among infants and young children and requires constant management [18,19].

One method for detecting EVs is polymerase chain reaction (PCR). Conventional PCR has the advantage of detecting low concentrations in environmental samples, rapid assay speed, and low cost [20]. However, real-time RT-qPCR has been widely applied in viral diagnostics because of its improved sensitivity, nucleotide-labeling chemistry, and advances in oligoprobe hybridization technology. Furthermore, this method also allows detection and monitoring in real time [21,22].

To date, several studies have reported the development of detection methods for environmental and patient fecal samples [23,24]. However, studies aimed at detecting viruses in water samples are rare. The lack of a standardized detection method complicates water source identification in the event of a waterborne diseases caused by an enterovirus. There is also a lack of comparative studies on the detection sensitivity and PCR primer concentrations for EVs. Water samples in which EVs are commonly found contain heavy metals and phenolic compounds. These compounds act as PCR inhibitors; therefore, PCR methods with high specificity and sensitivity are required [25,26].

In this study, we aimed to optimize an enterovirus detection method by comparing the primers and PCR conditions of existing methods, therefore the only sensitivity of test methods was evaluated to find the most detectable primer/probe set and PCR conditions. This study proposed a method that can be applied to drinking water to rapidly detect EVs in cases of food poisoning.

2. Materials and methods

2.1. Virus culture and RNA extraction

There are many types of enteroviruses, but we chose only Coxsackievirus B5 and Echovirus 30, which are provided by domestic distributors (Korea Bank for Pathogenic Virus). The strains for the development of the enterovirus detection test method, Coxsackievirus B5 (1.6×10^9 pfu/mL) and Echovirus 30 (5.0×10^{10} pfu/mL), were obtained from the Korea Bank for Pathogenic Virus. African green monkey kidney (Vero) cells were obtained from the Korean Cell Line Bank. All the viruses were inoculated into Vero cells and cultured serially to obtain sufficient viral amounts. RNA extraction from coxsackievirus B5 and Echovirus 30 was performed according to the manufacturer's instructions using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) (Fig. 1).

2.2. Primer selection for enterovirus detection

2.2.1. Conventional RT-PCR primers

Based on a literature survey of domestic and foreign enterovirus gene detection methods, conventional RT-PCR primers, target gene sites, RT-nested PCR, and PCR final base pair sizes are listed. Among them [16,23,24,27–31,40], 11 primer sets were selected. Experiments were conducted using primer concentrations based on three previous studies with excellent detection limits. The information for each primer is shown in Table 1.

The RT-PCR mixture for the MFDS test was prepared using the Superscript IV One-step RT-PCR Kit Ver. 2 (Thermofisher Scientific, USA); 5 μL of extracted viral RNA, 12.5 μL of 2X Master mix, 2 μL each of 10 pmol of forward and reverse primers, 0.2 μL of SuperScript™ RT (Reverse Transcription) mix at 50 units/μL, and 1 μL of the remaining distilled water (D.W.) for a total volume of 25 μL. Nested RT-PCR mixture consisted of 4 μL of 10 mM dNTPs, 5 μL of 10 × Buffer (with MgCl2), 1 μL of 5 unit/μL Top DNA polymerase (Bioneer, Korea), 2 μL each of 10 pmol of forward and reverse primers, and 30 μL of remaining D.W. for a total volume of 50 μL. All primers were synthesized by Bioneer.

RT-PCR amplification of the above method was performed as follows: cDNA synthesis step at 42 °C for 45 min, pre-heating at 94 °C

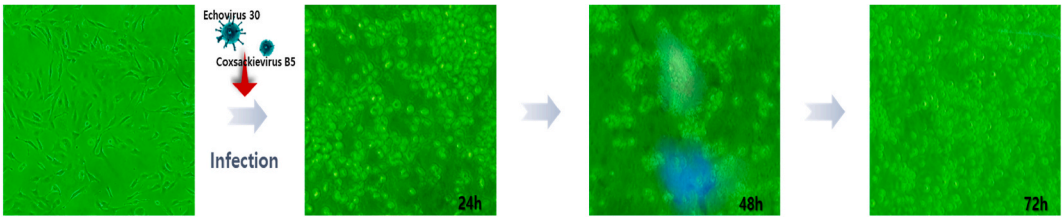

Fig. 1. Propagation of Enterovirus in cell culture. (20× magnification). *Enterovirus = Coxsackievirus B5, Echovirus 30. Images of Vero cells (20× Magnification) taken after infection enterovirus 0 h–72 h.

Table 1
Information about conventional RT-PCR primer sets for detection of Enterovirus.

