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Development of a One-Step Duplex RT-PCR Method for the Simultaneous Detection of VP3/VP1 and VP1/P2B Regions of the Hepatitis A Virus

Mi-Ju Kim[1], Shin-Young Lee[1], Hyun-Joong Kim[1], Jeong Su Lee[2], In Sun Joo[2], Hyo Sun Kwak[2], and Hae-Yeong Kim[1*]

[1]*Graduate School of Biotechnology and Institute of Life Sciences & Resources, Kyung Hee University, Yongin 17104, Republic of Korea*
[2]*Division of Food Microbiology, National Institute of Food and Drug Safety Evaluation, Cheongju 28159, Republic of Korea*

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**Corresponding author*
Phone: +82-31-201-2660;
Fax: +82-31-204-8116;
E-mail: hykim@khu.ac.kr

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The simultaneous detection and accurate identification of hepatitis A virus (HAV) is critical in food safety and epidemiological studies to prevent the spread of HAV outbreaks. Towards this goal, a one-step duplex reverse-transcription (RT)-PCR method was developed targeting the VP1/P2B and VP3/VP1 regions of the HAV genome for the qualitative detection of HAV. An HAV RT-qPCR standard curve was produced for the quantification of HAV RNA. The detection limit of the duplex RT-PCR method was 2.8×10^1 copies of HAV. The PCR products enabled HAV genotyping analysis through DNA sequencing, which can be applied for epidemiological investigations. The ability of this duplex RT-PCR method to detect HAV was evaluated with HAV-spiked samples of fresh lettuce, frozen strawberries, and oysters. The limit of detection of the one-step duplex RT-PCR for each food model was 9.4×10^2 copies/20 g fresh lettuce, 9.7×10^3 copies/20 g frozen strawberries, and 4.1×10^3 copies/1.5 g oysters. Use of a one-step duplex RT-PCR method has advantages such as shorter time, decreased cost, and decreased labor owing to the single amplification reaction instead of four amplifications necessary for nested RT-PCR.

Keywords: Hepatitis A virus (HAV), simultaneous detection, epidemiological study, one-step duplex reverse transcription-polymerase chain reaction (RT-PCR), reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Introduction

Hepatitis A virus (HAV) is the leading cause of foodborne diseases worldwide. HAV is transmitted primarily by either the fecal-oral route or from person-to-person in infectious hepatitis outbreaks [4]. One of the major HAV transmission routes is the consumption of contaminated foods such as seafood (shellfish and oysters), fruits (strawberries and blueberries), and vegetables (lettuce and green onions), to name a few [1, 4, 6, 9, 10, 14, 15]. The International Standard Procedures for viral detection from fruits, vegetables, and other foods have not yet been validated [1]; therefore, many scientists have examined methods to facilitate rapid and sensitive HAV detection through improving the extraction and concentration of virus in various foods [3, 17]. Although the amplification of HAV RNA by molecular detection methods like reverse transcription-polymerase chain reaction (RT-PCR) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) are known as the most sensitive and rapid methods for HAV detection compared with culture methods, the development of efficient, sensitive, and direct HAV detection methods from food matrices remains challenging. HAV is reported to have a low infectious dose (as low as 10 to 100 virus particles, which is hard to detect even using molecular detection methods) through epidemiological studies [1, 9, 18] and the preparation (extraction and concentration) of HAV from food is difficult to standardize by simple routine methods owing to differences in the food matrices [14, 19]. Moreover, recent studies suggested that ingredients in food matrixes can inhibit HAV molecular detection methods [9, 10, 20]; for

example, sugars changed biochemically by fruit ripening inhibit the extraction and concentration of HAV. To overcome these difficulties, various studies have focused on modifying sampling and HAV concentration methods, the preparation of RNA, and other molecular biological techniques pursuing sensitive, reliable, direct, and routine detection to prevent HAV infection from food products [1, 3, 7, 8, 12, 17, 19, 20].

