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Evaluation and Application of Reverse Transcription Loop-Mediated Isothermal Amplification for Detection of Noroviruses

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A one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of norovirus (NV) was developed. In order to design primer sets for the detection of a wide range of NVs, NVs were categorized into three groups, that is, genogroup I (GI), prevalent GII, and minor GII; three sets of primers were developed for each group. Clinical specimens of patients suffering from enteric RNA viruses, such as NV, group A and C rotavirus, and sapovirus were examined using these primer sets. Various genotypes of NVs were detected in clinical specimens from patients infected with NV where no false positive reaction was observed with other enteric RNA viruses. Additionally, 88 samples of acute gastroenteritis outbreaks were analyzed by an RT-LAMP assay and compared with the results of routine RT-PCR. The results of the RT-LAMP assay corresponded well to that of RT-PCR. These findings suggest the practical application of the RT-LAMP assay for the detection of NVs in clinical specimens. Consequently, the RT-LAMP system and conventional detection kits (NVGI and NVGII detection kits; Eiken Chemical Co., Ltd., Japan) were compared. The detection rate of the prevalent and minor GII primer sets was similar to that of the conventional NVGII kit, while the detection rate of the GI primer set is different because it can detect several genotypes better than the conventional NVGI kit. This is an initial report that the RT-LAMP system is able to detect NVs in clinical specimens within a wide range. ***J. Med. Virol. 79:326–334, 2007.***

KEY WORDS: norovirus; RT-LAMP; rapid diagnosis

INTRODUCTION

The genus *Norovirus* is a member of the virus family *Caliciviridae*. Noroviruses (NVs) have emerged as the single most common cause of outbreaks as well as sporadic cases of acute gastroenteritis in children and adults throughout the world [de Wit et al., 2001; Blanton et al., 2006; Infectious Agents Surveillance Report (IASR), 2005 (http: //idsc.nih.go.jp/iasr/); Wheeler et al., 1999; Kirkwood et al., 2005].

There is no consensus on a uniform classification scheme for NVs. However, five genogroups (G) including animal NVs (in pigs, cows, and mice) have been tentatively reported using the molecular characterization of complete capsid gene sequences [Green et al., 1995; Vinje et al., 2000; Fankhauser et al., 2002; Karst et al., 2003; Oliver et al., 2003; Zheng et al., 2006]. Three of these five Gs contain human strains (genogroup I (GI), GII, and GIV) [Zheng et al., 2006]. The National Institute of Infectious Diseases (NIID, Tokyo, Japan) group proposed slightly different NV genotyping using a precise scheme based on variability of the capsid N-terminal/shell (N/S) domain gene [Kageyama et al., 2004]. They identified a total of 31 genotypes (14 genotypes in GI and 17 genotypes in GII). Frequently detected GII genotypes such as GII.2, 3, 4, 6, and 12 (prevalent GII) are classified as the same genotypes in GII for both classifications [Kageyama et al., 2004; Zheng et al., 2006], whereas infrequently detected GII genotypes (minor GII) are classified mainly into different genotypes in GII according to both classifications. Zheng et al. [2006] categorized GII.17 of Kageyama's classification in GIV. All genotypes used in this report follow Kageyama's classification [Kageyama et al., 2004].

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Routine laboratory diagnosis of NV infection is based primarily on RT-PCR using several pairs of primers. For interpretation of RT-PCR products, methods other than agarose gel electrophoresis are necessary in order to prevent false-positive results. DNA sequencing and hybridization is used commonly for confirmation of NV RT-PCR products; however, both methods are time-consuming and tedious, requiring more than 2 days for completion.

Recently, rapid and sensitive nucleotide amplification-based detection systems other than routine RT-PCR, such as real-time RT-PCR using TaqMan probes [Kageyama et al., 2003] have been reported. However, these methods require high-precision instrumentation.

The loop-mediated isothermal amplification (LAMP) assay originally described [Notomi et al., 2000] is based on the principle of autocycling strand displacement DNA synthesis for the detection of a specific DNA sequence with specific characteristics: (1) all reactions can be conducted under isothermal conditions ranging from 60 to 65°C; (2) the specificity of the reaction is extremely high because it uses six primers [Nagamine et al., 2002] recognizing eight distinct regions on the target nucleotides; and (3) a simple detection method such as visual judgment using Fluorescent Detection Reagent (Eiken Chemical Co., Ltd.) is possible. The LAMP assay is also very effective for RNA template detection using reverse transcriptase (RTase) together with DNA polymerase. Detection of RNA viruses are reported as such, West Nile virus [Parida et al., 2004], Severe Acute Respiratory Syndrome Coronavirus [Hong et al., 2004], and Dengue virus [Parida et al., 2005] using a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay. Therefore, the RT-LAMP assay can be applied for the detection of NVs in many laboratories engaged in the detection of NV.