No.	References	Target gene	Primer name	Sequence (5′—3′)	Product size (bp)
1	Baasner et al. [24]	5′-UTR	EV2b +	GGCCCCTGAATGCGGCTAAT	107
			Entero b −	ACACGGACACCCAAAGT	
2	Cho et al. [27]		EV1	CAAGCACTTCTGTTTCCCCGG	362
			EV3	CTTGCGCGTTACGAC	
3	Nijhuis et al. [28]		RHI-3	CAAGCACTTCTGTTTCCCCGG	298
			RHI-4	CATTCAGGGGCCGGAGGA	
4	Ji et al. [23]		EV1 +	CGGCCCCTGAATGCGGC	190
			EVU +	CCCCTGAATGCGGCTAAT	
			EV2 −	CACCGGATGGCCAATCCA	
			RFLP-3 +	AAGCACTTCTGTTTCCC	298
			RFLP-4 −	ATTCAGGGGCCGGAGGA	
			MD91+	CCTCCGGCCCCTGAATGCGGCTAAT	661
			EVP4+	CTACTTTGGGTGTCCGTGTT	
			OL68-71-	GGTAAYTTCCACCACCAICC	
5	Fujimoto et al. [29]		E2+	CCTCCGGCCCCTGAATG	154
			E1−	CACCGGATGGCCAATCCA	
			R1−	ATTGTCACCATAAGCAGCCA	
6	Fugimoto et al. [40]		P-2	CCTCCGGCCCCTGAATGCGGCTAAT	756
			E33	TCCGGGAATTTCCAGTACCA	
			EVP	CTACTTTGGGTGTCCGTGTT	656
			OL68-71R	GGGAACTTCCAGTACCAYCC	
7	CDC/WHO/ROE (2015)	VP3/VP1	AN89	CCAGCACTGACAGCAGYNGARAYNGG	348–393
			AN88	TACTGGACCACCTGGNGGNAYRWACAT	
8	Kim et al. [16]	VP1	224N-1F	GCRATGTTRGGRACWCATGT	814
			224N-2F	GCSATGTTRGGMACRCAYGT	
			224N-14R	GGRTTBGWKGANGTYTGCCA	
			89N-2F	CCHGCDCTHACCGCWGTGGARACDGG	371–350
			89N-17F	CCMATMCTHCAAGCHGCHGAGAYYGG	
			89N-14R	GGRSCNCCDGGWGGYACAWACAT	
			89N-19R	GGHGCVCCYGGYGGYACRTACAT	
9	MFDS (17162MFDS034) (2017)	RPOL	RPOL-2S	WGCMTTTGAYYAYWCIGGITAYGAYGC	190
			RPOL-2A	RGTGCCWGAICAICCIGAKGGCAT	

*MFDS, Ministry of Food and Drug Safety; CDC, Centers for Disease Control and Prevention; WHO, World Health Organisation; ROE, Regional Office for Europe; RPOL, RNA Polymerase Large Subunit.

for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 40 s, and final extension step 72 °C for 5 min. Nested RT-PCR was performed with a pre-heating of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 40 s, and final extension step 72 °C for 5 min.

PCR amplification was performed in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, USA). RT-PCR and Nested PCR products (3 μL) were electrophoresed on a 2% agarose gel at 120 V for 30 min, and the results were visualized using a GelDoc Go Gel Imaging System with Image Lab Touch Software (Bio-Rad, USA).

The virus copy number was determined by the 10-fold step dilution method of the RNA sample and was observed in the concentration range of 10^3 to 10^{12} copies/mL.

2.2.2. *Real-time RT-qPCR primers and probes*

Real-time RT-PCR primers/probes and target gene sites were considered and listed based on a literature survey of domestic and foreign enterovirus gene detection methods. Among these, primer/probe sets from 11 studies [31–38,40,50] were selected. Comparative experiments of the 11 primer/probe sets were conducted by applying the PCR conditions of the reference test method and the PCR conditions of the Guidance documents of food poisoning [31]. Comparative experiments using different primer concentrations were conducted based on the two studies that showed high detection limits. The primer/probe information for each sample is listed in Table 2.

Real-time RT-qPCR was performed using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, USA) with the following composition: 5 μL viral RNA, 12.5 μL 2 × RT-PCR buffer, and 5 μL enhancer. RT-PCR buffer (2 × , 5 μL), 1.5 μL of Enhancer, 0.5 μL of 25 × Enzyme mix, 1 μL each of 20 pmol of forward and reverse primers, 0.5 μL of 10 pmol of probe, and 3 μL of remaining D.W. for a total volume of 25 μL. All primers and probes were synthesized and used by Bioneer.

PCR amplification was performed using the 7500 Real-Time PCR System (Applied Biosystems). The PCR conditions were as follows: cDNA synthesis at 45 °C for 30 min, pre-heating at 95 °C for 10 min, denaturation for 45 cycles at 95 °C for 15 s, and annealing and extension at 56 °C for 1 min. Each RT-qPCR sample was analyzed in triplicate.

Virus copy number was then determined by a 10-fold step dilution of the RNA sample and observed in the concentration range of 10^3 to 10^{12} copies/mL.

Table 2
Information about real-time RT-qPCR primer sets for detection of Enterovirus.

