Application of a Modified Loop-Mediated Isothermal Amplification Kit for Detecting Norovirus Genogroups I and II

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Norovirus is a major etiologic agent in worldwide outbreaks of gastroenteritis associated with food as well as person-to-person transmission. The ubiquitous nature of Norovirus necessitates simple and rapid detection methods with high accuracy and sensitivity. To this end, several investigators have evaluated the usefulness of commercial reverse-transcription loop-mediated isothermal amplification (RT-LAMP) kits for detecting Norovirus genogroups I (GI) and II (GII). In previous studies, the conventional Loopamp kit for Norovirus GII showed a relatively high detection rate, while that for Norovirus GI showed a relatively low detection rate. In the present study, clinical Norovirus specimens were used to compare the detection rate of a modified Loopamp kit for Norovirus GI with the rates of the conventional Loopamp kit for Norovirus GI and an ''in-house'' RT-LAMP GI primer set, methods which had a high detection rate. Results from the present study showed that the modified Loopamp kit for Norovirus GI had a higher detection rate for two viral genotypes (GI.3, GI.11). On comparison with an ''in-house'' GII primer set using genotype GII.4 viruses circulating recently, the detection rate by the Loopamp kit for Norovirus GII was found to be higher, with a 98% detection rate. These results indicate the applicability of the modified LAMP kit for GI and the conventional LAMP kit for GII for detection of Noroviruses in clinical samples. *J. Med. Virol.* **81:2072–2078, 2009.** © 2009 Wiley-Liss, Inc.

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INTRODUCTION

Acute gastroenteritis is a common ailment in humans and remains a major public health concern worldwide. Norovirus, a member of the genus *Norovirus*, in the family *Caliciviridae*, is considered to be a common cause of outbreaks and sporadic cases of acute gastroenteritis among all age groups. Transmission occurs by ingestion of contaminated food or water, exposure to contaminants (fomites, aerosolized vomitus), and person-to-person contact. Known to have high transmissibility and a low infectious dose, Norovirus has been recognized as the primary source of outbreaks in a range of environments, including nursing homes, hospitals, schools, hotels, and cruise ships [Vinje and Koopmans, 1996; Cheesbrough et al., 2000; Hedlund et al., 2000; Marks et al., 2000; Pang et al., 2000; Johansson et al., 2002; Lopman et al., 2002; Marks et al., 2003]. However, an optimum detection method that is both simple and swift to administer while maintaining accuracy and sensitivity has yet to be used widely.

Noroviruses can be divided into five genogroups based on the genome sequence of the RNA-dependent RNA polymerase and the capsid regions [Vinje et al., 2000, 2004; Zheng et al., 2006]. Of the five identified genogroups, three distinct strains (GI, GII, and GIV) have been associated with human gastroenteritis, containing at least 8 (GI), 17 (GII), and 1 (GIV) genotype [Zheng et al., 2006].

In outbreak settings, a diagnostic method that surpasses present methods in simplicity, speed,

sensitivity, and accuracy is important for preventing the further dissemination of the virus into the population at large. Enzyme immunoassays (EIAs) have been improved with the development of a multivalent antibody panel by pooling monoclonal antibodies to recombinant Norovirus capsid proteins, thereby allowing detection of a broad range of Norovirus genotypes [Kitamoto et al., 2002; Shiota et al., 2007]. A rapid and simple diagnostic kit based on immunochromatography which was developed using rabbit polyclonal antibodies against the recombinant Norovirus capsid was found to be predominantly type-specific. However, several studies have shown that these immunological assays lack sensitivity when compared with the gene amplification method of reverse transcription-polymerase chain reaction (RT-PCR) [Dimitriadis et al., 2006; Wilhelmi de Cal et al., 2007; Takanashi et al., 2008].

With regard to methods of target gene amplification for detecting Norovirus in clinical samples, RT-PCR combined with DNA sequencing has been used as the standard method at our institution, and was used as the reference detection method in the present study. Real-time RT-PCR is considered to be as reliable as RT-PCR with hybridization or sequencing.

