



Updating a New Semi-nested PCR Primer Pair for the Specific Detection of GII Norovirus in Oysters

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Abstract

Oysters are major transmission vectors of noroviruses (NoVs) in the environment. Outbreaks of NoVs are often associated with the consumption of NoV-contaminated oysters. Laboratory confirmation of suspected oyster samples is a critical step in the surveillance and control of NoVs. Because of non-specific amplification, false-positive results are frequently obtained by semi-nested RT-PCR with the presently widely used primer set (G2SKF/G2SKR). Here, a novel universal PCR primer set N (NG2OF/NG2OR) specific for genogroup II (GII) NoVs was designed based on all GII NoV sequences available in public databases. Specific products were obtained with the primer set N when the NoV-positive oysters, spiked with each of five representative genotypes of GII NoVs (GII.17, GII.13, GII.4, GII.3, and GII.12), were subjected to analyzing. No products were detected with the primer set N for the NoV-negative oysters, while the primer set C gave various non-specific bands. Twenty-three out of 156 fresh oyster samples were NoV-positive with both the primer set N and the classic primer set, while eight were NoV-positive solely with the primer set N. Compared with the classic primer set, the newly designed primer set N had a higher detection rate and improved specificity for GII NoVs in oyster samples. These results show that the novel PCR primer pair is specific and applicable for the detection of GII NoVs in oysters.

Keywords GII norovirus · Semi-nested RT-PCR · Primers · Oysters

Introduction

Noroviruses (NoVs) are one of the main causes of human non-bacterial acute gastroenteritis (AGE) (de Graaf et al., 2016). In children less than five years of age, NoVs account for about one-fifth of all AGE cases globally (Ahmed et al., 2014). NoVs belong to the family *Caliciviridae* (de Graaf et al., 2016; Robilotti et al., 2015). The NoV genome includes a single-stranded, positive-sense RNA, which

contains three open reading frames (ORFs): ORF1 encodes six non-structural proteins, including RNA-dependent RNA polymerase (RdRP); ORF2 encodes the major capsid protein (VP1); and ORF3 encodes the minor structural protein (VP2) (de Graaf et al., 2016; Vinjé, 2015). Currently, NoVs are divided into 10 genogroups (GI–GX). Five genogroups (GI, GII, GIV, GVIII, and GIX) infect humans (Chhabra et al., 2019). Among these, GII is the most prevalent human NoV worldwide (Mattison et al., 2018), including China (Qin et al., 2017; Yu et al., 2014).

Transmission of NoVs can be by diverse routes, including fecal–oral (through contaminated food or water), and direct or indirect contact with contaminated surfaces (de Graaf et al., 2016). Oysters are recognized as common transmission vectors of NoVs, and outbreaks are often associated with the consumption of NoV-contaminated oysters (Bitler et al., 2013; Mathijs et al., 2012). As filter feeders, oysters can accumulate NoVs from the surrounding water at concentrations as high as 10^3 – 10^7 genome copies per gram in digestive tissue (Sarmiento et al., 2020; Tan et al., 2018). More than 80% of human NoV genotypes have been detected in oyster samples or oyster-related NoV outbreaks (Yu et al.,

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2015). Eating raw or insufficiently heated oysters presents a potentially high risk for NoV infection (Alfano-Sobsey et al., 2012; Cheng et al., 2005). Oyster-related AGE has been documented worldwide (Iritani et al., 2014; McIntyre et al., 2012; Meghnath et al., 2019; Nenonen et al., 2009; Pu et al., 2018). Therefore, effective and accurate monitoring of NoV contamination in oyster samples is of great importance.