No.	References	Target gene	Primer name		Sequence (5′—3′)
1	Coudray-Meunier (2015)	5′-UTR	F		GCCCCTGAATGCGGC
			R		GATTGTCACCATAAGCAGC
			P		FAM-GGAACCGACTACTTTGGGTGTCCGT-BHQ1
2	MFDS (17162MFDS034) (2017)		F		CCCCTGAATGCGGCTAATC
			R		GATTGTCACCATAAGCAGC
			P		FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA
3	Donaldson et al. [33]		F		GGCCCCTGAATGCGGCTAAT
			R		CACCGGATGGCCAATCCAA
			P		CGGAACCGACTACTTTGGGTGTCCG
4	Hymas et al. [34]		Hymas1	EV1	GGCCCCTGAATGCGGCTAAT
				EV2	CAATTGTCACCATAAGCAGCCA
				EV probe	CTTTGGGTGTCCGTGT
			Hymas2	EV-F	AATAAATCATAAGAAGAGYCTATTGAGCTA
				EV-R	AATAAATCATAAGGATTRGCCGCATTC
				EV-probe	TCCGGCCCCTGAATGC
5	Fujimoto et al. [40]		F		TCCTCCGGCCCCTGA
			R		GATTGTCACCATAAGCAGCCA
			P		CGGAACCGACTACTTTGGGTGTCCGT
6	Zhang et al. [35]		F		TACTTTGGGTGTCCGTGTTT
			R		TGGCCAATCCAATAGCTATATG
			P		AYTGGCTGCTTATGGTGACRAT
7	Dierssen et al. (2007)		EQ-1		ACATGGTGTGAAGAGTCTATTGAGCT
			EQ-2		CCAAAGTAGTCGGTTCCGC
			EP		TCCGGCCCCTGAATGCGGCTAAT
8	Bragstad et al. (2014)		F		GGTGYGAAGAGTCTATTGAGC
			R		CACCCAAAGTAGTCGG
			probe-1		FAM-CCGGCCCCTGAATG-BHQ1
			probe-2		ROX-CGCAAGTCCGTGGCGGAA-BHQ1
9	Azzouzi et al. [38]		Pan-EV F		GCGATTGTCACCATWAGCAGYCA
			Pan-EV R		GGCCCCTGAATGCGGCTAATCC
			Pan-EV probe		FAM-CCGACTACTTTGGGWGTCCGTGT-BHQ1
10	CDC/FDA (2015)	VP1	AN887		CAAACTCGCACAGTGATAAAYCARCA
			AN893		GTATTATTACTACTACCATTCACNGCNAC
			AN890		FAM-GTCCATTTGAAAAAGTTCTTGTC-BHQ1

*MFDS, Ministry of Food and Drug Safety; CDC, Centers for Disease Control and Prevention; FDA, Food and Drug Administration.

2.3. Enterovirus genotyping (sequence analysis)

Sequence analysis was performed to confirm the genotype of the EVs using the selected gene detection method. The PCR amplification products were purified using the AccuPrep® PCR/Gel Purification Kit (Bioneer, Korea). Sequences were analyzed on a 3500 Series Genetic Analyzer (Applied Biosystems) using the Big Dye Terminator v3.1 Sequencing kit (Applied Biosystems). For phylogenetic analysis, four genotype sequences of EVs A, B, C, and D collected from the NCBI were used as reference groups. SeqMan software (DNASTAR, USA) was used to compare the reference sequences with the analyzed sequences. Similarity was checked using the Basic Local Alignment Search Tool (BLAST) and phylogenetic analysis was performed using MegAlign software 5 (DNASTAR, USA).

2.4. Specificity analysis of optimal methods for EV detection

To verify the specificity of the enterovirus gene detection method, food poisoning viruses (other than EVs) and bacteria were used to confirm the non-specific detection. Five bacterial species (*Vibrio parahaemolyticus, Staphylococcus aureus, Clostridium perfringens, Escherichia coli*, and *Bacillus cereus*) were identified using VITEK MS (Biomerieux). Three viruses (Achivirus, Coxsackievirus, and Echovirus) were isolated by cell culture. The other virus (Norovirus, hepatitis A virus, and rotavirus) were performed with RNA isolated from our division. Nucleic acids of the cultured bacteria and viruses were extracted and applied to selected EVs using conventional and real-time RT-PCR methods.

2.5. Positive control synthesis

Positive controls were designed using primers and probes for the selected enterovirus gene detection method and foreign gene sequence (Enteric Adenovirus/NCBI # DQ315364). They were designed to be commonly used for both conventional RT-PCR and real-time RT-qPCR and to have a base pair size different from that of the enterovirus. Synthesis of the positive control gene was performed by Bioneer (Daejeon, Korea).

2.6. Confirmation of the applicability of the optimal test method for enterovirus detection in water

To verify the applicability of the detection method, EVs were artificially inoculated into 600 mL of water and then subjected to a desalting-concentration process using a housing (Pentek 158110, USA) containing a NanoCeram® VS 2.5-5 (Argonide Corp., USA) filter. Enterovirus genes were extracted from the depuritized samples, and the final selected conventional RT-PCR and real-time RT-qPCR were performed to confirm their applicability. The validation experiment was performed in triplicates. A positive control of the enterovirus produced was used as a positive control.

3. Results

3.1. Confirmation of enterovirus detection based on conventional RT-PCR and setting of conditions

A comparison experiment was conducted to verify the detection of EVs using the 11 selected reference methods. The PCR conditions for the selected references were used for the conventional RT-PCR (Table 1). After confirming the detection, we selected eight primer set references that confirmed the detection of both Coxsackievirus B5 and Echovirus 30 among the 11 primer set reference methods.