Several methods for target gene amplification, including isothermal amplification methods include nucleic acid sequence-based amplification (NASBA) [Moore et al., 2004], transcription-reverse transcription concerted (TRC) assay [Ishiguro et al., 2003], and loop-mediated isothermal amplification (LAMP) assay [Notomi et al., 2000] were reported recently. With regard to application of the RT-LAMP assay for detection of Norovirus [Fukuda et al., 2006; Yoda et al., 2007], additional primers are required for detection of other RNA viruses, such as West Nile virus [Parida et al., 2004], severe acute respiratory syndrome coronavirus [Hong et al., 2004], human influenza A virus (limited to detection of H1–H3) [Poon et al., 2005] due to the diversity of Noroviruses as well as the broad detection targets.

In a previous study, three primer sets (namely GI, prevalent GII, and minor GII) were developed for the RT-LAMP method of detecting Norovirus and compared with the conventional RT-LAMP kit (Eiken Chemical Co., Ltd, Tokyo, Japan) [Yoda et al., 2007]. Results showed that the "in-house" GI primer set was superior to that used in the conventional Loopamp kit for Norovirus GI. The "in-house" prevalent and minor GII primer sets for the detection of GII provided detection rates similar to those attained with the conventional kit for Norovirus GII [Yoda et al., 2007].

GII.4 is the most frequent genotype of Norovirus strains detected, the ubiquity of which accounts for the many new variants discovered. Although previous evaluations of conventional Loopamp kits (Eiken Chemical Co., Ltd) for Norovirus detection showed that the Loopamp kit for Norovirus GII had a relatively high detection rate [Iturriza-Gomara et al., 2008], the reactivity toward the common GII.4 strains in Japan are not known. Therefore, the detection rate of the Loopamp kit for Norovirus GII was re-examined and compared with the detection rate of the "in-house" GII primer set, using primarily clinical specimens shown to contain common circulating GII.4 strains.

The detection rate of the modified Loopamp kit for Norovirus GI developed by the Eiken Chemical Co., Ltd was compared with the detection rates of the conventional Loopamp kit for GI and the "in-house" GI primer set that showed a high detection rate in a previous study, using clinical samples.

MATERIALS AND METHODS

Clinical Specimens

Clinical specimens used in the present study were 171 samples obtained from 63 gastroenteritis outbreaks and sporadic cases of gastroenteritis submitted to the Osaka Prefectural Institute of Public Health (OPIPH) for investigation into the causative agent of acute gastroenteritis outbreaks between 2006 and 2008. RT-PCR combined with DNA sequencing detected either Norovirus GI or GII (not both) in 134 samples (49 GI and 85 GII), and no Norovirus in the remaining 37 samples, including 1 A rotavirus-positive specimen, 3 sapovirus-positive specimens, 2 enteric adenovirus-positive specimens, and 3 astrovirus-positive specimens. All specimens had been stored until use as 10% suspensions in saline at 4°C.

RNA Extraction

Following centrifugation, viral RNA was extracted from 200 µl of supernatant using an automated RNA extractor (Magtration System 6GC or 12GC; Precision Science System Co., Ltd, Matsudo City, Chiba, Japan) according to the manufacturer's protocol. A 50-µl volume of eluate was obtained and stored at −80°C until use.

RT-PCR

RT-PCR was conducted in accordance with a standard protocol [Kojima et al., 2002] using two sets of primer pairs (G1 SKF and G1 SKR for GI, and G2 SKF and G2 SKR for GII). Briefly, cDNA was synthesized in $1\times$ Ex Taq buffer containing 12.5 U of Moloney murine leukemia virus (MuLV) RTase (Applied Biosystems, Foster City, CA), 5 nmol dNTP (Takara Bio, Inc., Otsu, Shiga, Japan), and 10 U of RNase Inhibitor (Takara Bio, Inc.) with either 50 pmol reverse primers, G1 SKR, or G2 SKR at 42°C for 30 min. Following synthesis, PCR was performed, with the first denaturation at 94°C for 3 min followed by 30 cycles of denaturation (94°C for 30 sec), primer annealing (51°C for 1 min), and extension (72°C for 1 min) in a 50-µl total reaction volume of $1\times$ Ex Taq buffer containing 1.25 U of Takara Ex Taq (Takara Bio, Inc.), 200 pmol dNTP, 25 pmol of each primer, and 10 µl of cDNA. After RT-PCR, 5 µl of the product was analyzed using 1.5% agarose gel electrophoresis, and the products were visualized by ethidium bromide staining.