HAV is a single-stranded 7.5 kb RNA virus that belongs to the genus *Hepatovirus* of the *Picornaviridae* family and is the causative agent of acute viral hepatitis [4, 14, 21]. The HAV genome consists of a 5′ untranslated region (UTR), an open reading frame (ORF), and a 3′ UTR. The single ORF encoding the polyprotein is divided into three segments, P1, P2, and P3, where P1 encodes the structural proteins (VP1, VP2, VP3, and the putative VP4) and P2 and P3 encode nonstructural proteins [4, 14, 21]. The capsid gene VP1 and junction VP1/P2B are used to genotype HAV strains, which are classified into seven genotypes. Genotypes I, II, III, and IV originate from humans, whereas IV, V, and VI originate from simians [14]. Conserved and varied RNA sequences in the VP1/P2B region of HAV have been used as representative target sequences for the detection, genotyping, and epidemiological studies of HAV [14], and a recent molecular epidemiological study based on sequencing of the VP1/P2B junction reported that this area is stable for the accurate representation of phylogenetic relationships among outbreak and sporadic HAV strains [21].

The aim of this study was to develop a qualitative RT-PCR method for the simultaneous detection of VP1/VP3 and VP1/P2B junctions in HAV for food safety and epidemiological investigations. We tested the one-step RT-PCR method with various artificially HAV-spiked food matrices, including oysters, fresh lettuce, and frozen strawberries, to evaluate the ability of the method to detect HAV.

Materials and Methods

Viruses and Purification of Viral RNA

Hepatitis A virus HM175 strain grown in fetal Rhesus Kidney-4 derived (FRhK-4) cells was obtained from the Ministry of Food and Drug Safety (MFDS) in Korea. All stocks prior to use were stored at −80°C in aliquots. Viral RNA was extracted from 140 µl of the HAV solution using the QIAamp Viral RNA Mini kit (Qiagen, USA) according to the manufacturer's instructions. RNA was eluted in a 60 µl final volume and immediately used for RT-qPCR (RT-PCR) or stored at −80°C. The FRhK-4 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum, and were incubated at 37°C with 5% CO_2 in a conventional incubator.

Spiking HAV to Food Samples

The oyster digestive diverticula (1.5 g), fresh lettuce (20 g), and frozen strawberries (20 g) were purchased from local markets and spiked with 50 µl of HAV solution. The HAV solution was previously quantified using the RT-qPCR method and was serially diluted in diethylpyrocarbonate (DEPC)-treated water from 3×10^7 to 3×10^3 copies per 50 µl, just before spiking to food samples. The artificially contaminated food samples were air dried in a hood for 60 min and non HAV-spiked food samples were used as negative controls.

Extraction and Concentration of HAV Particles from Spiked Food Samples

The isolation and concentration of HAV from food samples followed recommended guidelines for the detection of foodborne virus established by the MFDS, with slight modifications (http://www.foodsafetykorea.go.kr). Each oyster digestive diverticula was added to 1 ml of proteinase K solution (25 mg/ml) and homogenized using a blender for 1 min. The homogenate was placed in a shaking incubator at 320 rpm for 60 min at 37°C and then reacted in a preheated water bath for 15 min at 60°C. After centrifugation for 5 min at 4,000 ×*g* (Eppendorf, Germany), the supernatant was used to extract viral RNA. For fresh lettuce and frozen strawberries, each was added to 200 ml of phosphate-buffered saline (pH 7.4) and placed in a shaking incubator for 1 h. After centrifugation at 15,700 ×*g* for 20 min, the eluate was added to a 40% PEG8000 (Sigma-Aldrich, USA) and 3 M NaCl solution, up to 45% (v/v) and 15% (v/v), respectively. The mixture was placed in a shaking incubator at 4°C for 16 h to concentrate the viral RNA, and was then centrifuged at 15,700 ×*g* for 20 min and the portion of supernatant was wasted. After vortexing, the same volume of chloroform (Sigma-Aldrich) was added to the supernatant and centrifuged at 10,000 ×*g* for 30 min. To perform the second virus concentration, the supernatant was transferred to a new tube and the 40% PEG8000 and 3M NaCl solution, up to 45% (v/v) and 15% (v/v), respectively, was added again. The eluate was placed in a shaking incubator at 4°C for 3 h and then centrifuged at 15,700 ×*g* for 30 min at 4°C. The pellet was suspended with DEPC-treated water, and 140 µl was used for the extraction of viral RNA. All experiments were done in triplicate. Each (140 µl) HAV-containing suspension was extracted using the QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. HAV RNA was eluted in a 30 µl final volume and 5 µl was used immediately for RT-qPCR or stored at −80°C. The eluted HAV RNA oyster samples were diluted 10-fold in water and a 5 µl aliquot was used for RT-qPCR.

