Research Article

Development of Enhanced Primer Sets for Detection of Norovirus

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Norovirus (NV) is a major viral pathogen that causes nonbacterial acute gastroenteritis and outbreaks of food-borne disease. The genotype of NV most frequently responsible for NV outbreaks is GII.4, which accounts for 60–80% of cases. Moreover, original and new NV variant types have been continuously emerging, and their emergence is related to the recent global increase in NV infection. In this study, we developed advanced primer sets (NKI-F/R/F2, NKII-F/R/R2) for the detection of NV, including the variant types. The new primer sets were compared with conventional primer sets (GI-F1/R1/F2, SRI-1/2/3, GII-F1/R1/F2, and SRII-1/2/3) to evaluate their efficiency when using clinical and environmental samples. Using reverse transcription polymerase chain reaction (RT-PCR) and seminested PCR, NV GI and GII were detected in 91.7% (NKI-F/R/F2), 89.3% (NKII-F/R/R2), 54.2% (GI-F1/R1/F2), 52.5% (GII-F1/R1/F2), 25.0% (SRI-1/2/3), and 32.2% (SRII-1/2/3) of clinical and environmental specimens. Therefore, our primer sets perform better than conventional primer sets in the detection of emerged types of NV and could be used in the future for epidemiological diagnosis of infection with the virus.

1. Introduction

Norovirus (NV), belonging to family Caliciviridae, is a major cause of acute viral gastroenteritis [1]. Although symptoms, which typically appear between 12 and 48 h, are generally mild and self-limiting, they can be severe in immunocompromised groups such as infants and the elderly [2, 3]. Viral infection is primarily related to foodborne illness, but person-to-person contact and waterborne outbreaks are also important vehicles for transmission [4–7].

The NV genome is composed of approximately 7.7 kb of single stranded positive sense RNA (+ssRNA), which includes three open reading frames (ORFs): ORF1, ORF2, and ORF3 [8]. Six nonstructural proteins in a polyprotein are encoded by ORF1, including an RNA-dependent RNA polymerase (RdRp) [9]. ORF2 and ORF3 encode major structural capsid protein (VP1) and minor structural capsid protein (VP2), respectively [10]. VP1 consists of a shell domain (S) and two protruding (P) domains [11]. The PI domain, a protruding flexible hinge region, is located between the S and P2 domains [12]. The P2 domain is a hypervariable region that binds to host cell [13]. The stability of VP1 is increased by VP2, which prevents its degradation [14].

NV is classified into six groups, genogroups I to VI (GI to GVI), based on the amino acid sequences of the RdRp and VP1 [5, 15, 16]. The genogroups GI, GII, and GIV are found in humans [5]. Outbreaks appear more frequently in GI, GII than GIV [17–20]. In particular, GII.4 has emerged continuously every 2–3 years in an evolved form [21]. Consequently, it accounts for 87% of the NV outbreaks that occur globally [22–24].

In the Republic of Korea, NV GI.4 Sydney type emerged between 2012 and 2013, during which time it accounted for 60.4% of NV GI.4 diagnoses [25]. Detection of the NV GIH...
Table 1: Description of NV GI sequences for genetic analysis.