Semi-Nested PCR to Analyze Cross-Reactivity

Semi-nested PCR was conducted in combination with RT-PCR [same as above RT-PCR procedure except 5′-primers; COG1F (5′-CGYTGGATGCGNTTYCATGA-3′) for GI and COG2F (5′-CARGARBCNATGTTYAGRTG-GATGAG-3′) for GII]. PCR was performed, with the first denaturation at 94°C for 3 min followed by 30 cycles of denaturation (94°C for 30 sec), primer annealing (55°C for 30 sec), and extension (72°C for 1 min) in a 50-µl total reaction volume of 1× Ex Taq buffer containing 1.25 U of Takara Ex Taq (Takara Bio, Inc.), 200 pmol dNTP, 25 pmol of each primer, and 1 µl of RT-PCR product. Five microliters of the semi-nested PCR product was analyzed using 1.5% agarose gel electrophoresis, and the products were visualized by ethidium bromide staining.

Sequencing and Phylogenetic Analysis of Noroviruses in Specimens

Using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), all Norovirus RT-PCR products of the appropriate size (330 bp for GI and 344 bp for GII) were sequenced in both directions using the same primers used for the RT-PCR, as described previously [Cauchi et al., 1996]. The data sequences obtained were compared with those of the reference Norovirus strains obtained from GenBank, and subsequently classified into 31 genotypes, as reported previously [Kageyama et al., 2004].

RT-LAMP Assay

Loopamp assay (conventional GI, GII, and improved GI). A Loopamp assay (Eiken Chemical Co., Ltd) was carried out in a 25 µl total reaction volume according to the manufacturer's instructions using a Loopamp real-time turbidimeter LA-320C (Teramecs, Kyoto, Japan). Briefly, after mixing 2× buffer, primer mixture, and distilled water, the tubes were heated at 95°C for 5 min and then held on ice for a further 5 min. The enzyme mixture and 5 µl of the sample were then added to the reaction tube. However, 2 µl of the sample was used for detection sensitivity test exceptionally. The reaction mixture was incubated at 63°C for 60 min in an LA-320C turbidimeter. Positive (from each kit) and negative controls (RNA extract from saline) were used in each test. Although none of the sequence data for the primers were public knowledge, the target region was the ORF1−ORF2 junction.

OPH primer sets (GI and mixed GII, that is, previous prevalent GII and minor GII). An RT-LAMP assay was performed using an "in-house" primer set (OPH primer set) in 25 µl of the reaction mixture with a DNA amplification kit (Eiken Chemical Co., Ltd), as described previously [Yoda et al., 2007]. The target region of this primer set was the ORF1−ORF2 junction. To detect GII strains, a mixed primer set was used. The mixed primer set contained all primers of a prevalent GII primer set (12 primers) and minor GII primer set (15 primers). The reaction mixture contained 12.5 µl of 2×

reaction mix, 50 pmol of each inner primer, 5 pmol of each outer primer, 25 pmol of each loop primer, 3.75 U avian myeloblastosis virus (AMV) RTase (Invitrogen, Carlsbad, CA), 8 U Bst DNA polymerase large fragment (New England Biolabs, Ipswich, MA), and the specified amounts (2−5 µl) of target RNA. The mixture was incubated at 62°C for 60 min in an LA-320C turbidimeter (Teramecs). Positive and negative controls were the same as those used in the Loopamp assay, except for the controls for GII. Because the positive control used in the Loopamp kit did not work for GII, positive samples tested beforehand were used instead. The reaction was considered to be positive when the turbidity at 650 nm became ≥0.1 within 60 min of reaction initiation, with an exponential increase of turbidity.

To assess the detection limits of the RT-LAMP assay, the experiment was conducted in triplicate using a serial 10-fold dilution of 2 µl of samples for all of the RT-LAMP systems. The detection limit was defined as the lowest positive concentration of genome copies, with the sample considered positive if all three samples tested positive.