This study describes (a) the evaluation of a newly developed RT-LAMP assay for the detection of NVs and compares the results with that of the routine RT-PCR assay, (b) comparisons between the RT-LAMP assay and conventional RT-LAMP detection kits for NVs (Eiken Chemical Co., Ltd.), and (c) application of the RT-LAMP assay for outbreaks of acute gastroenteritis.

MATERIALS AND METHODS

Clinical Specimens

A total of 182 samples from 14 public health centers were submitted to Osaka Prefectural Institute of Public Health (OPIPH) for investigation of the causative agent of acute gastroenteritis (outbreaks and sporadic cases) during 2004–2006. Ninety-four samples had been analyzed previously for bacterial incubation and enteric virus (such as NV, group A and C rotavirus, sapovirus, astrovirus, and enteric adenovirus). These 94 samples were used to evaluate both of the RT-LAMP systems (OPIPH system and the conventional RT-LAMP kit) and the results were compared with routine RT-PCR assays (Table II). Subsequently, the newly developed RT-LAMP assay was used as one of the methods for investigating 8 acute outbreaks of gastroenteritis composed of 88 clinical specimens. Five of those outbreak incidents were suspected as cases of person-to-person transmission and one incident was a food-borne illness. Before the investigation, the causes of the other two cases were not clear (Table IV). The specimens had been stored as 10% suspensions in saline at 4°C.

RNA Extraction

Using an automated RNA extractor, Magtration System 6GC or Magtration System 12 GC (Precision Science System Co. Ltd., Matsudo City, Chiba, Japan), viral RNA was extracted from 200 μl of centrifuged samples according to the manufacturer's protocol. A 50 μl volume of eluted solution was obtained after 30 min and was stored at −80°C.

Primer Design

NV-specific RT-LAMP primers were designed in the relatively conserved region (RNA polymerase region to N-terminal capsid region, ORF1 to ORF2). Due to their diversity, the NVs were categorized into three categories, that is, GI (GI.1, 2, 3, 4, 5, 6, 8, 9, and 12), prevalent GII (GII.2, 3, 4, 6, and 12), and minor GII (GII.1, 5, 7, 8, 14, 16, and 17). The nucleotide sequences of strains including prototypes based on published sequences were aligned with the available sequences of other strains* (Genbank accession numbers are described at the end of this paragraph) using DNASIS software (Hitachi Software, Tokyo, Japan). A set of six primers was designed as shown in Figure 1; outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and loop primers (FLP and BLP). The FIP primer consisted of the F1c sequence, a TTTT spacer, and the F2 sequence. In the same way, BIP contained the B1c sequence, a TTTT spacer, and the B2 sequence. Two additional loop primers were designed to accelerate the amplification reaction. The primers recognized eight distinct regions on the target sequence (Fig. 1) and were designed by employing the LAMP primer design support software program version 2 (Net Laboratory, Japan: http://venns.netlaboratory.com). In addition to the general criteria described [Notomi et al., 2000], terminal dimer formation, 3′ hairpin formation, and self-complementary were avoided. Special attention was given to adjust the melting temperature (Tms) of the primers in such a way that the Tms were in the following order: F1c and B1c > F2 and B2 > F3 and B3. All 3 primer sets were composed of more than 6 primers (12–15 primers) in order to address the variations of the NV sequences. Table I lists the details of the oligonucleotide primers used for the LAMP assay. All primers were OPC cartridge purified grade and synthesized by Nihon Gene Research Laboratories, Inc. (NGR, Inc., Sendai, Japan).

The scheme of Kageyama et al. [2004] was used in this report for the NV classification. *GI.1, Norwalk virus: AF093797, KY-89/89/JP: L23828; GI.2, Southampton/91/UK: L07418; GI.3, Desert ShieldDSV395: U04469; GI.4, Chiba407/1987/JP: AB042808; GI.5, Apalachicola Bay/318/1995/US: AF414406; GI.6, BS5/98/DE:

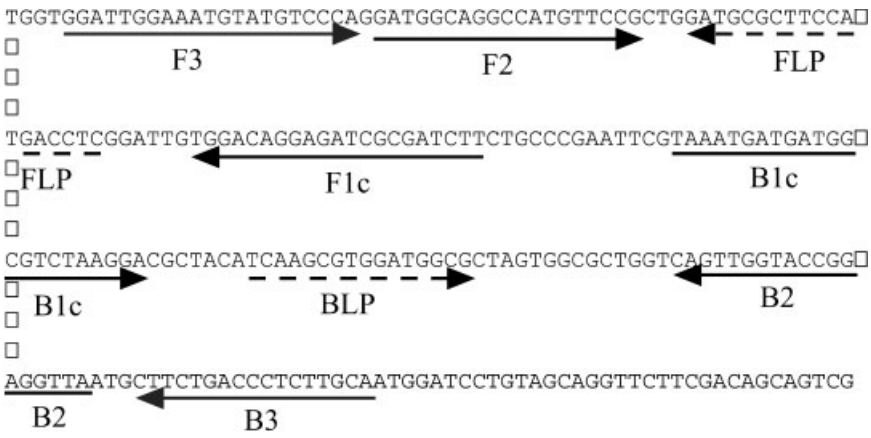

Fig. 1. Nucleotide sequences of GI.1, Norwalk virus (AF093797, nt 5,251–5,490) used for designing the primers. Among the GI primer set, the outer primers, F3 (F3-1), B3 (B3-2), the inner primers, FIP: F1c + TTTT + F2, BIP (BIP-2): B1c + TTTT + B2-2, FLP, BLP (BLP-1), and two additional loop primers, FLP and BLP (BLP-1) were actually used for the GI.1 genotype, and are shown in this figure as the primer location example.