To compare the detection limits, we used Coxsackievirus B5 RNA with an initial copy number of 9.2×10^{12} copies/mL and Echovirus 30 RNA with an initial copy number of 9.1×10^{12} copies/mL to confirm the detection limits. As a result of checking the detection limit, the method of Nijhuis et al. (2002), Ji et al. (2014), and Kim et al. (2009) showed a higher detection limit than other reference methods [28,23,16]. In the case of Nijhuis et al. [28], Coxsackievirus B5 was detected up to 10^5 copies/mL and Echovirus 30 was detected up to 10^7 copies/mL. The method of Ji et al. [23] detected Coxsackievirus B5 up to 10^6 copies/mL and Echovirus 30 up to 10^5 copies/mL. Using the method of Kim et al. [16], we detected Coxsackievirus B5 up to 10^5 copies/mL and Echovirus 30 up to 10^6 copies/mL (Fig. 2, Table 3). Comparative experiments were conducted with primer concentrations of 10 and 20 pmol for the three references with high detection limits, and the results showed that the detection limit was generally higher at a primer concentration of 10 pmol. Among the three methods, we found that the limit of detection of enterovirus in the test method was high, ranging from 10^5 copies/mL to 10^6 copies/mL (Fig. 3, Table 4). Among the two references, the gene detection method that targets the VP gene [16], which has a high detection limit and is easy to analyze phylogenetically, was selected. The PCR conditions and primer sequences for the final selected conventional EV RT-PCR test method are shown in Table 5.

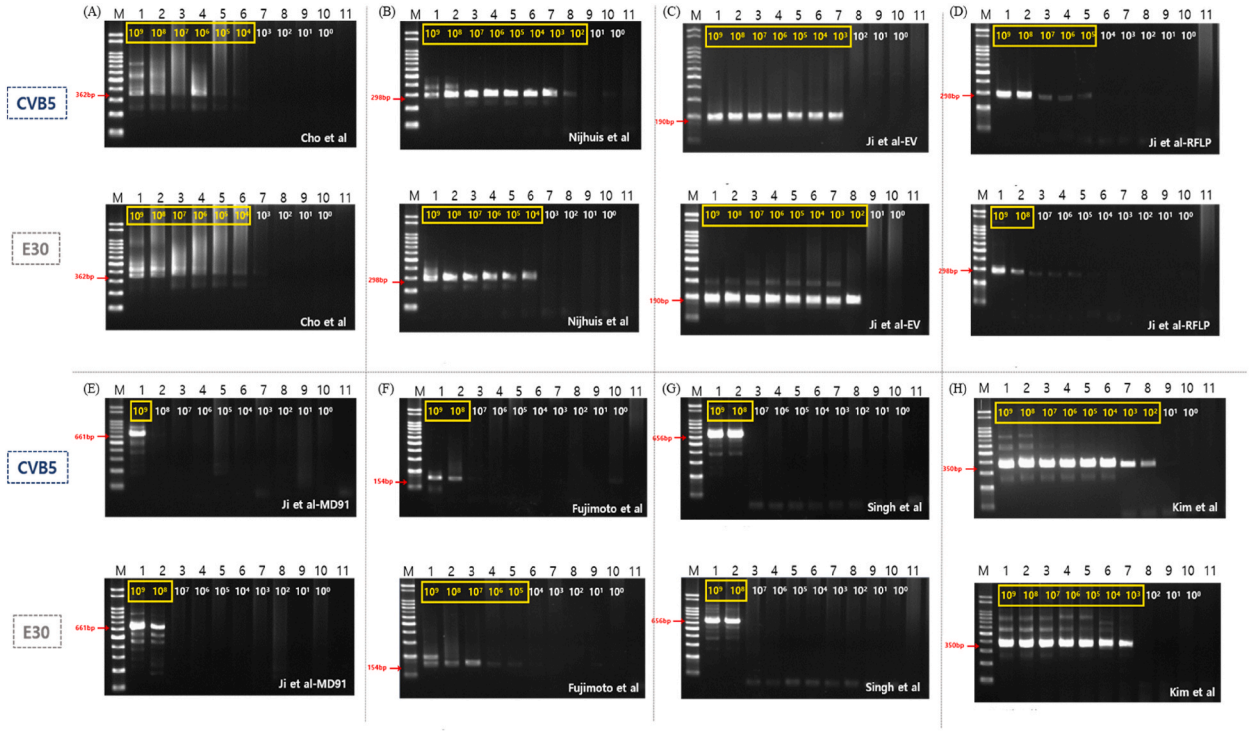

Fig. 2. Comparison of conventional RT-PCR detection limits. The 10-fold serially diluted Coxsackievirus B5 and Echovirus 30 RNAs was amplified using the reference detection methods. Lanes: M, 100 bp DNA marker; 1–10, Coxsackievirus B5 (CVB5) and Echovirus 30 (E30) 10^{12}–10^3 copy/mL; 11, Negative control.

Table 3
Comparison of eight reference detection methods.

References	Target gene	Product size	LOD (copy/mL)	
			Coxsackievirus B5	Echovirus 30
Cho et al. [27]	5′- UTR	362	10^7	10^7
Nijhuis et al. [28]		298	10^5	10^7
Ji et al. [23]		190	10^6	10^5
		298	10^8	10^8
		661	10^{12}	10^{11}
Fujimoto et al. [29]		154	10^{11}	10^{10}
Fujimoto et al. [40]		656	10^{11}	10^{11}
Kim et al. [16]	VP1	350	10^5	10^6

*LOD, limit of detection.