| Accession number (GenBank) | Genotype | Strain                                      | Accession number (GenBank) | Genotype | Strain                                      |
|----------------------------|----------|---------------------------------------------|----------------------------|----------|---------------------------------------------|
| EU085529                   | GI.1     | P774.Delsjo2004/Gothenburg/Sweden           | KF039731                   | GI.1     | CH4X0533/2009/USA                           |
| FJ515294                   | GI.2     | Leuven/2003/BEL                             | KF039732                   | GI.1     | CHA5A010/2009/USA                           |
| JN1796918                  | GI.2     | Roosendaal029/2006/NL                       | KF039733                   | GI.1     | CHA9A004_20110426/2011/USA                 |
| JN183159                   | GI.9     | S48/2008/Lilla                              | KF039734                   | GI.1     | CHA6A014/2009/USA                           |
| JN183161                   | GI.7     | S24/2008/Lilla Edet                         | KF039735                   | GI.1     | CHA3A007/2008/USA                           |
| JN603244                   | GI.3     | S29/2008/Lilla Edet/Sweden                  | KF039736                   | GI.1     | CHA7A011/2010/USA                           |
| JN603245                   | GI.4     | S50/2008/Lilla Edet/Sweden                  | KF039737                   | GI.1     | CHA6A003_20091104/2009/USA                 |
| JQ388274                   | GI.6     | Kingston/ACT160D/2010/AU                    | KF306212                   | GI.2     | Jingzhou/2013401/CHN                        |
| JQ743331                   | GI.4     | 1999                                        | KF429761                   | GI.1     | 8MoIII/1972/USA                             |
| JQ743332                   | GI.2     | 10360/2010/VNM                              | KF429765                   | GI.1     | 8W1968/USA                                  |
| JQ911594                   | GI       | 8FIIa/1968/USA                              | KF429770                   | GI.1     | 8McIII/1973/USA                             |
| JX023285                   | GI.1     | TCH-099/USA/2003                           | KF429773                   | GI.1     | 8CKIIc/1974/USA                             |
| KC998959                   | GI.6     | CHA7A009/2010/USA                           | KF429774                   | GI.1     | 8UIII/1973/USA                              |
| KF039725                   | GI.1     | CHA6A003_20091031/2009/USA                 | KF429783                   | GI.1     | 8K/1979/USA                                 |
| KF039726                   | GI.1     | CHA2A014/2008/USA                           | KF429789                   | GI.1     | 8MC/1978/USA                                |
| KF039727                   | GI.1     | CHA2A014/2008/USA                           | KF586507                   | GI.9     | CAIQ12II0628                                |
| KF039728                   | GI.1     | CHA2A014/2008/USA                           | L07418                     |          |                                             |
| KF039729                   | GI.1     | CHA6A007/2010/USA                           | M87661                     |          |                                             |
| KF039730                   | GI.1     | CHA9A004_20110419/2011/USA                 |                            |          |                                             |

Strain is difficult with existing RT-PCR primer sets (GII-F1/R1, SRII-1/2/3) because of the continuous variation of the strain [26, 27]. In addition, GI-F1/R1 primer set does not always have sufficient specificity to detect NV because false-positive detection commonly occurs [28]. Therefore, the aim of this study was to develop primer sets for efficiently detecting NV GI and GII, including detection of newly emerged strains that could not previously be identified with conventional primer sets. Once new primer sets were developed, we evaluated their efficiency using an RT-PCR assay to test clinical and environmental specimens.

2. Materials and Methods

2.1. Collection of Clinical and Environmental Samples. Two sample types, clinical and environmental specimens, were used for detection of NV GI and GII. Eighty-six unknown samples were used for detection of NV GI. They included 22 clinical samples from Gyeonggi Institute of Health Environment (GIHE) that were originally obtained during 2012 and 2013 and 24 clinical and 40 environmental samples from Waterborne Virus Bank (WAVA) originally obtained from 2006 to 2013. To identify NV GII, we used 134 unknown samples that included 35 clinical samples from GIHE, originally collected during 2012 and 2013, and 32 clinical and 67 environmental samples from WAVA, collected from 2006 to 2013.

All stool specimens were collected from patients who suffered from diarrhea caused by acute gastroenteritis. Environmental specimens were collected from groundwater in the Republic of Korea during June and October 2013. All samples were stored at −80°C until use.

2.2. Ethical Clearance. All clinical samples were obtained during the medical treatment of patients with acute gastroenteritis. All patients provided written informed consent, which has been kept on file at the GIHE and WAVA. Human rights were not abused nor were ethical issues encountered during the study. All experimental work and collection of samples were supervised and approved by the Institutional Review Board (IRB) of Songeui Medical Campus, The Catholic University of Korea (approval number MC14SISI0039).

2.3. Primer Design. In order to design new primer sets, 37 sequences of NV GI (Table 1) and 52 sequences of NV GII (Table 2) were obtained from NCBI and imported into EditSeq and MegAlign in DNASTAR software (DNASTAR, USA).

We designed 3 candidates for the NV GI primer set and 5 candidates for the NV GII primer set in the conserved regions of ORF 1 and ORF 2. Among them, NKI and NKII (Table 3) which have outstanding efficiency were selected. Inner primers were designed to detect NV from water samples because
NV generally presents with a low titer in water [29]. They were constructed based on conserved sites from primer sets NKI-F and NKII-R. We named the inner primers NKI-F2 and NKII-R2 and used them for nested PCR.