AF093797; GI.8, WUG1/00/JP: AB081723; GI.9, SaitamaSzUG1/99/JP: AB039774; GII.1, Hawaii/71/US: U07611; GII.2, Melksham/89/UK: X81879, SnowMountain/76/US: AY134748; GII.3, SaitamaU201GII/98/JP: AB039782, Mexico/89/MX: U22498, Arg320/95/AR: AF190817; GII.4, Lordsdale/93/UK: X86557, Camberwell/94/AU: AF145896; GII.5, MOH/99: AF397156, WhiteRiver/290/94/US: AF414423; GII.6, Saitama U3/GII/97/JP: AB039776; GII.8, Saitama U25GII/98/JP: AB039780; GII.12, Gifu'96: AB045603, Saitama U1GII/97/JP: AB039775; GII.16, Saitama T53GII/02/JP: AB112260; GII.17, FortLauderdale/560/1998/US: AF414426, Saint Cloud/624/1998/US: AF414427).

RT-PCR

RT-PCR was performed using two sets of primer pairs (G1SKF and G1SKR for GI and G2SKF and G2SKR for GII) in accordance with the standard protocol [Kojima et al., 2002]. Briefly, following cDNA synthesis with either of 50 pmol reverse primers, G1SKR or G2SKR at 42°C for 30 min, with 5 nmol dNTP and 10 U of RNasin (Takara BIO, Inc., Otsu City, Shiga, Japan) in 1× Ex Taq buffer (Takara BIO, Inc.) using Moloney murine leukemia virus (MuLV) RTase (Applied Biosystems, Foster City, CA), PCR consisted of 30 cycles of denaturation (94°C for 30 sec), primer annealing (51°C for 1 min), and extension (72°C for 1 min) after the first denaturation at 94°C for 3 min with Takara Ex Taq (Takara BIO, Inc.) using 10-μl of cDNA and 25 pmol of each primer with 200 pmol dNTP in a 50-μl total reaction volume. After the RT-PCR was performed, 5 μl of the product was analyzed by 1.5% agarose gel electrophoresis and the products were visualized by ethidium bromide staining.

Sequencing and Phylogenetic Analysis of NVs in Specimens

All extracts that resulted in NV RT-PCR products of the appropriate size (330 bp for GI and 344 bp for GII) were sequenced with the same primers that were used for RT-PCR in both directions by using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) as described previously [Cauchi et al., 1996]. The phylogenetic category was determined by using the blast search at http://www.ddbj.nig.ac.jp/search/blast-j.html.

RT-PCR Other Than Routine Detection

RT-PCR was performed in a similar way as the routine procedure, except with different forward primers (GII-FP: 5′-CAACCATGARRACCCVWCYGA-3′ or GII-FP2: 5′-GGACTAGRGGVCCYAAYCATG-3′ for GII and GI-FP: 5′-ACTTAGAARRMRDRTNGATGG-3′ for GI). The thermal profile for PCR was 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min except for the first five cycles. For the first five cycles, the annealing temperature was 37°C instead of 55°C. A final extension after the 35 cycles was at 72°C for 1 min. The amplified products were analyzed by 1% agarose gel electrophoresis, and the positive product was used for sequencing (using the same primers as RT-PCR). The accession numbers of the sequences that were used for the primer design were: AB248831, AB248832, AB248833, AB248834, AB248835, AB248836, AB248837, AB248838, AB248839, AB248840, AB248841, AB248842, AB248843, AB248844, AB248845, and AB248846.

RT-LAMP

The RT-LAMP reaction was preformed in a 25-μl total reaction mixture volume with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) containing 50 pmol each of inner primers FIP(s) and BIP(s), 5 pmol each of outer primers F3s and B3s, 25 pmol each of loop primers FLP(s) and BLP(s), 1.4 mM deoxynucleoside triphosphate, 0.8 M betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, 3.75 U avian myeloblastosis