Monoplex and Duplex RT-PCR for Qualitative HAV Detection

Two primer pairs (BR-5/BR-9 and HAV1/HAV2), designed for the VP1/P2B and VP3/VP1 junctions of the HAV genome, were

Table 1. Primers and probes used for the detection of HAV.

Target region on HAV genome	Method	Primers and probes	Sequence 5′ to 3′	Position	Amplicon (bp)	References
VP1/P2B	RT-PCR	BR-5	TTGTC TGTCA CAGAA CAATC AG	2950-2972	361	[16]
		BR-9	AGTCA CACCT CTCCA GGAAA ACTT	3310-3286		
VP3/VP1	RT-PCR	HAV1	GCTCC TCTTT ATCAT GCTATG GAT	2172-2196	244	[2]
		HAV2	CAGGA AATGT CTCAG GTACT TTCT	2415-2391		
5′ UTR	RT-qPCR	Primer forward	GCGGC GGATA TTGGT GAG	458-476	78	[5]
		Primer reverse	CAATG CATCC ACTGG ATGAG A	535-515		
		Probe	FAM-TTAAG ACAAA AACCA TTCAA CGCCG GAG-TAMRA	480-507		

FAM, 6-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine.

used with monoplex and duplex RT-PCR for the qualitative detection of HAV (Table 1). The primers were purchased from Bionics (Korea) and were used at 800 nM for VP1/P2B and 200 nM for VP3/VP1. Monoplex RT-PCR was carried out with 2 µl of extracted viral RNA, 400 µM of each dNTP, 800 nM of each forward and reverse primer, 10 units of AMV reverse transcriptase (Promega, USA), 5 units of Top DNA polymerase (Bioneer, Korea), 5 µl of 10× buffer, and DEPC-treated water in a final reaction volume of 50 µl.

The RT-PCR amplification was performed in a thermocycler (Model PC 808; ASTEC, Japan) with an initial cDNA synthesis step at 42°C for 40 min, followed by pre-denaturation for 2 min at 94°C and then 45 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, extension for 1 min at 72°C, and post-elongation for 7 min at 72°C. For duplex RT-PCR, the same mixture and PCR conditions as for single RT-PCR were used except for the addition of both primer pairs (BR-5/BR-9 and HAV1/HAV2) in a single test, to simultaneously detect the two HAV regions. The limit of detection (LOD) values of monoplex and duplex RT-PCR were estimated with quantified HAV RNA samples (quantified by RT-qPCR with internal RNA standard materials), serially diluted from 1.4×10^5 to 1.4×10^{-1} copies/µl. Amplified products (6 µl) were separated on a 3% agarose gel in 0.5× Tris-acetate-EDTA buffer, stained with ethidium bromide, and visualized under UV irradiation.

RT-qPCR for Quantification of HAV RNA

Quantitative detection of HAV RNA was performed by RT-qPCR with a primer pair and TaqMan probe that were designed for the 5′ UTR of the HAV genome (Table 1). The primers and probe were synthesized from Bionics and Bioneer, respectively. The RT-qPCR was performed using AgPath-ID One-step RT-PCR Reagents (Ambion, USA) and was carried out with 400 nM of each forward and reverse primer, 200 nM probe, 12.5 µl of 2× RT-PCR buffer, 1.5 µl of enhancer, 1 µl of 25× enzyme mix, and DEPC-treated water in a final reaction volume of 25 µl.