2.4. Viral RNA Extraction. Viral RNA was extracted from clinical and environmental specimens with the QIAamp viral RNA extraction kit (Qiagen, Germany) according to the manufacturer’s instructions. Each viral RNA sample was eluted with 60 μL of elution buffer and stored at −80 °C until use in the RT-PCR assay.

2.5. RT-PCR and Seminested PCR. Extracted RNA was amplified by both RT-PCR and seminested PCR in S1000 Thermal Cycler (BIO-RAD, Singapore). In RT-PCR, extracted RNA was reverse-transcribed and amplified using the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) according to the manufacturer’s protocol. The total reaction mixture volume of 25 μL contained the following: 5× QIAGEN OneStep RT-PCR buffer (5 μL), 20 pmol primers (1 μL each) (NKI-F/R, NKII-F/R, SRI-1/2, SRII-2/3, GI-F2/R1, and GII-F1/R1), 10 mM dNTP mix (1 μL), enzyme mix (reverse transcriptase and Taq polymerase, 1 μL), extracted viral RNA template (3 μL), and RNase-free water (13 μL) (Welgene, Republic of Korea). RT-PCR conditions were as follows: reverse transcription at 50 °C for 30 min, initial PCR activation at 95 °C for 15 min, 39 cycles of denaturation at 94 °C for 30 s, annealing at each optimal annealing temperature (Tables 4 and 5) for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

For seminested PCR, the RT-PCR product (3 μL), 20 pmol primers (1 μL each) (NKI-F2/R, NKII-F/R2, SRI-2/3, SRII-2/3, GI-F2/R1, and GII-F2/R1), and RNase-free water (15 μL) were added to a Maxime PCR PreMix Kit (t-StarTaq) (iNtRON Biotechnology, Republic of Korea). PCR conditions were as follows: initial denaturation at 94 °C for 2 min, 20 cycles of denaturation at 94 °C for 30 s, annealing at each optimal annealing temperature (Tables 4 and 5) for 10 s, extension at 72 °C for 20 s, and a final extension at 72 °C for 5 min.

2.6. Sequence Analysis of PCR Products. The products of RT-PCR and seminested PCR were analyzed using 2% agarose gel electrophoresis in TAE buffer with DNA SafeStain solution (Lamda Biotech, USA) and were purified in agarose gel with a HiYield Gel/PCR DNA Fragments Extraction Kit (RBC,
Table 3: The nucleotide sequences of NV GI (a) and NV GII (b) strains were compared with sequences of designed primer sets.

(a) GI

| Primer     | NKI primer set |
|------------|----------------|
|            | NKI-F (forward) | NKI-R (reverse) |
|            | G   | T | A | A | A | T | G | A | T | G | C | G | T | C | T | A | A |
| M87661 (GI) |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| KF039732 (GI.1) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| KF306212 (GI.2) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JN603244 (GI.3) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JN603245 (GI.4) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JQ388274 (GI.6) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JN183161 (GI.7) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| KF586507 (GI.9) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |

(b) GII

| Primer     | NKII primer set |
|------------|-----------------|
|            | NKII-F (forward) | NKII-R (reverse) |
|            | C   | T | Y | A | G | C | A | R | A | G | T | G | A | C | Y |
| X86557 (GII) |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JN797508 (GII.1) |   |   |   | A |   |   |   |   | T |   |   |   |   |   |   | 2 |
| KF429769 (GII.2) |   |   |   | A |   |   |   |   | T |   |   |   |   |   |   | 0 |
| KF306213 (GII.3) |   |   |   | A |   |   |   |   | T |   |   |   |   |   |   | 2 |
| JX459907 (GII.4) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| KC464499 (GII.12) |   |   |   | A |   |   |   |   | T |   |   |   |   |   |   | 2 |
| KC597139 (GII.17) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JN899245 (GII.21) |   |   |   | A |   |   |   |   |   |   |   |   |   |   |   | 1 |

| Primer     | NKII-R (reverse) |
|------------|-----------------|
|            | T   | C | G | A | C | G | C | A | T | C | T | T | C | A | C |
| X86557 (GII) |     |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JN797508 (GII.1) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| KF429769 (GII.2) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| KF306213 (GII.3) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JX459907 (GII.4) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| KC464499 (GII.12) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| KC597139 (GII.17) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
| JN899245 (GII.21) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 0 |
Table 4: Information of NV GI primer sets.