TABLE I. Details of RT-LAMP Assay Primer Sets Used for Detection of Noroviruses

Names of primer sets (genotypes)	Target position[a]	Primer[b]	sequence (5′ → 3′)
Prevalent GII	4,973–5,179 Lordsdale (201–207 bp)	F3-1	GAAGGTGGCATGGAYTTTTA
		F3-2	AGTGGTGGTCTGGARTTTTA
		B3-1	TCHAGWGCCATBRYCTCRTT
		B3-2	ACSGGYTCAAGAGCCATG
		FIP	AGATTGCGATCGCCCTCCCATTTTGCCCAGACAAGARSCMATGTT
		BIP-1	TGDGAATGAAGATGGCGTCGATTTTTTGRCCTCTGGKACGAGGTT
		BIP-2	TGTGAATGAAGATGGCGTCGATTTTYYTCTGGGACGAGRCMGGC
		BIP-3	TGTGAATGAAGATGGCGTCGATTTTGACCTCATTATTACTTTCTGGC
		FLP	ATCHGAGAAYCTCATCCA
		BLP-1	GATGGGTCCGCAGCCAA
		BLP-2	ATSACGCCGCTCCATC
		BLP-3	TSACGCCGCTCCATCTAA
Minor GII	4,961–5,189 Saitama U25 (229–241 bp)	F3-1	ACWGARYTSAARGARGGTGG
		F3-2	GGCATGGTWATCAAAGAAG
		F3-3	GAGATYAAGAGTGGTGGTC
		B3-1	AGCCCCMRCCACRGGYTC
		B3-2	AGCGCCYGCAACKGGG
		FIP-1	CCCAHGTGCTSARGTCTGAGAATTTTTGGATTTTTACGTGCCMAGRCA
		FIPN-2	CCCAHGTGCTSARGTCTGAGAATTTTCATGAGTTTTATGTGCCAAAGT
		BIPN-1	TGTGAATGAAGATGGCGTCGATTTTTCWTTKTKRMYCTCTGGKACGA
		BIPN-2	TGTGAATGAAGATGGCGTCGATTTTGYCTCAKTATTRCTYTCTGGC
		FLP-1	ATCTCATCCAYCTRAACAT
		FLP-2	CATCCAYCTGAACATGGC
		FLP-3	CTCATCCACCTGAACATAG
		BLP-1	RTGACGCMGCTCCATC
		BLP-2	ATGACGCTACTCCATCAA
		BLP-3	TGACGCCGCTCCATCTAA
GI	5,247–5,456 Norwalk virus (202–209 bp)	F3-1	GGTTTRGARATGTATGTSCCA
		F3-2	AAGGGGGSYTTGARATSTAC
		B3-1	CATTGRWATWGGSTCAGCT
		B3-2	TGCAAGAGGGTCAGAAG
		B3-3	GWGGCAGTGGTTCAGCA
		FIP	GAGATYGCGRTCYCCTGTCCATTTTGVTGGCAGGCCATGTTCCG
		BIP-1	TAAATGATGATGGCGTCTAAGGATTTTRCCTCYGGTACYAGCTGGC
		BIP-2	TAAATGATGATGGCGTCTAAGGATTTTTAACCTCCGGYACSAGYTGA
		FLP	GAGRTCATGGAASCGCATC
		BLP-1	CCAAGCGYRGATGGCG
		BLP-2	ACGCCCCAMCAAACATG
		BLP-3	ACGCCCCAMCATCSCCT

[a]Target position was indicated using representative viruses. Genbank accession numbers: Lordsdale, X86557; Saitama U25, AB039780; Norwalk Virus, NC001959.
[b]F3, forward outer primer; B3, backward outer primer; FIP, forward inner primer; BIP, backward inner primer; FLP, forward loop primer; BLP, backward loop primer.

virus (AMV) RTase (Invitrogen, Carlsbad, CA), 8 U Bst DNA polymerase large fragment (New England Biolabs, Ipswich, MA), and the specified amounts (2–4 μl) of target RNA. The mixture was incubated at 62°C for 60 min in a Loopamp real-time turbidimeter (LA-320; Teramecs, Kyoto City, Japan). Real-time measurement of turbidity is available using an inexpensive photometer [Mori et al., 2001] since the amplification of DNA is directly correlated with the production of magnesium pyrophosphate leading to turbidity. The reaction was considered to be positive when the turbidity became 0.1 within 60 min. To facilitate the field application of the RT-LAMP assay, the results of amplification by the RT-LAMP assay were obtained through naked-eye inspection using Fluorescent Detection Reagent (FDR) (Eiken Chemical Co., Ltd.). Briefly, the RT-LAMP assay was performed in the same way as described above except with the addition of 1.5 μl of FDR to the mixture before the reaction. The reaction mixture was incubated at 62°C for 60 min and then heated at 80°C for 2 min to terminate the reaction using heat blocks and observed with the naked eye. FDR (calcein) binds with the manganese ion and remains quenched. When the LAMP amplification reaction occurs, a by-product, pyrophosphate, deprives FDR (calcein) of the manganese ion, which results in the emission of fluorescence. When the free calcein binds to the magnesium ion in the reaction mixture, the fluorescence emission becomes stronger.