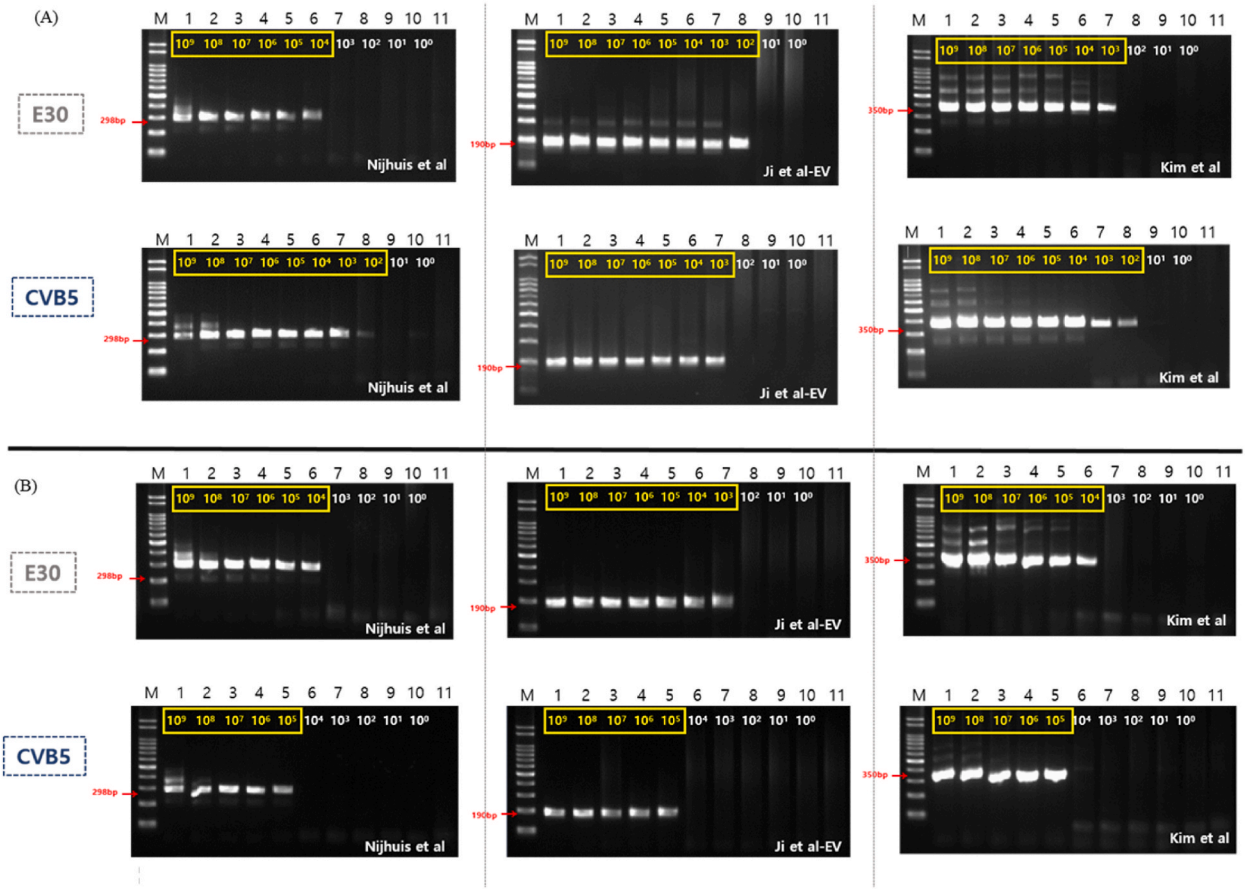

Fig. 3. Comparison of conventional RT-PCR detection limit by primer concentration. (A) The 10-fold serially diluted Echovirus 30 (E30) and Coxsackievirus B5 (CVB5) RNAs were amplified with 10 pmol primers by reference detection methods. Lanes: M, 100 bp DNA marker; 1–10, Echovirus 30 and Coxsackievirus B5 10^{12}–10^3 copy/mL; 11, Negative control (**B**) The 10-fold serially diluted Echovirus 30 (E30) and Coxsackievirus B5 (CVB5) RNAs were amplified with 20 pmol primers by reference detection methods. Lanes: M, 100 bp DNA marker; 1–10, Echovirus 30 and Coxsackievirus B5 10^{12}–10^3 copy/mL; 11, Negative control.

3.2. Comparison of detection limits of enterovirus based on real-time RT-qPCR and setting of conditions

The primer/probe sets of the 11 selected reference methods were used to detect EVs. Among the 11 primer/probe sets, EVs were detected in the primer/probe sets of the six reference methods. To compare the detection limits, Coxsackievirus B5 RNA with an initial copy number of 9.2×10^{12} copies/mL and Echovirus 30 RNA with an initial copy number of 9.1×10^{12} copies/mL were used. Among the six reference methods, the primer/probe sets of Hymas et al. [34] and Azzouzi et al. [38] presented uniquely high detection limits when tested under the guidance documents of food poisoning PCR conditions (Table 6). The detection limits of EVs were compared by

Table 4
Limit of detection of Coxsackievirus B5 and Echovirus 30 by using conventional RT-PCR.

Reference	Target gene	Product size	LOD (copy/mL)			
			Coxsackievirus B5		Echovirus 30	
			Primer [10 pmol]	Primer [20 pmol]	Primer [10 pmol]	Primer [20 pmol]
Nijhuis et al. [28]	5′- UTR	298	10^5	10^8	10^7	10^7
Ji et al. [23]		190	10^6	10^8	10^5	10^4
Kim et al. [16]	VP1	350	10^5	10^8	10^6	10^7

*LOD, limit of detection.

Table 5
Selected sets of primers for the detection of Enterovirus.