Laboratory confirmation of suspected oyster samples is a critical step in the surveillance and control NoV contamination. Among the molecular detection methods available for NoVs, real-time RT-PCR is relatively mature and highly sensitive for human samples (Jothikumar et al., 2005; Zhou et al., 2017); however, the resultant amplification products are too short (Anonymous, 2013; Kageyama et al., 2003) to be used for subsequent sequencing and genotyping, making investigation of NoV genotypes in oysters difficult. In addition, false-positives are common. With the development of next-generation sequencing, amplicon and transcriptomic sequencing have been increasingly used in oyster NoV research (Desdouts et al., 2020; Imamura et al., 2016a, 2016b; Strubbia et al., 2020). Metagenomic sequencing is time-consuming and costly, making it unsuitable for routine monitoring of NoVs in oysters. Nested RT-PCR, which amplifies target fragments over two rounds of PCR, each using different primers pairs, can improve the detection specificity for samples with low template concentrations (Medici et al., 2005). Two pairs of genogroup-specific primers were initially designed for the detection of GII noroviruses in human stool samples by using RT-PCR (G2SKF/G2SKR) (Kojima et al., 2002) and real-time RT-PCR (COG2F/COG2R) (Kageyama et al., 2003), respectively. Soon after, three of these four primers were combined in semi-nested RT-PCR (COG2F/G2SKR for RT-PCR and G2SKF/G2SKR for semi-nested PCR) to increase the detection sensitivity for oyster samples (Nishida et al., 2003), which were named as set C (C, the abbreviation for classic) in this study. Presently, these primer pairs have been widely used for routine detection of GII NoVs (Ji et al., 2020; Parrón et al., 2019; Zhang et al., 2020), including by semi-nested PCR methods (Bhavanam et al., 2020; Iritani et al., 2010; Ueki et al., 2005).

However, in our study, detection of NoV contamination in oysters by using the primer set C resulted in many non-specific PCR products (Figure S1), which were amplified from the DNAs of oysters and their intestinal bacteria (Table S1). Apparently, the specificity of the primer set C is interfered with by digestive tissue and intestinal bacterial community of oysters.

In this study, we sought to improve specificity for the detection of GII NoVs in oyster samples by designing a novel semi-nested RT-PCR primer set. Comparing with the classic primer set C, our newly designed primer set N (N, the abbreviation for new) can dramatically improve the detection specificity and detection rate for GII NoVs in commercial

oyster samples, and represents an alternative to better detect the contamination level of the GII NoVs in oysters.

Materials and Methods

Primer Design and In Silico Analysis

All NoV sequences available online were downloaded from NCBI nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide/>) on December 14, 2020 and then genotyped by using the Norovirus Typing Tool v.2.0 (<https://www.rivm.nl/mpf/typingtool/norovirus>). Sequences containing ORF1 only or having fewer than 1000 nucleotides (nt) were removed from analysis (Guo et al., 2018; Jia et al., 2020). The most abundant genotype was GII.4, with a sequence abundance of 52.7%. Conserved regions of GII.4 NoV sequences were identified using ClusterW (<https://www.geneious.com>). Primer candidates were designed based on these conserved regions (Fig. 1). Primer specificity was verified in silico using Primer-BLAST against the GenBank nucleotide database. Mismatches in conserved regions were evaluated using the program of Map primers (Geneious Primer version 2019.0.3) (Fig. 1).

Oyster Samples

Commercial oyster samples (*Crassostrea gigas*, body length of 9–12 cm, $n = 156$) were purchased fresh from the Luchaogang aquatic products market in Shanghai, China, from October 2020 to January 2021. The oyster samples were transported on ice, and dissected on the day of sampling.

RNA Extraction

The entire digestive gland tissue (approximately 400 mg) of each oyster was dissected and homogenized by using a Fast-Prep-24 (MP Biomedicals, USA) at a speed of 6 m/s for 30 s. Approximately, 50 mg of the homogenized digestive tissue was subjected to total RNA extraction using the Animal Tissue Total RNA Extraction Kit (Generay Biotech, Shanghai) according to the manufacturer's instructions. Extracted RNA was stored at -80°C until use.