RT-LAMP Conventional NVGI (EC NVGI) and EC NV GII Detection Kit

All procedures were performed in a 25-μl total reaction mixture volume according to the manufacturer's protocol (Eiken Chemical Co., Ltd.). Briefly, after mixing 2× buffer, primer mixture, and distilled water (DW), the tubes were heated at 95°C for 5 min and then kept on ice for 5 min, followed by the addition of the enzyme mixture and 5 μl of sample. Amplification of the targeted DNA fragments was performed at 63°C for 60 min in a Loopamp real-time turbidimeter.

Real-Time Quantitative RT-PCR

cDNA synthesis was performed as described in the RT-PCR section. Real-time RT-PCR was conducted as described previously [Kageyama et al., 2003], with slight modifications. In brief, a 50-μl reaction volume was used containing 5 μl cDNA solution, 25 μl TaqMan Universal PCR Master Mix (Applied Biosystems), a set of primers, and probes. In the detection of NV GI, 400 nM of each of primers COG1F and COG1R and a mixture of fluorogenic probes [15 pmol of RING1 (a)-TP and 5 pmol RING1 (b)-TP] were used. To detect NV GII, 400 nM of each of primers COG2F and COG2R and a fluorogenic probe [15 pmol of RING2 (a)] were used. PCR amplification was performed in triplicate with the ABI Prism 7000 sequence detector (Applied Biosystems), and data were collected and analyzed with Sequence Detector software version 1.2.3 (Applied Biosystems).

In G-specific operations, an NV GI- or GII-specific standard curve was generated by a 10-fold serial dilution (10^6–10^1 copies) of purified NV GI (GI.9) or GII (GII.3) cDNA plasmids as previously described [Kageyama et al., 2003].

RESULTS

Primer Design

The success of RT-LAMP assay amplification relies on the specificities of the designed primer sets. The target range of the primers was selected on the basis of relatively conserved regions of the NVs. The potential target regions were selected in the RNA polymerase and the N-terminal of the capsid regions according to nucleotide sequence comparison. Because of the diversity of the NVs, the initial primer set for prevalent GII was targeted to GII.4, minor GII was targeted to GII.5 and 7, and GI was targeted to GI.1. Each primer set was synthesized for each target genotype, and then improved for other genotypes within each group. The optimal reaction temperature was set to 62°C. Table I lists details of the final primer sets and locations.

Detection of Several Genotypes of NV Using Newly Developed RT-LAMP

Comparative evaluation of RT-LAMP with routine RT-PCR was performed to examine the rate of genotype detection. Genotypes were determined using RT-PCR followed by nucleotide sequencing. Ninety-four samples were chosen from different incidents (Table II). There are several minor sequence differences even within the same genotype. However, the results of the RT-LAMP assay corresponded well with that of RT-PCR, as shown in Table II.

Comparison of Reactivity Between Newly Developed RT-LAMP and Conventional RT-LAMP Kit Using Various NV Genotypes

The reactivity of the RT-LAMP assay developed in this study was compared with that of EC NV GI and EC NV GII detection kits (Eiken Chemical Co., Ltd.); the results are listed in Table II. Prevalent GII and minor GII primer sets have a detection rate similar to that of the EC NV GII detection kit. However, the rate of the GI primer set appeared to surpass that of the EC NV GI detection kit because the primer set can detect several genotypes (GI.3, 11, and 12) better than the EC NV GI detection kit.

Specificity of the RT-LAMP

The specificity of NV RT-LAMP was examined using other RNA virus-infected specimens such as group A rotavirus, group C rotavirus, and sapovirus that had previously been tested for NVs by RT-PCR and had produced negative results for NVs. The specific reaction of the OPIPH RT-LAMP assay was demonstrated as indicated in Table II.

TABLE II. Comparative Results and Specificities of the RT-LAMP and RT-PCR Assay Systems for Detection of NVs

Primer set	Virus genotype	No. of samples	RT-LAMP (OPH)[a]	RT-LAMP (Eiken)[b]	No. from different incidents
GI	GI.1	1	1	1	1
	GI.3	7	7	3	3
	GI.4	3	3	3	1
	GI.8	4	4	4	2
	GI.11	2	2	0	1
	GI.12	8	8	2	2
Prevalent GII	GII.2	10	10	10	4
	GII.3	10	10	10	5
	GII.4	10	10	10	5
	GII.6	10	10	10	5
	GII.12	2	2	2	1
Minor GII	GII.1	5	3	4	1
	GII.5	4	4	4	2
	GII.7	3	3	3	1
Grp A rota[c]		6	0	NA[d]	2
Grp C rota[c]		5	0	NA[d]	1
Sapovirus[c]		4	0	NA[d]	1

[a]OPH, Osaka Prefectural Institute of Public Health.
[b]Eiken, Eiken Chemical Co., Ltd. Detection kit for NVs (GI or GII).
[c]The specimens were examined for NVs and produced negative results using RT-PCR. Regarding RT-LAMP analysis, three primer sets were used; Grp, group.
[d]NA, not attempted.