Target gene	PCR Type	Primer	Polarity[a]	Sequence (5′—3′)[b]	Size (bp)
VP1	RT-PCR	224N-1F	+	GCRATGTTRGGRACWCATGT	814
		224N-2F	+	GCSATGTTRGGMACRCAYGT	
		224N-14R	−	GGRTTBGWKGANGTYTGCCA	
	Nested PCR	89N-2F	+	CCHGCDCTHACCGCWGTGGARACDGG	350
		89N-17F	+	CCMATMCTHCAAGCHGCHGAGAYYGG	
		89N-14R	−	GGRSCNCCDGGWGGYACAWACAT	
		89N-19R	−	GGHGCVCCYGGYGGYACRTACAT	

[a] +, Forward primer; −, Reverse primer.
[b] Mixed based; R = A + G, Y = C + T, S = G + C, W = A + T, K = G + T, M = A + C, B = C + G + T, D = A + G + T, H = A + C + T, V = A + C + G, N = any base.

Table 6
Variation of detection against primer concentration by real-time RT-qPCR.

Origin	Target gene	PCR condition	Coxsackievirus B5				Echovirus 30			
			10 pmol		20 pmol		10 pmol		20 pmol	
			Ct value	LOD (copy/mL)	Ct value	LOD (copy/mL)	Ct value	LOD (copy/mL)	Ct value	LOD (copy/mL)
Hymas et al. [34]	5′-UTR	Guidance documents of food poisoning	16.6 ± 0.1 ~38.3 ± 0.3	10^7	12.4 ± 0.1 ~38.9 ± 0.1	10^4	15.4 ± 0.1 ~36.2 ± 0.7	10^8	11.1 ± 0.2 ~38.3 ± 0.1	10^4
Azzouzi et al. [38]			18.1 ± 0.1 ~33.9 ± 0.4	10^7	17.2 ± 0.1 ~33.5 ± 0.1	10^7	15.1 ± 0.1 ~36.6 ± 0.1	10^6	14.4 ± 0.1 ~39.5 ± 0.1	10^6

*LOD, Limit of detection.

selecting primer concentrations of 10 and 20 pmol for the two references. The test method of Azzouzi et al. [38] showed similar detection limits based on primer concentration. The other method by Hymas et al. [34] showed a higher detection limit with a primer concentration of 20 pmol (Table 6). Therefore, the PCR conditions of the Guidance documents of food poisoning [31], which detected EV up to 10^4 copies/mL and showed the highest detection limit with an initial Ct value of 11.09 ± 0.2, were finally selected. The PCR conditions and primer sequences for the final selected EV real-time RT-PCR test are shown in Table 6.

3.3. Analysis of detected EV genetic information

The EV sequences were analyzed using the selected gene detection method [16]. When sequenced by the selected gene detection method, the detected enterovirus genotype was found to be the same as the Coxsackievirus B5 and Echovirus 30 genotypes. The phylogenetic tree showed that the enterovirus genotypes detected in the Enterovirus B reference group were aligned to Coxsackievirus B5 and Echovirus 30 (Fig. 4A). In addition, the Enterovirus A, B, C, and D reference groups were aligned to Coxsackievirus B5 and Echovirus 30 (Fig. 4B).

3.4. Specificity analysis of the optimal PCR method

As a result of the specificity test, we found that only enterovirus appeared as a band in conventional RT-PCR. No nonspecific gene amplification was observed for other bacteria or viruses (Fig. 5A). The RT-qPCR amplification curve showed that only EVs were