Quantitative Real-Time RT-PCR for Determining Detection Limits

cDNA synthesis was performed as described in the RT-PCR section. RT-PCR was conducted as described previously, with slight modifications [Kageyama et al., 2003]. Briefly, a 50 µl reaction volume was used, containing 25 µl TaqMan Universal PCR Master Mix (Applied Biosystems), a set of primers, and probes with 5 µl cDNA solution. Norovirus GI detection was conducted using 20 pmol each of the primers COG1F (5′-CGYTGGATGCGNTTYCATGA-3′) and COG1R (5′-CT-TAGACGCCATCATCATTYAC-3′) and a mixture of fluorogenic probes (20 pmol of RING1 [a]-TP and 5 pmol of RING1 [b]-TP). PCR amplification was conducted in triplicate using an ABI Prism 7000 sequence detector (Applied Biosystems), and data were collected and analyzed with Sequence Detector software version 1.2.3 (Applied Biosystems).

RESULTS

Comparison of Genogroup-Specific RT-LAMP Assays With Fecal Specimens

A total of 134 clinical specimens diagnosed previously as Norovirus-associated acute gastroenteritis by RT-PCR with sequence confirmation were used for the evaluation of GI- or GII-specific RT-LAMP primer set assays. Evaluation results were compared to different primer sets of the RT-LAMP system (Tables I and II).

With regard to the 49 GI-positive specimens, the modified Eiken GI kit and OPH GI primer set showed high concordance (92% for both primer sets), whereas the conventional Eiken GI kit showed moderate concordance (80%). The main difference in detection rates between the modified and conventional Eiken GI kits was observed in genotypes GI.3 and GI.11. The detection

TABLE I. Comparison of RT-LAMP Assays to Detect Norovirus GI Strains Using Different Primer Sets

Genotype	Number of samples	Modified Eiken GI	OPH GI	Eiken GI
GI.2	5	5/5	5/5	5/5
GI.3	11	9/11	9/11	5/11
GI.4	22	22/22	22/22	21/22
GI.5	1	1/1	1/1	1/1
GI.8	8	6/8	6/8	6/8
GI.11	2	2/2	2/2	1/2
Total	49	45/49	45/49	39/49

OPH, primer set developed at Osaka Prefecture Institute of Public Health.

TABLE III. Reactivity of Different Primer Sets Towards the Individual Clinical Samples

Character of the sample	Modified Eiken GI	OPH GI	Eiken GI
GII.3, GI.4	+	−	+
GII.4-1	+[a]	−	−
GII.4-2	−	−	+[a]
GII.4-3	−	−	+[a]
GII.4-4	−	−	+[a]
Total	2/5	0/5	4/5

OPH, primer set developed at Osaka Prefecture Institute of Public Health.
[a]Considered as cross-reactivity between different NV genogoups.

rates using the modified Eiken GI kit for these two genotypes were 82% and 100%, respectively, while detection rates for these genotypes using the conventional kit were 45% and 50%, respectively (Table I).

With regard to the 85 GII-positive specimens, the concordances of the conventional Eiken GII kit and the OPH mixed primer set were 94% and 76%, respectively (Table II). The differences noted between these two detection systems were primarily due to the poor detection rate of the OPH mixed primer set towards the commonly circulating GII.4 strains (78%). Although not indicated in Table II, the detection rates of the OPH mixed primer set were 88% and 73% for the specimens in financial years 2006 and 2007, respectively. In contrast, the detection rates of the conventional Eiken GII kit were higher for those same 2 years (100% and 98%).

Reciprocity of the reaction of the Primer Sets Towards Different Genogroups

The modified Eiken GI kit was positive with two samples containing different genogroups of Noroviruses, and the conventional Eiken GI kit was positive with four samples, while the OPH GI primer set did not react with these samples (Table III). Positive reactivity with a single sample was observed with the conventional Eiken GII kit but not with the OPH mixed primer set (Table IV). This reciprocity of the reactivity was analyzed by semi-nested PCR followed by sequencing.