The RT-qPCR was performed using the 7500 real-time PCR system (Applied Biosystems, USA). The reaction was initiated at 42°C for 30 min for the synthesis of cDNA, followed by denaturation at 95°C for 10 min and then 45 cycles of 95°C for 15 sec and 60°C for 60 sec. The RT-qPCR of each RNA sample was performed in triplicate.

The copy number of HAV RNA in samples was quantified with the internal standard curve, which was plotted by 10-fold diluted RNA standard materials (from 5×10^6 copies/5 µl to 5×10^2 copies/5 µl) using RT-qPCR.

Sequencing of PCR Products

To confirm the identity of the amplified RT-PCR products, the PCR products were separated by agarose gel electrophoresis. Amplicons were purified from the gel using the QIAQuick Gel Extraction kit (Qiagen) and sequenced. Sequencing was performed using an automated DNA sequencer (Applied Biosystems) with primers (HAV1/HAV2 and BR-5/BR-9). The identity of the viral sequences was verified by a BLAST search against the NCBI nucleotide database.

Results

Quantification of Prepared HAV RNA Sample

In vitro synthesized standard RNA material from constructed DNA was diluted 10-fold from 5×10^6 copies to 5×10^2 copies and averaged standard curves were generated by RT-qPCR experiments that were repeated six times. The slope of the produced standard RNA material regression line was -3.36 with high correlation coefficient ($R^2 > 0.994$) and efficiency (98.61%).

The concentration of extracted RNA from an HAV HM175 sample was quantified using RT-qPCR with the internal standard curve plotted by diluted RNA standard. The extracted and quantified HAV RNA was serially diluted 10-fold from 1.4×10^5 to 1.4×10^{-1} copies/µl and used to evaluate the limit of detection (LOD) of the monoplex and duplex RT-PCRs.

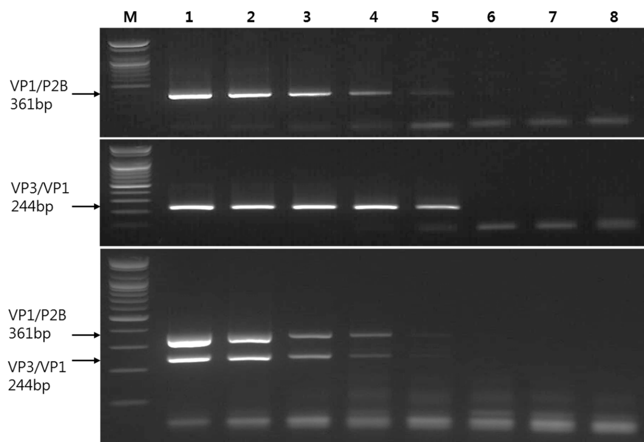

Fig. 1. The limit of detection: one-step monoplex and duplex RT-PCR for HAV detection.

The 10-fold serially diluted HAV RNAs were amplified with an expected 361 bp amplicon for the VP1/P2B region and a 244 bp amplicon for the VP3/VP1 region. Lane M, 100 bp DNA marker; Lanes 1 to 7, 2.8×10^5 to 2.8×10^{-1} copies of positive control; Lane 8, negative control.