| Primers  | Sequence (5' -3') | Location | Region | Annealing temperature (°C) | Polarity | Reference |
|---------|-------------------|----------|--------|---------------------------|----------|-----------|
| NKI-F   | GTA AAT GAT GAT GGC GTC TAA | 5354–5373 | Capsid | 51 | + | This study |
| NKI-R   | ACC CAD CCA TTR TAC ATY TG | 5649–5668 | Capsid | 55 | – | |
| NKI-F2  | GAT GGC GTC TAA GGA CGC | 5363–5380 | Capsid | 51 | + | |
| GI-F1   | CTT GCC GAA TTY GTA AAT GAT GAT | 5342–5365 | Capsid | 55 | – | [26] |
| GI-R1   | CCA ACC CAR CCA TTR TAC ATY TG | 5649–5671 | Capsid | 55 | – | |
| GI-F2   | ATG ATG ATG GCG TCT AAG GAC GC | 5358–5380 | Capsid | 55 | – | |

| Primers  | Sequence (5' -3') | Location | Region | Annealing temperature (°C) | Polarity | Reference |
|---------|-------------------|----------|--------|---------------------------|----------|-----------|
| SRI-1   | CCA ACC CAR CCA TTR TAC AT | 5652–5671 | Capsid | 55 | – | |
| SRI-2   | AAA TGA TGA TGG GTG GTA T | 5356–5373 | Capsid | 55 | – | |
| SRI-3   | AAA AYR TCA CCG GGC GTA T | 5578–5596 | Capsid | 55 | – | |

aThe means of alphabet sequence are the following: Y = C, T; R = A, G; K = G, T; D = A, G, T.
bLocation is based on accession number M87661 (Norwalk virus).

Table 5: Information of NV GII primer sets.

| Primers  | Sequence (5' -3') | Location | Region | Annealing temperature (°C) | Polarity | Reference |
|---------|-------------------|----------|--------|---------------------------|----------|-----------|
| NKII-F  | CTY AGG CAR ATG TAC TGG ACY | 4805–4825 | RdRp   | 55 | + | This study |
| NKII-R  | TCG ACG CCA TCT TCA TTC AC | 5081–5100 | RdRp   | 55 | – | |
| NKII-R2 | GGA GCC AGA TTT CAG TCG C | 5060–5078 | RdRp   | 55 | – | |
| GII-F1  | GGG AGG GCC ATC GCA ATC T | 5049–5067 | RdRp   | 55 | – | |
| GII-R1  | CCR CCI GCA TRI CCR TTR TAC AT | 5367–5389 | Capsid | 55 | – | [26] |
| GII-F2  | TTT GCA ATG AAG ATG GCC TCG ART | 5079–5102 | Capsid | 55 | – | |
| SRII-1  | CGC CAT CTT CAT CCA A A | 5078–5096 | RdRp   | 55 | – | |
| SRII-2  | TWC TCT TTY TTY TAT GGT GAT GAT GA | 4583–4605 | RdRp   | 55 | – | [27] |
| SRII-3  | TTW CCA AAC CAA CWC GCT G | 4767–4785 | RdRp   | 55 | – | |

aThe means of alphabet sequence are the following: Y = C, T; R = A, G; I = inosine; W = A, T.
bLocation is based on accession number X86557 (Lordsdale virus).

Taiwan). For sequencing, PCR products were sent to Cosmo Genetech (Republic of Korea), and returned sequences were analyzed using the basic local alignment search tool (BLAST) in NCBI.

2.7. Statistical Analysis. An efficiency test of both GI primer sets (GI-F1/R1/F2, SRI-1/2/3, and NKI-F/R/F2) and GII primer sets (GII-F1/R1/F2, SRII-1/2/3, and NKII-F/R/R2) was statistically analyzed using SPSS 20.0 [30].

2.8. Nucleotide Sequence Registration. The NV GI and NV GII sequences that were submitted to GenBank in NCBI (http://www.ncbi.nlm.nih.gov/) were isolated from stool and groundwater samples.