TABLE II. Comparison of RT-LAMP Assays to Detect Norovirus GII Strains Using Different Primer Sets

Genotype	Number of samples	Eiken GII	OPH mixed GII
GII.2	2	2/2	1/2
GII.3	8	7/8	7/8
GII.4	58	57/58	45/58
GII.5	1	1/1	0/1
GII.6	1	1/1	1/1
GII.7	3	3/3	2/3
GII.13	12	9/12	9/12
Total	85	80/85	65/85

OPH, primer set developed at Osaka Prefecture Institute of Public Health.

The modified and the conventional Eiken GI kits detected GI.4 Norovirus in GI.4 and GII.3 mixed infection, but the OPH GI primer set could not detect GI.4 Norovirus in the same sample. GI.4 Norovirus detection was confirmed by semi-nested PCR followed by sequencing. However, the semi-nested PCR for GI Noroviruses could not detect any specific bands in the rest of four samples (Table III). The reactivity of the modified and the conventional Eiken GI kits was taken as cross-reactivity between different Norovirus genogroup (Table III).

The genotype, GII.4 Norovirus was detected by the conventional Eiken GII kit, but not with the OPH mixed primer set. This detection was due to mixed Norovirus infection with GI.5 and GII.4. The existence of GII.4 Norovirus in this sample was confirmed by sequencing of semi-nested PCR product.

None of the five RT-LAMP primer sets reacted nonspecifically with any of the 37 Norovirus-negative clinical specimens, including group A rotavirus-positive, sapovirus-positive, enteric adenovirus-positive, and astrovirus-positive specimens.

Comparison of the Sensitivity of Detection of Norovirus GI-Specific Primer Sets

As sequence data for the primer sets used in the Eiken GI kits were not public, clinical specimens were used. The sensitivity of each RT-LAMP assay for detecting Norovirus GI RNA (GI.4 and GI.8) was determined using serial 10-fold dilutions of the extracted RNA previously quantified in triplicate by real-time RT-PCR assay. Sensitivity detection data for the primer sets are shown in Table V. The modified Eiken GI kit showed the highest sensitivity for GI.4 and GI.8, the conventional

TABLE IV. Reactivity of Different Primer Sets Towards the Individual Clinical Sample

Character of the sample	Eiken GII	OPH mixed GII
GI.5, GII.4	+	−

OPH, primer set developed at Osaka Prefecture Institute of Public Health.

TABLE V. Comparison of Detection Sensitivity of Each Primer Set

Genotype	Modified Eiken GI	OPH GI	Conventional Eiken GI
GI.4	8×10^0	8×10^3	8×10^0
GI.8	8×10^{-1}	8×10^1	8×10^0

The detection sensitivity is indicated as number of copies of Norovirus genome.

Eiken GI kit moderate sensitivity, and the OPH GI primer set the lowest sensitivity (Table V).

DISCUSSION

In the present study, the modified Loopamp kit for Norovirus GI was compared with the conventional Loopamp kit for Norovirus GI and the "in-house" GI primer set (OPH GI primer set). With regard to Norovirus GI detection, both the modified kit and the OPH GI primer set showed higher concordance than the conventional kit. Further, the detection rates for two particular GI genotypes (GI.3 and GI.11) were higher with the modified kit and OPH primer set than with the conventional kit. Of the three primer sets examined, the modified Eiken GI kit had the highest detection sensitivity for GI.4 and GI.8. These results suggest that the modified Eiken GI kit may be useful for detecting Norovirus in clinical specimens.

Accurate diagnosis of viral gastroenteritis is essential not only for preventing the further dissemination of the infection, but also for relieving the burden in society and providing treatment for patients, particularly during outbreaks. In this regard, given its simplicity of execution and short diagnosis time, the highly sensitive RT-LAMP assay appears to be suitable for detecting Norovirus.

The modified Eiken GI kit examined in the present study showed high concordance (92%) for Norovirus GI detection with the results of a reference RT-PCR combined with sequencing assay. In contrast, the conventional Eiken GI kit showed only moderate concordance (80%). Detection rates for GI.3 and GI.11 were higher with the modified Eiken GI kit than with the conventional Eiken GI kit (from 45% to 82%, and from 50% to 100%, respectively). The detection rates were similar between the modified Eiken GI kit and the OPH GI primer set. However, the modified kit showed the highest detection sensitivity among the three primer sets for GI.4 and GI.8, detecting 8×10^0 and 8×10^{-1} copies, respectively. Taken together, these data suggest the usefulness of the modified Eiken GI kit in detecting Norovirus in clinical specimens.