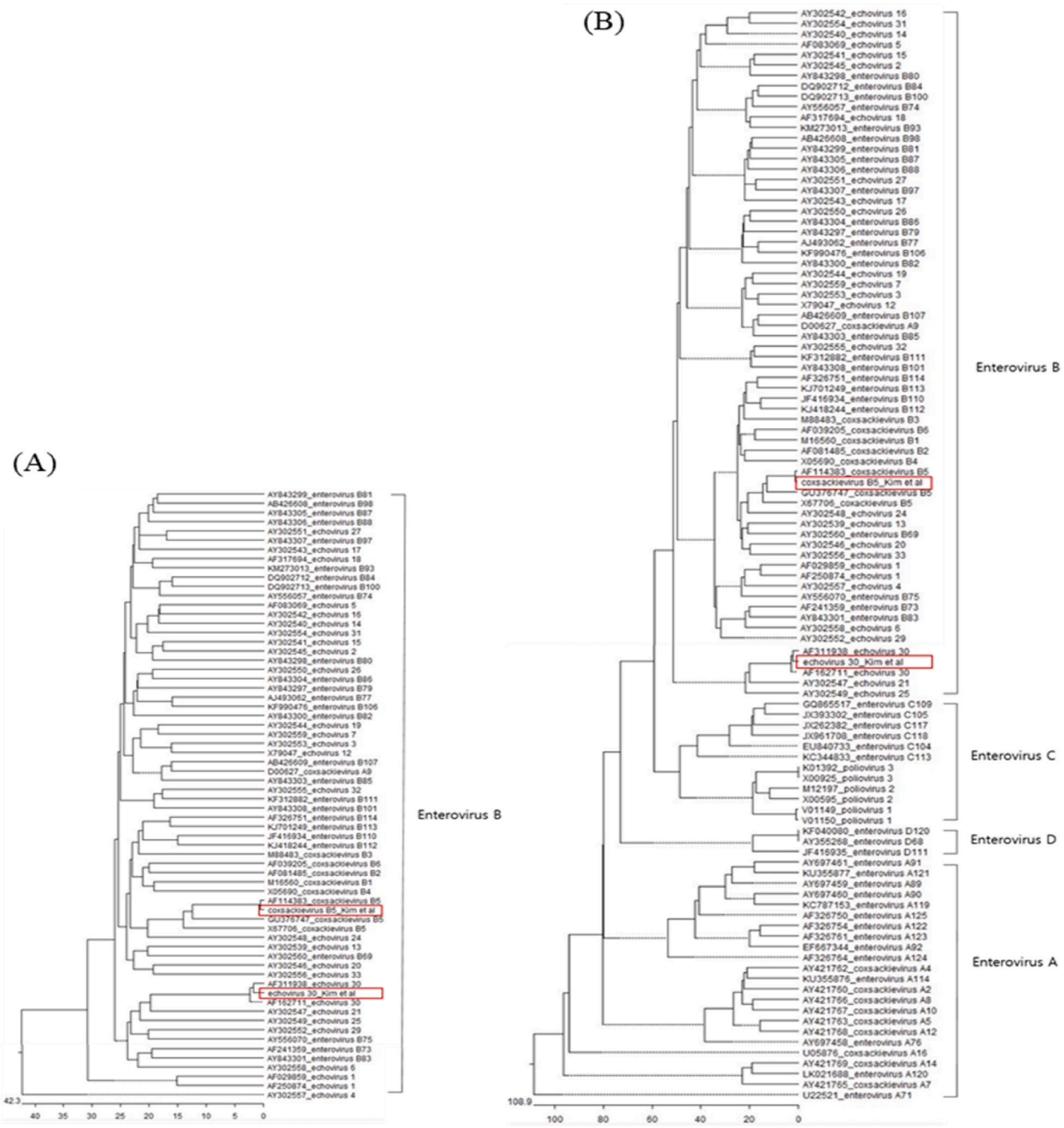

Fig. 4. Phylogenetic tree analysis of Enterovirus using the optimized RT-PCR detection method. (A)The phylogenetic tree showed that the enterovirus genotypes detected in the Enterovirus B reference group were aligned to Coxsackievirus B5 and Echovirus 30. (B) Enterovirus A, B, C, and D reference groups were aligned to Coxsackievirus B5 and Echovirus 30.

amplified (Fig. 5B). This confirms that the selected detection method is valid for EV detection.

3.5. Verification of the applicability of the optimal PCR method in water sample

Finally, the selected gene detection method was used to detect Enterovirus in drinking water to verify the applicability of the test method. Conventional RT-PCR and real-time RT-qPCR amplification curves confirmed the EV detection (Fig. 6A and B). These findings suggest that the selected gene detection method can detect EVs, even when applied to water.

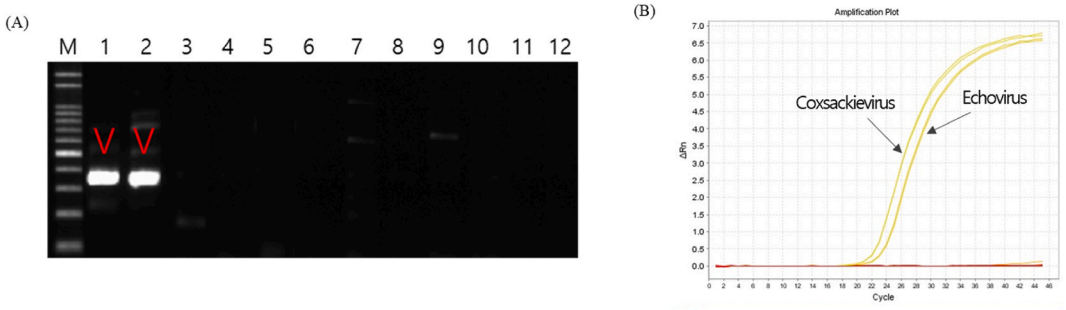

Fig. 5. Specificity verification for optimized conventional RT-PCR and real-time RT-qPCR methods to detect Enterovirus. (A) The specificity of Conventional RT-PCR was verified using the other viruses and bacteria. Lanes: M, 100 bp DNA marker; 1, Coxsackievirus B5; 2, Echovirus 30; 3, Rotavirus; 4, HAV; 5, Norovirus; 6, Aichivirus; 7, *Vibrio parahaemolyticus*; 8, *Staphylococcus aureus*; 9, *Clostridium perfringens*; 10, *Escherichia coli*; 11, *Bacillus cereus*; 12, negative control. (**B**) Specificity of real-time RT-qPCR for other viruses and bacteria.