Sensitivity and Specificity Evaluation of Primer Sets

Oyster digestive tissue was confirmed as NoV-negative by semi-nested RT-PCR with primer set C. These oysters were then used for primer sensitivity and specificity assessment. Firstly, digestive tissues were spiked, respectively, with NoV using NoV-positive stool samples containing five GII genotypes (GII.17, GII.13, GII.4, GII.3, and GII.12) frequently

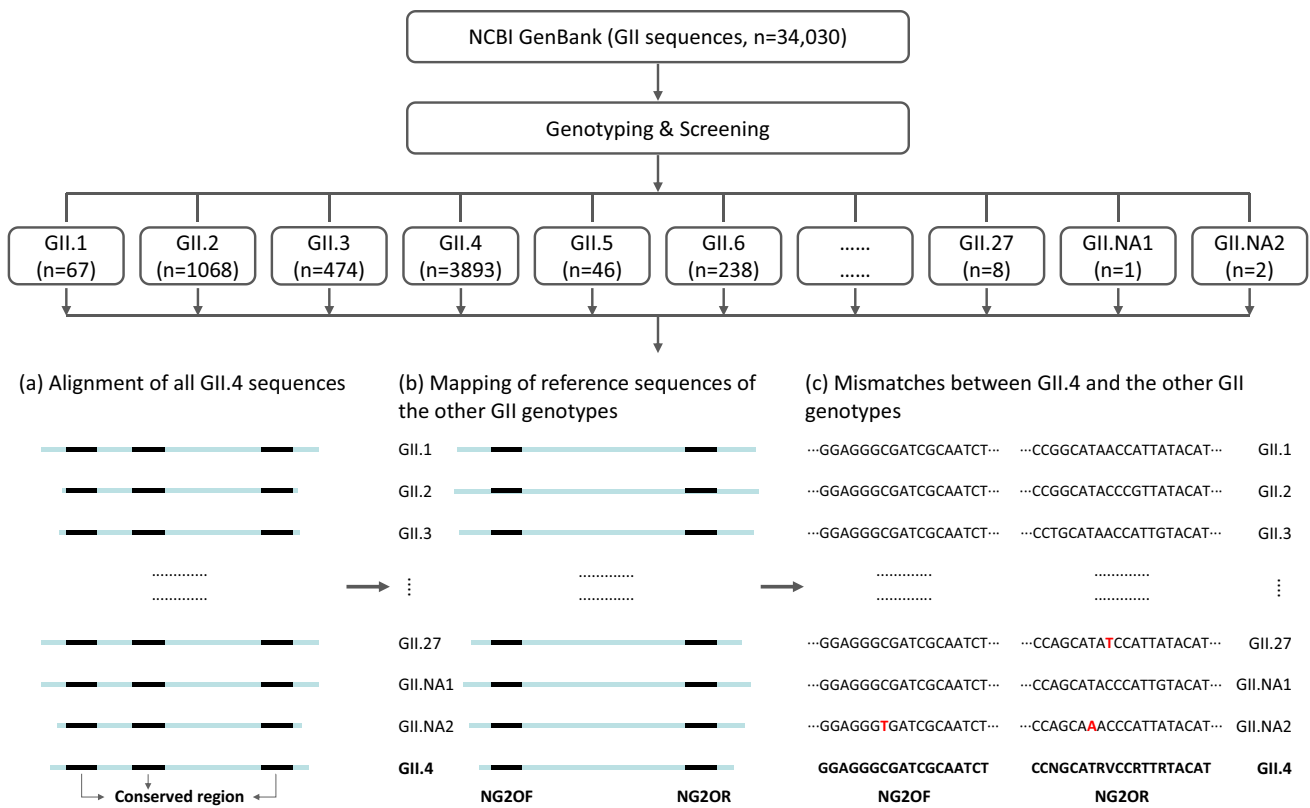


Fig. 1 Schematic of design and in silico evaluation of the semi-nested PCR primers for GII noroviruses. Removal of short sequences yielded 7385 sequences ranging from 1000 to 7618 nucleotides, which belong to GII.1 (*n*=67), GII.2 (*n*=1068), GII.3 (*n*=474), GII.4 (*n*=3893), GII.5 (*n*=46), GII.6 (*n*=238), GII.7 (*n*=42), GII.8 (*n*=49), GII.9 (*n*=25), GII.10 (*n*=8), GII.11 (*n*=8), GII.12 (*n*=66), GII.13 (*n*=105), GII.14 (*n*=53), GII.16 (*n*=20), GII.17 (*n*=1086), GII.18 (*n*=3), GII.19 (*n*=2), GII.20 (*n*=5), GII.21 (*n*=77), GII.22