One-Step Monoplex and Duplex RT-PCR Limits of Detection for Qualitative HAV Detection

The concentration of each primer pair was optimized based on the amplified band intensities resulting from duplex RT-PCR assay and were finally established at 800 nM for the BR-5/BR-9 primer pairs and 200 nM for the HAV1/HAV2 primer pairs in RT-PCR. The mono and duplex RT-PCRs for HAV detection amplified the expected RT-PCR products of 361 bp (BR-5/BR-9 primer pairs from the VP1/P2B region) and 244 bp (HAV1/HAV2 primer pairs from the VP3/VP1 region) with HAV RNA (Fig. 1). Sequencing results of the RT-PCR products confirmed that the expected sequences and regions of HAV RNA were amplified using the mono and duplex RT-PCR methods (data not shown). The LOD for both monoplex and duplex RT-PCR methods was 2.8×10^1 copies; however, the amplified band intensity of the duplex RT-PCR was weaker than that of the monoplex RT-PCR.

Application of the One-Step Duplex RT-PCR and RT-qPCR Methods to Food Samples

The duplex RT-PCR of HAV was evaluated with various HAV-spiked food matrices to confirm its epidemiological application in HAV foodborne outbreaks. The selected food matrices for HAV spiking were oysters, fresh lettuce, and frozen strawberries, which have been implicated in HAV foodborne outbreaks by previous reports [6, 13, 15]. HAV solution (10-fold serially diluted HAV in DEPC-treated water in 50 μl) was used to spike each food sample and the final isolated (eluted) volumes of HAV-containing

Table 2. Result of qualitative RT-PCR and quantitative RT-qPCR with various artificially HAV-spiked food matrices.

	Inoculated amount of HAV (copies)	Duplex RT-PCR		Quantitative PCR (copies)	Recovery (%)
		VP3/VP1 junction	VP1/P2B junction		
Fresh lettuce (20 g)	3×10^7	+ + +	+ + +	+, 7.3×10^4	1.74
	3×10^6	+ + +	+ + +	+, 2.7×10^4	6.37
	3×10^5	+ + +	+ + +	+, 9.4×10^2	2.24
	3×10^4	+ − −	+ − −	+, 2.9×10^1	0.71
	3×10^3	− − −	− − −	ND	ND
Frozen strawberry (20 g)	3×10^7	+ + +	+ + +	+, 7.3×10^4	1.74
	3×10^6	+ + +	+ + +	+, 9.7×10^3	2.28
	3×10^5	+ − −	+ − −	+, 2.0×10^3	4.72
	3×10^4	− − −	− − −	ND	ND
	3×10^3	− − −	− − −	ND	ND
Oysters (1.5 g)	3×10^7	+ + +	+ + +	+, 5.4×10^5	31.88
	3×10^6	+ + +	+ + +	+, 7.8×10^3	47.30
	3×10^5	+ + +	+ + +	+, 4.1×10^3	24.51
	3×10^4	+ − −	+ − −	ND	ND
	3×10^3	− − −	− − −	ND	ND

The 10-fold serially diluted HAV solutions (from 3×10^7 copies to 3×10^3 copies) were spiked on 20 g of fresh lettuce, 20 g of frozen strawberries, and 1.5 g of oysters. ND, Not detected.

suspensions were 1 ml from fresh lettuce and frozen strawberries, and 2.5 ml from oysters. A 140 μl aliquot of each HAV-containing suspension (according to the manufacturer's instructions) was used for HAV RNA extraction. The qualitative (one-step duplex RT-PCR) detection was carried out in triplicate and the results are shown in Table 2. The HAV one-step duplex RT-PCR LOD for each food model was 9.4×10^2 copies/20 g fresh lettuce, 9.7×10^3 copies/20 g frozen strawberries, and 4.1×10^3 copies/1.5 g oysters. All experiments were confirmed to lack cross contamination with a negative food control. The HAV recoveries were 0.7–6.4% in fresh lettuce, 1.7–4.7% in frozen strawberries, and 24.5–47.3% in oysters.