Comparison of Sensitivity Between Two RT-LAMP Systems Using Six NV Genotypes

To compare sensitivity in detecting NVs among genotypes between the newly developed RT-LAMP system (OPIPH system) and the EC NV detection kits, relatively often detected GI genotypes in OPIPH (GI.3 and GI.8) and prevalently detected GII genotypes (GII.2, 3, 4, and 6) were selected. Since the sequence of the primer sets of the EC NV detection kits were not public, the clinical specimens were used and were estimated as NV copies of the specimens by real-time PCR (triplicated) before the comparison experiment. The entire sensitivity test was conducted in triplicate using serial 10-fold dilutions of 4 μl of samples for the OPIPH and EC NV detection kits. The detection limit was defined the last positive copies (the sample was considered positive if all three samples tested positive) as shown in Table III. The representative results of the sensitivity test for detection of the NV genotypes are shown in Figure 2a,b. Figure 2a,b indicates the newly developed RT-LAMP reaction using GII.3 and GI.3, respectively, and shows good reproducibility of both reactions within the detectable range. The OPIPH system required a slightly longer time to become positive than did that of the EC NV detection kits. However, the sensitivity of the newly developed primer sets for the detection of all genotypes is similar or better than that of the EC NV detection kits.

Naked-Eye Observation of RT-LAMP Results Using FDR

The possibility of RT-LAMP detection of NVs using FDR was examined. A sensitivity test using GII.4 was performed in triplicate in the same way as for the FDR test (i.e., 5×10^3, 5×10^2, 5×10^1, 5×10^0 copies). The results of the representative tubes, that is, only positive and negative samples, are shown in Figure 3. A handy UV lamp (BLAK-RAY LAMP, Long wave UV-365 nm) was used. The degree of sensitivity to detect NVs using FDR is in agreement with that of real-time RT-LAMP, that is, all three tubes became positive in up to 5×10^1 copies, and only one tube became positive in 5×10^0 copies.

Diagnosis of Clinical Incidents Using Newly Developed RT-LAMP

To examine the possibility that the RT-LAMP assay is applicable for detecting outbreaks of NVs without non-specific reactions, the specimens of eight outbreaks were used, and the results of an RT-LAMP assay were

TABLE III. Sensitivity Test for Detecting Each Genotype of NVs by RT-LAMP (OPH and Eiken Kit)

G[a]	No. of copies in CS[b]	RT-LAMP system	Sensitivity
GI.3	8×10^5	OPH	8×10^1
		EC	8×10^4
GI.8	8×10^4	OPH	8×10^{-1}
		EC	8×10^{-1}
GII.2	7×10^4	OPH	7×10^0
		EC	7×10^1
GII.3	8×10^3	OPH	8×10^1
		EC	8×10^3
GII.4	5×10^6	OPH	5×10^1
		EC	5×10^1
GII.6	2×10^5	OPH	2×10^2
		EC	2×10^2

[a]Genotypes.
[b]CS, clinical sample; OPH, Osaka Prefectural Institute of Public Health; EC, Eiken Chemical Co., Ltd.

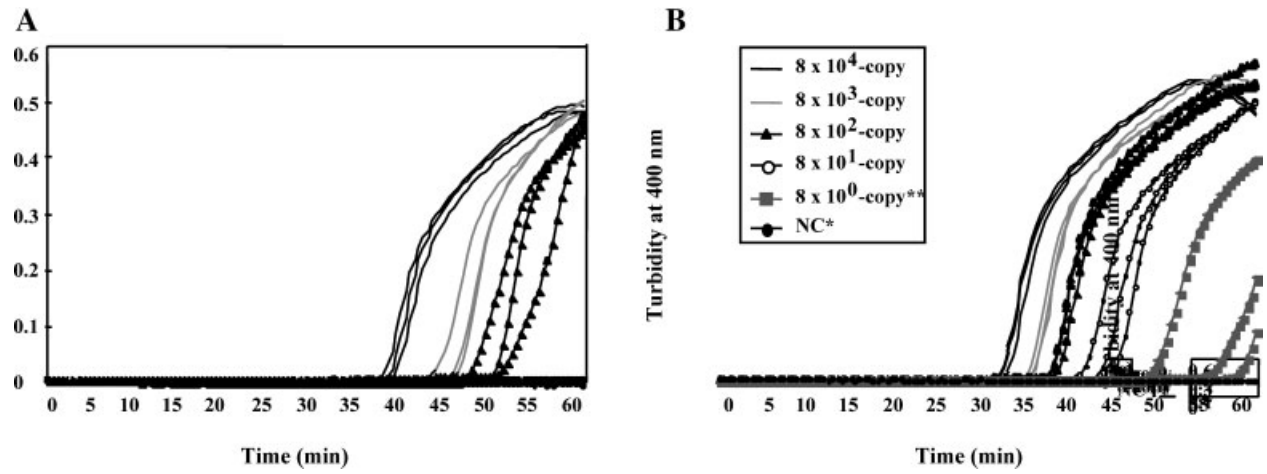

Fig. 2. Sensitivity test to detect NV using RT-LAMP assay (triplicated) measured by real-time turbidimeter (LA-320; Teramecs). The curves from left to right indicate decreasing concentrations of virus copies [8,000 to 80 in (**a**), and 80,000 to 8 in (**b**)]. a: Detecting GII.3 using the newly developed prevalent GII primer set. b: Detecting GI.3 using the newly developed GI primer set. *NC: negative control. **The turbidity of the third sample of eight copies in (b) was 0.08, so it was judged as negative (see Table III).