(*n*=2), GII.23 (*n*=6), GII.24 (*n*=5), GII.25 (*n*=7), GII.26 (*n*=19), GII.27 (*n*=8), GII.NA1 (*n*=1), and GII.NA2 (*n*=2) based on capsid genotyping. **a** The GII.4 sequences were used for multiple sequence alignment analysis to find conserved regions. **b** The conserved regions of GII.4 were mapped to the reference sequences of each genotype to select the best primers. **c** Mismatches between the primer set N and the reference sequences of each genotype were identified and are in red bold text

detected in oysters. Digestive tissue was contaminated at a rate of 10⁵ genome copies of NoVs per 50 mg of tissue. Secondly, stool samples containing rotavirus, sapovirus, astrovirus, or GI.4 NoV, were added to NoV-negative oyster digestive tissues as controls.

Semi-nested RT-PCR

RT-PCR (first-round) was performed in a reaction volume of 25 μL using a One Step RT-PCR Kit (Vazyme Biotech, Nanjing) according to the manufacturer’s protocol (Table 1). Reaction conditions were as follows: 50 °C for 30 min; 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The RT-PCR products were diluted ten times and used as templates for semi-nested PCR (Table 1). The second round was also carried out in a volume of 25 μL using a 2 × Taq Master Mix Kit (Novoprotein, Shanghai) according to the manufacturer’s manual. Reaction conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s,

55 °C for 30 s, and 72 °C for 30 s; hold at 72 °C for 15 min. The amplified products were analyzed by electrophoresis on a 2% agarose gel. The expected sizes of PCR products of the primer set C and primer set N were 344 and 337 bp, respectively.

Results

Primer Design and In Silico Analysis

The new primer set N (RT-PCR primers COG2F/NG2OR, semi-nested PCR primers NG2OF/NG2OR) was designed based on conserved regions of the GII.4 NoV genome. The primer sets C and N are shown in Table 1. When the primers were aligned with the 28 GII reference sequences, 13 and 19 mismatches were observed for the primer set N and primer set C, respectively (Fig. 2). These results indicate that both primer sets are specific for GII NoVs in silico.

Table 1 Semi-nested RT-PCR primer sets used for detection of GII noroviruses

	PCR round	Primer	Sequence ^c (5' → 3')	Position ^d	Product size (bp)
Primer set C	RT-PCR (1st round)	COG2F ^a	CARGARBCNATGTTYAGRTGGATGAG	4989	387
		G2SKR ^b	CCRCCNGCATRHCCRTTTRTACAT	5375	
	Semi-nested PCR (2nd round)	G2SKF ^b	CNTGGGAGGGCGATCGCAA	5032	344
		G2SKR	CCRCCNGCATRHCCRTTTRTACAT	5375	
Primer set N	RT-PCR (1st round)	COG2F	CARGARBCNATGTTYAGRTGGATGAG	4989	384
		NG2OR	CCNGCATRVCCRTTTRTACAT	5372	
	Semi-nested PCR (2nd round)	NG2OF	GGAGGGCGATCGCAATCT	5036	337
		NG2OR	CCNGCATRVCCRTTTRTACAT	5372	

^aKageyama et al. (2003)

^bKojima et al. (2002)

^cDegenerate bases are in bold. R = A, G; B = C, G, T; N = A, G, C, T; Y = C, T; H = A, C, T; V = A, G, C

^dNucleotide position for 5' end of primers and numbers refer to MG786781.1 (accession no.)

