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Rapid and Sensitive Detection of Norovirus Genomes in Oysters by a Two-Step Isothermal Amplification Assay System Combining Nucleic Acid Sequence-Based Amplification and Reverse Transcription–Loop-Mediated Isothermal Amplification Assays[▽]

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We developed a two-step isothermal amplification assay system, which achieved the detection of norovirus (NoV) genomes in oysters with a sensitivity similar to that of reverse transcription-seminested PCR. The time taken for the amplification of NoV genomes from RNA extracts was shortened to about 3 h.

Norovirus (NoV), which belongs to the family *Caliciviridae*, is a major etiological agent of acute gastroenteritis worldwide (1, 4, 9, 11, 14). Oysters are an important vehicle (2, 3, 16, 17, 19) and sometimes associated with food-borne outbreaks due to NoVs (2, 9, 20). The detection of NoVs in bivalves such as oysters is carried out usually by using reverse transcription (RT)-nested or -seminested PCR, because the number of genome copies is smaller in oysters than in fecal specimens (16, 17). Recently, the detection of NoV genomes in fecal specimens by isothermal amplification assays has been reported (5–8, 13, 15, 18, 21) and three detection kits for the detection of NoV genomes under isothermal conditions, based on RT–loop-mediated isothermal amplification (RT-LAMP; Eiken Chemical, Tokyo, Japan), nucleic acid sequence-based amplification (NASBA; Kainos Laboratories, Tokyo, Japan), and transcription-RT concerted amplification (TRC; Tosoh, Tokyo, Japan) assays have been commercially developed in Japan. They are used for the rapid detection of NoVs within 1 to 2 h. However, commercial kits for the sensitive detection of NoVs in foods such as bivalves have yet to be established.

In this paper, we report the application of a two-step isothermal amplification assay (NASBA-RT-LAMP assay) system combining the NASBA and genogroup-specific RT-LAMP assays for the rapid and sensitive detection of NoVs in oysters.

Fourteen lots of oyster samples (*Crassostrea gigas*) were collected from different oyster rafts in a nonapproved cultivation area for eating raw oysters in Hiroshima bay between December 2006 and March 2007, and three individual oysters per lot were used for tests. The stomach and digestive diverticula were homogenized by stomacher (Colworth, London, United Kingdom) with 10 ml of phosphate-buffered saline without magnesium or calcium (Nissui Pharmaceutical, Tokyo, Japan). After centrifugation at 3,000 × *g* for 30 min at 4°C, the supernatant was supplemented with a final concentration of 8% polyethylene glycol 6000 (Wako Pure Chemical, Osaka, Japan) and 0.4 M NaCl and incubated overnight at 4°C to precipitate viruses. The pellet was recovered by centrifugation at 6,000 × *g* for 30 min at 4°C and resuspended in 300 μl of RNase-free water. Then, the viral RNA was extracted from 140 μl of the suspension with a QIAamp viral RNA mini kit (Qiagen, Valencia, CA). The extracted RNA was kept at −80°C prior to use.

NASBA and genogroup-specific RT-LAMP assays were performed under the following conditions. The 13 oligonucleotide primers for the NASBA assay based on the sequence from the RNA-dependent RNA polymerase region to the capsid region were designed newly for the simultaneous amplification of genogroup I (GI) and GII in a single tube (Table 1). The NASBA assay as a first round of amplification was performed in 20 μl of reaction mixture with a NASBA amplification kit (Kainos) according to the manufacturer's protocol. Briefly, 10 μl of NASBA reaction mix containing the 13 newly designed primers and 5 μl of RNA extract were added to each tube. The final concentration of each primer is indicated in Table 1. The tube was incubated at 65°C for 5 min and then cooled to 41°C for 5 min. Next, 5 μl of enzyme mix (avian myeloblastosis virus reverse transcriptase, RNase H, and RNA polymerase) was added to each tube, which was incubated under isothermal conditions at 41°C for 90 min.

The genogroup-specific RT-LAMP assay as a second round of amplification was performed by adding 2 μl of NASBA amplification product under isothermal conditions at 62°C for 90 min as described previously (5). The RT-LAMP reaction was measured with a Loopamp real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan). The samples were taken to be negative for NoVs, when all of the duplicate tests were negative.

The RT-seminested PCR was used as the standard for the detection of NoV genomes, and McNemar's test in the software package Dr. SPSS II for windows (SPSS, Tokyo, Japan) was used to compare the NASBA-RT-LAMP assay and RT-seminested PCR. The RT-seminested PCR was performed based on protocols described elsewhere (5, 17). The amplified

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TABLE 1. Sequences of oligonucleotide primers for the NASBA assay

Primer[a]	Sequence (5′→3′)[b]	Polarity	Location	Final concn (μM)
G1F3N1	CCAGGYTGGCAGGCCATGTT	+	5270–5289[c]	0.2
G1F3N2	CCTGGKTGGCAGGCCATGTT	+	5270–5289[c]	0.2
G1F3N3	CCCGGCTGGCAGCCCATGTT	+	5270–5289[c]	0.2
G1F3N4	CCAGGRTGGCARGCCATGTT	+	5270–5289[c]	0.2
G1F3N5	CCGGGTTGGCAGGCAATGTT	+	5270–5289[c]	0.2
G1F3N6	CCAGGTTGGCAGGCTATATT	+	5270–5289[c]	0.2
G2F3N1	GGCATGGATTTCTACGTGCCCA	+	4979–5000[d]	0.2
G2F3N2	GGRATGGATTTTTACGTGCCAA	+	4979–5000[d]	0.2
G2F3N3	GGRATGGATTTYTATGTGCCRA	+	4979–5000[d]	0.2
G2F3N4	GGMATGGATTTTTACGTGCCCA	+	4979–5000[d]	0.2
G2F3N5	GGTCTGGARTTTTATGTGCCCA	+	4979–5000[d]	0.2
G1B31T7	aattctaatacgactcactatagggagagCCAACCCARCCA TTRTACA	−	5653–5671[c]	0.5
G2B31T7	aattctaatacgactcactatagggagagCCRCCNGCATRH CCRTTRTACA	−	5368–5389[d]	0.5