Despite for a lower detection sensitivity than the modified Eiken GI kit for GI.4 and GI.8, the OPH GI primer set had the same detection rate as the modified kit and a higher detection rate than the conventional kit. This discrepancy may be due to an excess of copies of the Norovirus genome in most of the clinical specimens

(Table I). The detection limit reported by Pang et al. [2004] was 25,000 copies of RNA transcripts per gram of stool for Norovirus GII and 2.5×10^7 copies/g of stool for Norovirus GI. If RNA extraction is 100% efficient, 8000 copies/tube would be equivalent to 1×10^7 copies/g of stool. Further, the sequence of the OPH GI primer set matches the target region of GI.4 better than the conventional Eiken GI kit, leading to higher detection rates (Table I). However, only highly sensitive GI detection kits could detect GI.4 Norovirus in certain mixed infection cases due to the small number of the Norovirus copies (Table III).

With regard to GII detection, the Eiken GII kit had a much higher concordance in detecting Norovirus GII (94%) than did the OPH mixed GII primer set (76%). This difference in detection rate was due to poor reactivity of the OPH mixed primer set towards the commonly circulating GII.4. The detection rates of the Eiken GII kit and the OPH mixed primer set for GII.4 were 98% and 78%, respectively. The high detection sensitivity (98%) of the Eiken GII kit for the common circulating GII.4 was in good agreement with the 100% detection rate reported previously [Iturriza-Gomara et al., 2008]. Although, the sensitivity for detecting GII.4 genotype of Norovirus was the same between the conventional GII kit and the "in-house" prevalent GII primer set [Yoda et al., 2007], the sensitivity for detecting GII.4 strains might be decreased with mixed primer sets due to interference of the primers [i.e., mixed prevalent GII primer set (12 primers) and minor GII primer set (15 primers)]. However, the reduced detection rate for GII.4 Norovirus in 2 years (88–73% in 2006–2007) indicated the emergence of mismatch between OPH mixed primer set and the circulating GII.4 strains. Because of the high sensitivity for detecting the GII.4 genotype, the conventional GII kit but not the OPH GII primer set might be able to detect GII Norovirus in GI.5 and GII.4 mixed infections (Table IV).

Cross-reactivity was observed in both of the Eiken GI kits towards the different Norovirus genogroups (Table III). In contrast, the OPH GI primer set did not cross-react with the different genogroup viruses. However, cross-reactivity is not a major concern, since these kits showed no non-specific reactions other than for Norovirus, as demonstrated in the present and previous studies [Yoda et al., 2007; Iturriza-Gomara et al., 2008].

Being a positive-sense single-stranded RNA virus, mutations in Norovirus are quite common, and new variants are reported frequently [Bull et al., 2006; Siebenga et al., 2007]. A recent study on the evolution of Norovirus in vivo in a single patient reported that the most cumulative nucleotide changes appeared within the protruding region of the capsid (P2 domain, amino acids 304–404) [Nilsson et al., 2003]. In contrast, the most highly conserved regions across all known Norovirus strains are located in the region from the C-terminal end of ORF1 to the N-terminal region of ORF2 [Katayama et al., 2002]. The primer sets used in the GI and GII Eiken detection kits were positioned in

the most conserved region, the ORF1−ORF2 junction, and appeared to be reasonable for use for detection.

Given the high prevalence of Norovirus-associated gastroenteritis worldwide over the last few years, a simple and accurate diagnosis provided by the RT-LAMP detection system is necessary to reduce the spread of this disease [Dingle, 2004; Lopman et al., 2004; Vipond et al., 2004; Widdowson et al., 2004; IASR, 2007]. Taking into account the results of this study and previous studies, the modified GI and GII Eiken kits provide a practical, effective method of detecting Noroviruses. However, considering the mutation of Noroviruses, further investigation into the detection rates of these kits is necessary.

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