compared with that of a routine RT-PCR assay. There are three primer sets, all of which were used in this experiment, as shown in Table IV. It became clear after the analysis that these eight outbreaks consisted of two non-NV incidents. However, the results of the RT-LAMP assay for the detection of NVs corresponded well with those of RT-PCR. The third incident consisted of patients and asymptomatic persons who were responsible for cooking and serving food. The results of RT-LAMP and RT-PCR indicated that this incident appeared to be a case of person-to-person infection rather than being foodborne. Based on an epidemiological study, the fourth incident was thought to be a foodborne outbreak. As a result of RT-PCR (including sequence analysis), it was judged to be a case of foodborne transmission, as shown in Table IV. The sixth case was an NV outbreak in an elementary school, and all five samples came from patients. In the seventh case, all the primer sets of the RT-LAMP assay showed no non-specific reaction because this case was caused by group C rotavirus. The last case occurred at a restaurant serving broiled meat and was foodborne caused by *Campylobacter* spp. In this case, the RT-LAMP method also showed no non-specific reaction. In all cases, the results of RT-LAMP agreed well with those of RT-PCR. Cross-reactivity between the prevalent GII primer set and minor GII primer set were observed. However, there was no cross-reactivity between the GI primer set and either of the GII primer sets in any of the cases, as shown in Table IV.

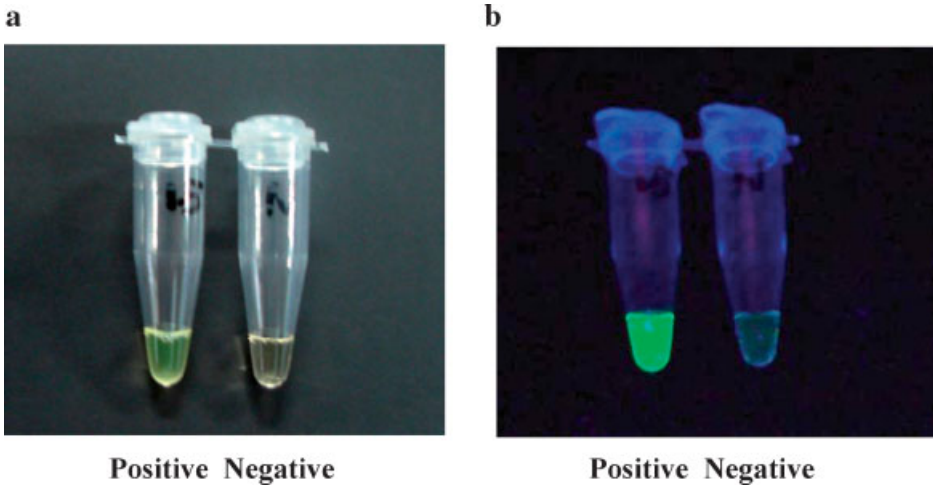

Fig. 3. Detection of NV RT-LAMP assays using Fluorescent Detection Reagent (FDR) instead of the real-time turbidimeter. **a**: Naked-eye inspection under normal light. The original faint orange color of the FDR changed to faint yellowish green in the case of positive amplification, whereas for a negative control without amplification, the original color is retained. **b**: Strong green fluorescence was visually observed under UV light [The same tubes were used as in (a)].

TABLE IV. Possibility of Field Application of RT-LAMP Compared With Routine RT-PCR

Case	No. of samples	RT-LAMP GI/ P.GII/M.GII	RT-PCR	Transmission[a] system	Location (category)	Causative agent
1	7	0/7/7	7	P	Kindergarten	NVGII.2
2	13	0/11/11	11	P	Child care nursery	NVGII.2,3
3	5	0/4/4	4	P	Day nursery (patients)	NVGII.2,4
	5	0/0/0	0		(Cooks and food servers)	
4	13	13/0/0	13	F	Nursing facilities (patients)	NVGI.8
	6	1/0/0	1		(Cooks and food servers)	NVGI.8
5	5	0/4/0	4	P	Junior high school	NVGII.3
6	5	0/5/5	3	P	Elementary school	NVGII.2
7	22	0/0/0	0	P	Elementary school	Group C rota
8	7	0/0/0	0	F	Shop selling broiled meat	*Campylobucter* spp.

[a]P, person-to-person; F, foodborne; Case No. 3, Among five cooks and food servers, none became positive for NV, so the case was considered to be person-to-person transmission; Case No. 4, Among cooks and food servers, the same genotype NV was detected. As a result, this case was considered as a foodborne outbreak.