3. Results

3.1. Selection of Primer Sets. Collected NV sequences (37 from NV GI, 52 from NV GII) were aligned using EditSeq and MegAlign in DNASTAR software. Results showed that conserved sequences were selected in ORF1 and ORF2.

From the 3 and 5 respective candidate NV GI and NV GII primer sets that were evaluated for efficiency using clinical samples, the most efficient primer sets were selected and used in this study (Table 3).

3.2. Detection of NV GI and GII. To evaluate the efficiency of the new primer sets (NKI-F/R/F2, NKI-F/R/R2) designed for this study, we used RT-PCR and compared them to conventional primer sets for NV GI (GI-F1/R1/F2, SRI-1/2/3) and GII (GII-F1/R1/F2, SRII-1/2/3). The ORF1 region was amplified by NKII-F/R/R2 and SRII-1/2/3, while the ORF2 region was amplified by NKI-F/R/R2, SRI-1/2/3, GI-F1/R1/F2, and GII-F1/R1/F2 (Figure 1).

For NV GI, 84 samples were confirmed with GI-F1/R1/F2, SRI-1/2/3, and NKI-F/R/R2 detecting 13, 6, and 22 samples, respectively. For NV GII, 134 samples were identified using GII-F1/R1/F2, SRII-1/2/3, and NKII-F/R/R2 to detect 31, 19, and 53 samples, respectively.

3.3. Comparison of Primer Sets. In total, 83 positive samples were identified, including 50 clinical (18 NV GI, 32 NV GII) and 33 environmental (6 NV GI, 27 NV GII) specimens. The most sensitive primer set for NV GI was NKI-F/R/R2, which detected 22/24 positive samples (91.7%). In comparison, the other primers detected 22/24 (91.7%) and 22/24 (91.7%) samples containing NV GI.

In detecting NV GII, the NKII primer set showed a superior diagnostic yield compared to the other sets. NKIIF/R/R2 identified 53/59 (89.3%) positive NV GII samples, whereas GII-F1/R1/F2 and SRII-1/2/3 identified 31/59 (52.5%) and
Table 6: Sensitivity of NV GI using each primer set.

| Positive specimens | NKI F/R/F2 | GI F/R/F2 | SRI 1/2/3 |
|--------------------|------------|-----------|-----------|
| GI.3               | 3          | 3         | —         |
| GI.4               | 6          | 2         | 4         |
| GI.6               | 5          | 4         | 1         |
| GI.8               | 1          | —         | 1         |
| GI.15              | 1          | —         | —         |
| GI                 | 1          | 1         | —         |
| Clinical samples   |            |           |           |
| (n = 18)           |            | 17 (94.4%)* | 10 (58.8%) | 6 (33.3%) |
| GI.3               | 4          | 3         | —         |
| GI.6               | 1          |           | —         |
| Environmental      |            |           |           |
| samples (n = 6)    |            | 5 (83.3%) | 3 (50.0%) | 0 (0%) |
| Total (n = 24)     |            | 22 (91.7%) | 13 (54.2%) | 6 (25.0%) |

All P values < 0.05 are by t-test except for environmental samples (GI F/R/F2 and NKI F/R/F2).

Environmental samples were not detected by SRI 1/2/3 primer.

* Percentage is NV detection/NV positive ratio.

Table 7: Sensitivity of NV GII using each primer set.

| Positive specimens | NKI F/R/R2 | GI F/R/F2 | SRI 1/2/3 |
|--------------------|------------|-----------|-----------|
| GII.2              | 2          | 1         | —         |
| GII.3              | 1          | —         | —         |
| GII.4              | 18         | 11        | 12        |
| GII.6              | 2          | 1         | —         |
| GII.13             | —          | 1         | —         |
| GII.16             | 1          | 1         | —         |
| GII.17             | 4          | 2         | —         |
| GII.21             | 1          | —         | —         |
| GII                | 1          | 1         | 1         |
| Clinical samples   |            |           |           |
| (n = 32)           |            | 30 (93.8%)* | 18 (56.3%) | 19 (59.4%) |
| GI.4               | 22         | 11        | —         |
| GII                | 1          | 2         | —         |
| Environmental      |            |           |           |
| samples (n = 27)   |            | 23 (85.7%) | 13 (48.1%) | 0 (0%) |
| Total (n = 59)     |            | 53 (89.3%) | 31 (52.5%) | 19 (32.2%) |

All P values < 0.05 are by t-test.