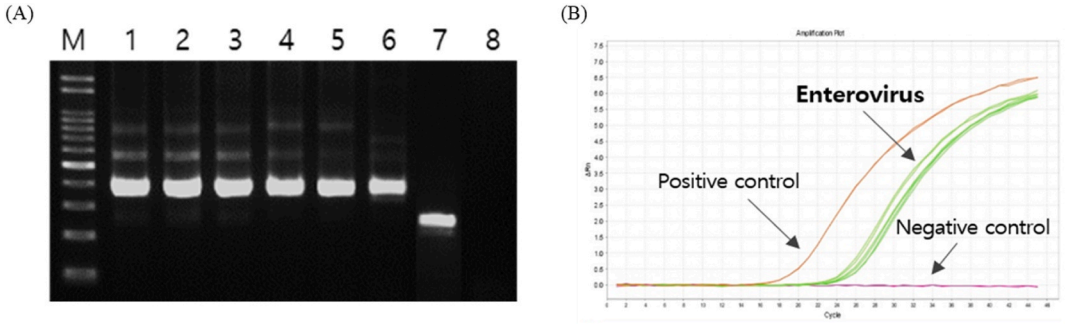

Fig. 6. Applicability verification for conventional RT-PCR and real-time RT-PCR methods to detect Enterovirus. (A) After artificially inoculating Enterovirus into food water, it was detected in triplicate by the optimized conventional RT-PCR method. Lanes: M, 100 bp DNA marker; 1–3, spiking sample of Coxsackievirus B5; 4–6, spiking sample of Echovirus 30; 7, positive control; 8, negative control. (B) Applicability test for real-time RT-qPCR method using spiking samples.

4. Discussion

Despite continuous reports of enteroviral infections [3,41–44], there is currently no test method for EVs other than the seven foodborne viruses listed in the Guidance documents of food poisoning. In this study, we aimed to develop more effective RT-PCR conditions to establish a test method for EVs.

EVs include Poliovirus, Coxsackievirus A and B groups, and Echovirus and Enterovirus groups, and are categorized into various genotypes and serotypes [45]. To select a target virus among EVs, we considered the detection frequency by referring to the 2017–2019 domestic pathogen surveillance status of enterovirus infections published by the Korea Centers for Disease Control and Prevention [18,19]. Among these, Coxsackievirus B5 and Echovirus 30, which are currently available for sale, were selected as the target viruses. Based on the results of a literature survey of domestic and foreign enterovirus gene detection methods, we conducted a study developed an enterovirus gene detection method that considers target genes.

Among the molecular diagnostic techniques used to detect foodborne illness viruses, real-time RT-qPCR is mainly used for reasons such as rapidity, high sensitivity, and specificity. Conventional RT-PCR can also be used for rapid detection and phylogenetic analysis through amplification of specific nucleic acid sites. Based on this, the primer set for each RT-PCR was selected through a survey of domestic and international literature, and a survey of test methods from overseas organizations. They were categorized according to the target region, final product size, and PCR conditions, and comparative experiments were conducted between the primers to confirm virus detection. We confirmed the presence of both Coxsackievirus B5 and Echovirus 30 (Table 3). The final test method was selected based on the best detection limit and locus, considering the copy number and Ct value. The conventional RT-PCR method was selected for the VP1 gene because it can detect specific serotypes and easily analyze viral genes [46]. The validity of the selected test method was verified by sequencing of two previously detected EVs. Phylogenetic analysis based on four genotypes of Enterovirus A, B, C, and D, which cause infections in humans and primates, confirmed that they are consistent with the Coxsackievirus B5 and Echovirus 30 genotypes (Fig. 4).

Food water is used in the manufacturing, processing, and cooking of food and is used by food manufacturers and collective feeding centers. Groundwater is primarily used as food water, and its risk as a vector for foodborne illnesses has been raised [47,48]. However, there is no current analysis of the detection of EVs in domestic groundwater, and it is necessary to study this. Therefore, this study aimed to obtain basic data for food safety management by identifying the current status of domestic enterovirus contamination in food

water. The final test method was applied to artificially inoculate EVs in drinking water to verify its applicability, and both test methods confirmed the detection of EVs. Specificity was verified for five types of food-poisoning viruses and bacteria using the enterovirus gene detection method. No specific gene amplification was found in any strain other than the EVs (Fig. 5).

The test method developed in this study is expected to be utilized not only for efficient detection of EVs in drinking water to prevent food poisoning, but also for environmental samples. However, further research is needed to reduce non-specific reactions in real-time RT-qPCR of environmental samples such as groundwater. Also, for the diagnostic test of EVs, additional studies are needed for detection in clinical samples as well as detection in water environments. Based on this, it will be utilized as a basic data for safety management by identifying the level of virus contamination in Korea through continuous monitoring.

Other EV diagnostic PCR kits have not been analyzed due to the different specimens used.

The following PCR kits were used to diagnose EV: BioMérieux (NucliSENS EasyQ Enterovirus v1.1 assay: cerebrospinal fluid from the patient), Bioneer (AccuPower EV Real Time RT-PCR kit: cerebrospinal fluid or feces from the patient), CERTEST BIOTEC S. L. (feces from the patient).

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Data statement

No Declaration
[39], [49]

CRediT authorship contribution statement

Boeun Yoo: Writing – original draft, Methodology, Investigation. **Mi-Gyeong Kim:** Writing – original draft, Methodology, Investigation. **A Young Min:** Methodology, Conceptualization. **Doo Won Seo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Seung Hwan Kim:** Supervision. **Soon Han Kim:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

RT-PCR	Reverse Transcription Polymerase Chain Reaction
PCR	Polymerase Chain reaction
HAV	Hepatitis A Virus
MFDS	Ministry of Food and Drug Safety
CDC	Centers for Disease Control and Prevention
WHO	World Health Organization
ROE	Regional Office for Europe
RPOL	RNA Polymerase Large Subunit
VP	Viral Protein
5′-UTR	Untranslated Region

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