[a] The primers G1B31T7 and G2B31T7 were modified from the primers G1SKR and G2SKR, respectively, as described by Kojima et al. (12).
[b] Lowercase letters indicate T7 promoter sequence. Mixed bases in degenerate primers are as follows: K, T or G; M, A or C; R, A or G; Y, C or T; H, not G; and N, any.
[c] Nucleotide positions according to those of Norwalk/68/US (GenBank accession no. M87661).
[d] Nucleotide positions according to those of Lordsdale/93/UK (GenBank accession no. X86557).

PCR products were purified from agarose gels with a DNA extraction kit (Millipore, Bedford, MA) and sequenced directly. The sequencing was carried out with a SequiTherm Excel II DNA sequencing kit LC for 25- and 41-cm gels (Epicentre Technologies, Madison, WI) and a LI-COR 4200 series sequencer (LI-COR, Lincoln, NE). The sequences were classified into 31 genotypes as described by Kageyama et al. (11). Additionally, for the measurement of the number of NoV genome copies in oysters, real-time quantitative RT-PCR was performed as described previously (5).

The sensitivity of the NASBA-RT-LAMP assay was examined in duplicate using serial 10-fold dilutions of RNA extracts prepared from four fecal specimens containing the genotype GI/4 or GII/4, which were predominant in Japanese oysters (16, 17). The NASBA-RT-LAMP assay had 100-fold more reactivity than the RT-LAMP assay for both genotypes (data not shown). Furthermore, the results of the NASBA-RT-LAMP assay using a total of 42 oysters in the 14 lots were compared to those of RT-seminested PCR, quantitative RT-PCR, NASBA, and RT-LAMP assays, as shown in Table 2. All of the 21 oysters tested in single assays of NASBA using dot plot hybridization with RING1(a)-TP and RING2-TP (10) and RT-LAMP gave negative results. However, the NASBA-RT-LAMP assay could detect NoV genomes in oysters with a sensitivity equivalent to that of the RT-seminested PCR, although 83.3% (35/42) of our oysters had less than 100 genome copies per oyster. Concordances of 75.0% (63/84) in both GI and GII between the NASBA-RT-LAMP assay and RT-seminested PCR, 73.8% (31/42) in GI, and 76.2% (32/42) in GII were observed (Table 2). No significant difference between the

TABLE 2. Comparison of assays for detection of norovirus genomes in oysters

Lot no.	Result for[a]: GI				GII			
	RT-seminested PCR	NASBA-RT-LAMP	NASBA or RT-LAMP	No. of copies/oyster	RT-seminested PCR	NASBA-RT-LAMP	NASBA or RT-LAMP	No. of copies/oyster
1	++−	++−	−−−	<100	−−+	+++	−−−	<100 ~ 1.6×10^2
2	−++	++−	−−−	<100	+++	+++	−−−	<100 ~ 1.1×10^2
3	+++	+−+	−−−	<100	+++	+++	−−−	<100 ~ 1.0×10^2
4	−−−	−−−		<100 ~ 1.1×10^2	−++	−−−		<100
5	++−	++−		<100 ~ 1.0×10^2	+−−	++−		<100
6	−−−	−−−		<100	+−+	+−−		<100
7	−−−	−−−		<100 ~ 1.2×10^2	−−−	−−−		<100
8	+++	−−+		<100	+++	−++		<100 ~ 1.3×10^2
9	+−−	+−−		<100	+−+	+−+		<100
10	−−−	−−−		<100	−−−	−−−		<100
11	−−−	+−+	−−−	<100	−−−	−−+	−−−	<100
12	−−−	−−−	−−−	<100	−−+	−−+	−−−	<100
13	++−	+−−	−−−	<100	−−−	−++	−−−	<100
14	+−−	−++	−−−	<100	+++	+++	−−−	<100

[a] The + (positive) and − (negative) symbols represent the same samples in order in RT-seminested PCR, NASBA-RT-LAMP, NASBA, and genogroup-specific RT-LAMP assays. Each symbol refers to the results from each oyster sample in a lot. NASBA-RT-LAMP is a two-step isothermal amplification assay system combining the NASBA and genogroup-specific RT-LAMP assays.

NASBA-RT-LAMP assay and RT-seminested PCR was found (McNemar's test; $P > 0.75$) Furthermore, the seven genotypes GI/4, GI/5, GI/7, GI/8, GI/10, GII/4, and GII/13 found in our oysters could be detected with the NASBA-RT-LAMP assay (data not shown).

The NASBA-RT-LAMP assay can amplify NoV genomes from RNA extracts in only about 3 h and is available to detect NoV genomes in bivalves such as oysters even in small laboratories because the system requires no precision instrument. Fortunately, the genogroup-specific RT-LAMP assay used for the second round of amplification is available commercially (Eiken).

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