Environmental samples were not detected by SRII 1/2/3 primer set.

* Percentage is NV detection/NV positive ratio.

4. Discussion

In the USA, estimated 570–800 people die annually from outcomes associated with NV. Outbreaks of the virus occur regularly, and new variant strains are identified worldwide every 2 to 3 years [3, 21, 31, 32]. Methods for detection of NV include RT-PCR, the enzyme-linked immunosorbent assay (ELISA), and transmission electron microscopy (TEM) [33, 34]. In previous studies, virus concentrations up to 10^2 virus-copies/mL were detected using RT-PCR compared to 10^5 virus-copies/mL with other methods [33]. The widespread use of RT-PCR for NV detection can be attributed to its superior sensitivity compared to other methods [35, 36]. For example, it is the recommended method for detection of NV in contaminated water, in which NV commonly exists at 4.91×10^2–3.51×10^3 copies/mL [29]. However, in order to minimize outbreaks and prevent potential deaths, improved
primer sets are required to accurately detect original and variant strains of NV.

It is possible that the primer sets used for detection of NV are different in each laboratory because a standard method for detection has not yet been established [37]. In previously published work, the primer sets GI-F1/R1/F2 and GII-F1/R1/F2, SRI-1/2/3 and SRII-1/2/3 have typically been used to detect NV [38, 39]. Indeed, GI-F1/R1/F2 and GII-F1/R1/F2 are recommended for detection of NV by the Centers for Disease Control and Prevention’s (CDC) of Republic of Korea (http://www.cdc.go.kr/CDC/contents/CdcKrContentView.jsp?cid=18302&menuId=HOME001-MNU175MNU834-MNU0839). Lee et al. (2011) used SRI-1/2/3, SRII-1/2/3, GI-F1/R1/F2, and GII-F1/R1/F2 primer sets when testing water samples collected in 2008 near groundwater in Republic of Korea [40]. They found that 117 sites were contaminated with NV GI and GII, and the study indicated that NV could more efficiently be detected with GI-F1/R1/F2 (35 samples) and GII-F1/R1/F2 (55 samples) compared to SRI-1/2/3 (27 samples) and SRII-1/2/3 (41 samples) [40]. Similarly, in the present study, the performance of SRI-1/2/3 and SRIII-1/2/3 in detection of NV was inferior to other primer sets. In particular, SRI-1/2/3 and SRIII-1/2/3 entirely failed to detect NV in water. Conversely, the majority of positive NV samples were detected using NKI-F/R/F2 (91.7%) and NKII-F/R/R2 (89.3%). We postulate that because the sequences of NKI-F/R/F2 and NKII-F/R/R2 were collected from 1968 to 2013, their design contained more conserved sequences in comparison to the GI-F1/R1/F2, GII-F1/R1/F2, SRI, and SRII primer sets and this could explain their superior performance.

NV GI.4 is known to be the most prevalent strain of NV [41], and since 2012 a new variant of NV GI.4 has caused many cases of gastroenteritis [42]. In detecting NV GI.4, an RT-PCR assay using a GII-F1/R1/F2 primer set or another forward primer (Cog2F, F2 FB, GV21) with a GII-F1/R1/F2 reverse primer has typically been used [25, 42–44]. However, these primer sets were designed before 2005 and are not suitable for detection of new NV mutant types that have continuously emerged since, such as the 2012 Sydney strain. Their unsuitability is probably explained by differences in sequence between the primers and the new variant types [45, 46].

In this study, NKII-F/R/R2, GI-F1/R1/F2, and SRII were used to detect the NV GI.4 variant. In total, 17 NV GI variant samples were confirmed using NKII-F/R/R2, SRII-1/2/3, and GI-F1/R1/F2. They detected 16, 12, and 10 NV GI samples, respectively. The GI-F1/R1/F2 primer set has been widely used for detection of NV GI.4 [42–44, 47]. However, in previous research, the efficiency of this primer set for detecting NV GI.4 was relatively low [25, 41]. Therefore, GI-F1/R1/F2 may be an inappropriate method for detection of the NV GI.4 variant.