Accession no.	Genotype	Primer Set C		No. of mismatch	Primer Set N		No. of mismatch
		G2SKF	G2SKR		NOG2F	NOG2R	
U07611.2	GII.1[P1]	CNTGGGAGGGCGATCGCAA	CCRCCNGCATRHCCRTTTRTACAT	0	GGAGGGCGATCGCAATCT	CCNGCATRVCCRTTTRTACAT	0
X81879.1	GII.2[P2]	-----	-----	0	-----	-----	0
U02030.1	GII.3[P3]	-----	-----	0	-----	-----	0
X76716.1	GII.4[P4]	-----	-----	0	-----	-----	0
KJ196288.1	GII.5[P5]	-----	-----	0	-----	-----	0
JX846927.1	GII.6[P7]	-----	-----	0	-----	-----	0
MH279833.1	GII.7[P7]	-----	G -----	1	-----	-----	0
AB039780.1	GII.8[P8]	-----	-----	0	-----	-----	0
AY038599.2	GII.9[P7]	-----	-----	0	-----	-----	0
AF504671.2	GII.10[P12]	-----	C ----- G -----	2	-----	-----	0
AB074893.1	GII.11[PNA4]	-----	-----	0	-----	-----	0
AB045603.2	GII.12[P12]	-----	-----	0	-----	-----	0
KJ196276.1	GII.13[P12]	-----	T -----	1	-----	-----	0
KM289171.1	GII.14[P14]	-----	T ----- G -----	2	-----	-----	0
AY502010.1	GII.16[P16]	-----	-----	0	-----	-----	0
AY502009.1	GII.17[P16]	-----	-----	0	-----	-----	0
AY823304.1	GII.18[P18]	-----	G -----	1	-----	-----	0
AY823306.1	GII.19[P11]	-----	C ----- G -----	2	-----	-----	0
EU072235.2	GII.20[P20]	-----	T -----	1	-----	-----	0
EU019230.2	GII.21[P21]	-----	T -----	1	-----	-----	0
AB083780.1	GII.22[P22]	-----	----- A -----	1	-----	-----	0
KT290889.1	GII.23[P23]	-----	-----	0	-----	-----	0
KY225989.1	GII.24[P24]	----- T -----	C ----- G -----	2	----- T -----	----- T -----	2
GQ856469.1	GII.25[P38]	-----	-----	1	-----	-----	0
KU306738.1	GII.26[P26]	-----	T -----	1	-----	-----	0
MG495077.1	GII.27[P27]	-----	-----	0	-----	-----	0
MG495079.1	GII.NA1[PNA1]	-----	-----	0	-----	----- T -----	1
MG706448.1	GII.NA2[PNA2]	----- T -----	C ----- A -----	3	----- T -----	----- A -----	2

Fig. 2 Mismatches between the semi-nested PCR primer sets and 28 GII norovirus reference sequences. Mismatches are in red bold text (Color figure online)

Sensitivity Evaluation

As shown in Fig. 3B, both primer sets gave the expected products in semi-nested RT-PCR of oyster tissue artificially contaminated with NoV genotypes GII.17, GII.13, GII.4, GII.3, and GII.12. Sequencing results confirmed that all the specific products were consistent with their corresponding genotypes, indicating similarity in the sensitivity of these two primer sets. However, the bands of GII.4 and GII.3 produced by the primer set N are weaker than that by the

primer set C, suggesting that the amplification efficiency of the primer set N in detecting GII.4 and GII.3 may be lower.

Specificity Evaluation

Semi-nested RT-PCR using the primer set N gave no obvious bands with any of the control samples (rotavirus, sapovirus, astrovirus, or GI.4 NoV) and NoV-negative oyster samples, and the background of the gel was relatively clean (Fig. 3A, C). In contrast, the primer set C gave various non-specific bands (100–1000 bp), including one near the expected size