DISCUSSION

Establishment of simple, rapid, and reliable methods for diagnosis of NV infections enables appropriate medical treatment as well as prevention of secondary infection. The RT-PCR method usually requires 2 days to confirm that a RT-PCR product is NV, while only 1 hr is required for an RT-LAMP assay. In this study, NVs were categorized initially into three groups for designing the appropriate primer sets. GI occupies about 10% of NV infection. Among GII NVs, genotypes GII.2, 3, 4, 6, and 12 are frequently detected, so this group was categorized as prevalent GII. In contrast, genotypes such as GII.1, 5, and 7 are not often detected; GII.8, 9, 10, 11, 13, 14, 15, 16, and 17 were not detected in OPIPH during 2004–2005. Therefore, these GII genotypes were categorized as minor GII. To separate the target genotypes into three groups, well-designed primer sets were developed, as shown in Table I. The detection rates of various genotype NVs between the OPIPH system and the conventional kit were compared using 79 clinical specimens from patients infected with NVs. The specificity of the OPIPH system were examined using 15 other enteric RNA viruses in outbreaks and/or sporadic cases. The results showed that the OPIPH system and the conventional kit indicated similar detection rates for GII NVs. The striking difference between the two systems was that the detection rate of GI NVs was much greater in the OPIPH system than in the conventional kit (Table II). Regarding the specificity of the OPIPH system, no non-specific reactions were observed against other enteric RNA viruses (Table II). The reaction of the new primer set showed good reproducibility within the sensitivity range, as indicated in Figure 2a,b. These figures demonstrated the reproducibility of the RT-LAMP reaction using more than 6 primers (12–15 primers) as primer sets.

There is a recent report of detecting NVs using RT-LAMP [Fukuda et al., 2006], and a commercialized NV detection kit using LAMP methods (Eiken Chemical Ltd.) was developed based on this system. The sensitivity obtained by Fukuda et al. [2006] was between 10^2 and 10^3 copies/tube, while those of a conventional kit and the OPIPH system showed 10^{-1}–10^3 copies/tube and 10^{-1}–10^2 copies/tube, respectively. When the sensitivities of the RT-LAMP system, the OPIPH system, and the conventional kit were compared using clinical specimens, the OPIPH system showed better sensitivity than that of the conventional kit. One hundred and 1,000 times greater sensitivity for GII.3 and GI.3, respectively, was observed for OPIPH system than for the conventional kit, where similar sensitivities were observed for other NVs in both systems (Table III). In other features, the conventional kit from Eiken Chemical Co. requires a heat treatment before 60 min incubation at 63°C to avoid non-specific reactions. In contrast, the RT-LAMP assay in the OPIPH system showed no non-specific reactions without heat treatment. The advantages of using OPIPH RT-LAMP systems compared with conventional NV detection kits are: (1) sensitivity is higher in some NV genotypes (GI.3 and GII.3); (2) specific GI genotypes (GI.3, 11, and 12) that cannot be detected by a conventional GI kit can be detected by the GI primer set developed in this study; and (3) additional treatment, that is, heating at 95°C for 5 min and being kept on ice for 5 min, is not required in the OPIPH system.

Practical applications of the RT-LAMP assay were evaluated using 88 specimens from 8 outbreaks including 2 non-NV incidents. The results of RT-LAMP corresponded well with that of RT-PCR with one exception, case No. 6, as shown in Table IV. In this case, the high sensitivity of RT-LAMP enabled detection of NVs in the patient specimens that could not be detected by routine RT-PCR. The data indicated the possibility of field application of RT-LAMP. The cross-reactivity between prevalent GII and minor GII (Table IV) is not a significant problem because the one-step RT-LAMP assay is for diagnosis and cannot be used to identify the NV genotype.

In conclusion, the RT-LAMP assay is a one-step, simple, and accurate method that does not require the use of expensive equipment. In addition to the above advantages, the availability of the results without opening the lid by either using FDR or a real-time turbidimeter reduces contamination. In this regard, it

will be a valuable assay method for the accurate and rapid detection of NVs in laboratories under all sorts of conditions including use in developing countries. An automated RNA extractor was used in this study, but a conventional RNA extraction kit such as a QIAamp viral RNA mini kit (QIAGEN) could be used for RNA extraction. The RT-LAMP assay will be the first candidate to be recommended for laboratories whose needs are the diagnosis or detection of NVs. RT-PCR assay should be used for subsequent molecular epidemiological analysis after the RT-LAMP assay. This RT-LAMP system was developed to meet the growing demand for detection of NVs. Although a conventional RT-LAMP kit for the detection of NVs has become available, the quality of this kit is unsatisfactory, especially for the detection of NV GI. Comparison of the OPIPH system with the conventional kit proved that the OPIPH system has advantages in diagnosing outbreaks of acute gastroenteritis, especially those caused by GI. Nevertheless, the RT-LAMP assay method for NV detection has just been developed and there is great diversity among NVs. Based on these factors, further studies are needed to evaluate the true value of the RT-LAMP assay for detecting NVs compared with routine RT-PCR system.

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