5. Conclusions

In our study, we showed that the NKi-F/R/F2 and NKII-F/R/R2 primer sets could be important for epidemiological diagnosis of NV in the laboratory because of their superior performance compared to other primer sets. Where contamination with NV was suspected, these primer sets could be applied to specimens taken from water sample with a low titer or clinical samples with a high titer. Additionally, our newly developed primer sets can be used to detect variant types of NV. Therefore, we recommend the use of NKi-F/R/F2 and NKII-F/R/R2 in future research.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] S. Maritschnik, E. E. Kanitz, E. Simons et al., “A food handler-associated, foodborne norovirus GI.4 Sydney 2012-outbreak following a wedding dinner, Austria, October 2012,” Food and Environmental Virology, vol. 5, no. 4, pp. 220–225, 2013.

[2] S. M. Griffin, N. E. Brinkman, E. J. Hedrick, E. R. Rhodes, and G. S. Fout, “Comparison of nucleic acid extraction and reverse transcription-qPCR approaches for detection of GI and GII noroviruses in drinking water,” Journal of Virological Methods, vol. 199, pp. 76–85, 2014.

[3] M. M. Patel, A. J. Hall, J. Vinje, and U. D. Parashar, “Noroviruses: a comprehensive review,” Journal of Clinical Virology, vol. 44, no. 1, pp. 1–8, 2009.

[4] K. Mattison, “Norovirus as a foodborne disease hazard,” Advances in Food and Nutrition Research, vol. 62, pp. 1–39, 2011.

[5] D.-P. Zheng, T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass, and S. S. Monroe, “Norovirus classification and proposed strain nomenclature,” Virology, vol. 346, no. 2, pp. 312–323, 2006.

[6] S. Svraka, E. Duizer, H. Vennema et al., “Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005,” Journal of Clinical Microbiology, vol. 45, no. 5, pp. 1389–1394, 2007.

[7] L. Barclay, G. W. Park, E. Vega et al., “Infection control for norovirus,” Clinical Microbiology and Infection, vol. 20, no. 8, pp. 731–740, 2014.

[8] K. Y. Green, T. Ando, M. S. Balayan et al., “Taxonomy of the caliciviruses,” The Journal of Infectious Diseases, vol. 181, supplement 2, pp. S322–S330, 2000.

[9] G. Belliot, S. V. Sosnovtsev, T. Mitra, C. Hammer, M. Garfield, and K. Y. Green, “In vitro proteolytic processing of the MD145 Norovirus ORF1 nonstructural polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells,” Journal of Virology, vol. 77, no. 20, pp. 10957–10974, 2003.

[10] M. E. Hardy and M. K. Estes, “Completion of the Norwalk virus genome sequence,” Virus Genes, vol. 12, no. 3, pp. 287–290, 1996.

[11] E. F. Donaldson, L. C. Lindesmith, A. D. Lobue, and R. S. Baric, “Viral shape-shifting: norovirus evasion of the human immune system,” Nature Reviews Microbiology, vol. 8, no. 3, pp. 231–241, 2010.
Y. Yan, H.-H. Wang, L. Gao et al., “A one-step multiplex real-time RT-PCR system for small round structured viruses and detection of enteric viruses in seafood,” *International Journal of Food Microbiology*, vol. 37, no. 1, pp. 27–36, 1997.

E. Wollants and M. van Ranst, “Detection of false positives with a commonly used Norovirus RT-PCR primer set,” *Journal of Clinical Virology*, vol. 56, no. 1, pp. 84–85, 2013.

L. Kittigul, A. Panjangampathanna, K. Pombubpa et al., “Detection and genetic characterization of norovirus in environmental water samples in Thailand,” *The Southeast Asian Journal of Tropical Medicine and Public Health*, vol. 43, no. 2, pp. 323–332, 2012.

S. M. Ahmed, B. A. Lopman, D. C. Payne et al., “Norovirus disease in the United States,” *Emerging Infectious Diseases*, vol. 19, no. 8, pp. 1198–1205, 2013.

L. Barclay, G. W. Park, E. Vega et al., “Infection control for norovirus,” *Clinical Microbiology and Infection*, vol. 20, no. 8, pp. 731–740, 2014.