Discussion

The duplex RT-PCR detection strategy in this study was designed to obtain complementary qualitative results for rapid and accurate HAV detection with the simultaneous amplification of two target regions of the HAV genome. Furthermore, the genotype information of HAV gained additionally after the DNA sequence analysis of two PCR products (VP1/P2B and VP3/VP1 junctions) enables the application of this duplex RT-PCR in epidemiological studies of HAV outbreaks. Although RT-qPCR is the most common DNA-based method to detect HAV, due to its higher sensitivity, speed, and specificity relative to qualitative RT-PCR, RT-qPCR is not suitable for epidemiological investigation of foodborne HAV outbreaks because the approximate 89 to 150 bp DNA fragments amplified by RT-qPCR are difficult to analyze genotypes via DNA sequencing [22]. Vaughan *et al*. [21] reported that the sequence of the VP1/P2B junction can be used by epidemiological studies to identify genotypes, track transmission, and assess phylogenetic relationships in HAV outbreaks. In this study, the VP1/P2B (361 bp) and VP3/VP1 (244 bp) junction region amplicons from duplex RT-PCR were sequenced and verified as HAV by a BLAST with the non-redundant (nr) nucleotide database. These results showed that the two duplex RT-PCR products were sufficient to be used in DNA sequencing and enable genotyping analysis to provide epidemiological information in HAV outbreaks.

Although the purification and detection methods of virus genomic materials have dramatically advanced over the last several decades due to the development of commercial protocols and kits, virus extraction and concentration from food matrices are still a critical step in the process to maintain consistent yield for molecular detection methods because the standardization of routine extraction and concentration methods is not suitable between different food matrices, and the recovery of virus from food matrices has high variation even within one individual food product [10, 18]. Previously, various methods for HAV extraction and concentration have been attempted from various food matrices [5, 18]. We employed two different food category-specific virus extraction and concentration methods (a Proteinase-K-based method for oysters was utilized to break polypeptides down into small units; a PEG-NaCl-based method for fresh lettuce and frozen strawberries was used to precipitate and collect RNA particles) considering the characteristics of the food matrix, to set up standard routine extraction and concentration methods of HAV for each category of food.

Previously reported LOD or the amount of HAV in food matrices used in our study were lower than 1.4×10^3 copies/ml fresh lettuce and frozen strawberry samples based on qualitative nested RT-PCR [6], between 10^3 and 10^2 viral particles/50 g strawberry and lettuce by real-time PCR [13], and from 1.1×10^2 to 4.1×10^6 RNA copies/g mussel digestive tissue [12]. In our study, the one-step duplex RT-PCR LODs were higher or in a similar HAV range relative to the previously reported values. Terio *et al*. [20] reported that a second amplification by nested RT-PCR produced a detection limit 1 logarithmic unit higher than RT-PCR. However, nested monoplex RT-PCR should be carried out in four amplification reactions to detect the two regions (the VP1/P2B and VP1/VP3 regions). Since the one-step duplex RT-PCR developed in this study amplifies two target bands in a single reaction, this assay has advantages such as decreased time, cost, and labor compared with previously used nested RT-PCR methods.

There were a few observable features in the results of RT-qPCR with HAV-spiked food matrices. The recovery yields (%) of HAV from fresh lettuce and frozen strawberries were not constant, even between samples with different HAV-spiked amounts. Relatively high levels of HAV-spiked samples (3×10^7 copies/20 g) showed low recovery yield relative to low levels of HAV-spiked samples (Table 2). In the performance of RT-qPCR from oysters, the eluted HAV RNA was diluted 10-fold for RT-qPCR because RNA directly eluted from the food matrix lacked amplification. We assume this is due to the presence of contaminating compounds that inhibit the reverse transcription step and/or the quantitative PCR step in RT-qPCR, as suggested in previous reports [9, 10, 11, 20]. As a result, lower amounts of eluted RNA or 10-fold diluted viral RNA were used in quantitative analysis to increase the sensitivity and reduce inhibition of HAV detection from food samples [1, 19]. The

key factor maintaining the efficiency of RT-qPCR is how to reduce and/or remove inhibitory compound(s) in the extraction and concentration and purification procedures.

In conclusion, we have developed a detection method that provides rapid and effective analysis to simultaneously detect VP3/VP1 and VP1/P2B junction regions from HAV in food samples.

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