H. F. Rabenau, M. Stürmer, S. Buxbaum, W. Preiser, and H. W. Doerr, “Laboratory diagnosis of norovirus: which method is the best?” *Interlaboratory*, vol. 46, no. 4, pp. 232–238, 2003.

J. Vinje, “Advances in laboratory methods for detection and typing of norovirus,” *Journal of Clinical Microbiology*, 2014.

E. de Bruin, E. Duizer, H. Vennema, and M. P. G. Koopmans, “Diagnosis of Norovirus outbreaks by commercial ELISA or RT-PCR,” *Journal of Virological Methods*, vol. 137, no. 2, pp. 259–264, 2006.

M. Swellam, M. S. Mahmoud, and A. A.-F. Ali, “Diagnosis of hepatitis C virus infection by enzyme-linked immunosorbent assay and reverse transcriptase-nested polymerase chain reaction: a comparative evaluation,” *IUBMB Life*, vol. 63, no. 6, pp. 430–434, 2011.

E. Guévermont, J. Brassard, A. Houde, C. Simard, and Y.-L. Trottier, “Development of an extraction and concentration procedure and comparison of RT-PCR primer systems for the detection of hepatitis A virus and norovirus GII in green onions,” *Journal of Virological Methods*, vol. 134, no. 1-2, pp. 130–135, 2006.

I. L. A. Boxman, J. J. H. C. Tilburg, N. A. J. M. Te Loeke et al., “Detection of noroviruses in shellfish in the Netherlands,” *International Journal of Food Microbiology*, vol. 108, no. 3, pp. 391–396, 2006.

H. K. Joung, S. H. Han, S.-J. Park et al., “ Nationwide surveillance for pathogenic microorganisms in groundwater near carcass burials constructed in South Korea in 2010,” *International Journal of Environmental Research and Public Health*, vol. 10, no. 12, pp. 7126–7143, 2013.

S.-G. Lee, W.-H. Jheong, C.-I. Suh et al., “ Nationwide surveillance of noroviruses in South Korea, 2008,” *Applied and Environmental Microbiology*, vol. 77, no. 4, pp. 1466–1474, 2011.

J.-G. Fu, J. Ai, X. Qi, J. Zhang, F.-Y. Tang, and Y.-F. Zhu, “Emergence of two novel norovirus genotype I1.4 variants associated with viral gastroenteritis in China,” *Journal of Medical Virology*, vol. 86, no. 7, pp. 1226–1234, 2014.

H. Mai, M. Jin, X. Guo et al., “Clinical and epidemiologic characteristics of norovirus GII.4 sydney during winter 2012–13 in Beijing, China following its global emergence,” *PLoS ONE*, vol. 8, no. 8, Article ID e71483, 2013.
[43] A. Thongprachum, W. Chan-it, P. Khamrin et al., “Molecular epidemiology of norovirus associated with gastroenteritis and emergence of norovirus GII.4 variant 2012 in Japanese pediatric patients,” Infection, Genetics and Evolution, vol. 23, pp. 65–73, 2014.

[44] J. Fonager, S. Barzinci, and T. K. Fischer, “Emergence of a new recombinant Sydney 2012 norovirus variant in Denmark, 26 December 2012 to 22 March 2013,” Eurosurveillance, vol. 18, no. 25, 2013.

[45] J.-S. Eden, M. M. Tanaka, M. F. Boni, W. D. Rawlinson, and P. A. White, “Recombination within the pandemic norovirus GII.4 lineage,” Journal of Virology, vol. 87, no. 11, pp. 6270–6282, 2013.

[46] V. Martella, M. C. Medici, S. de Grazia et al., “Evidence for recombination between pandemic GII.4 norovirus strains New Orleans 2009 and Sydney 2012,” Journal of Clinical Microbiology, vol. 51, no. 11, pp. 3855–3857, 2013.

[47] M. C. W. Chan, T. F. Leung, A. K. Kwok, N. Lee, and P. K. S. Chan, “Characteristics of patients infected with norovirus GII.4 Sydney 2012, Hong Kong, China,” Emerging Infectious Diseases, vol. 20, no. 4, pp. 558–661, 2014.