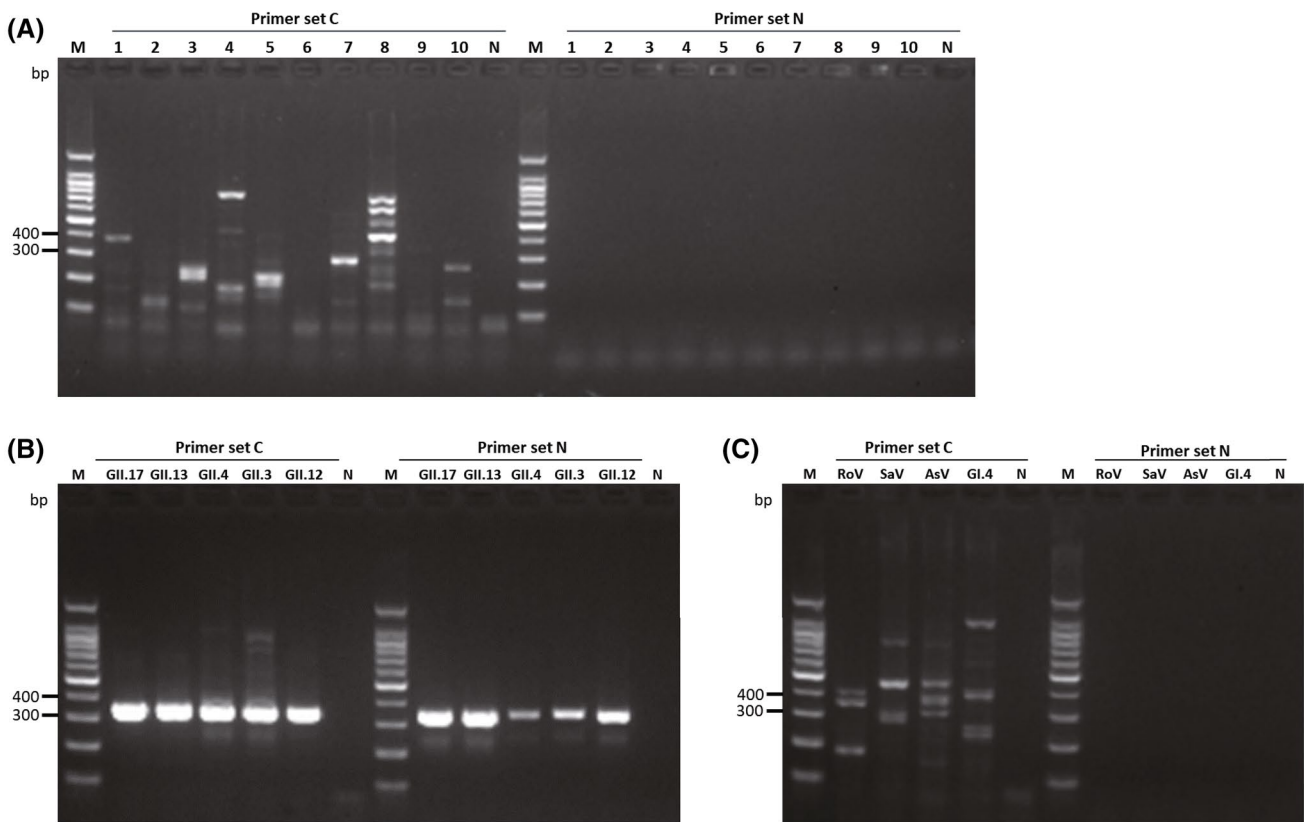


Fig. 3 Experimental evaluation of the performance of semi-nested RT-PCR primer sets. **A** Detection of norovirus-negative oyster samples. 1–10: Norovirus-negative oyster samples. **B** Sensitivity assess-

ment based on five representative GII noroviruses. **C** Specificity assessment based on other viruses. *RoV* rotavirus, *SaV* sapovirus, *AsV* astrovirus, *GI.4* norovirus, *M* 100 bp DNA ladder, *N* negative control

(344 bp). The non-specific amplicons (false-positive bands) were recovered, cloned, and sequenced; sequencing results based on BLASTN confirmed that these amplicons originated from the genome of oysters or their intestinal bacteria (Figure S1, Table S1). Taken together, these results indicate that the primer set N is highly specific, while the primer set C yields non-specific false-positive amplifications.

Detection in Oyster Samples

A total of 156 oyster samples were detected by semi-nested RT-PCR using the primer set C and set N. The detection rate of NoV-positive sample was higher with the primer set N (19.87%, 31/156; Table 2) than with the primer set C (14.74%,

23/156; Table 2). Twenty-three samples were NoV-positive with two primer sets, while eight were NoV-positive solely with the primer set N. Further sequencing confirmed that all eight samples were NoV-positive with three genotypes including GII.2 (*n* = 2), GII.3 (*n* = 4), and GII.17 (*n* = 2) (Table S2). These results suggest the higher detection efficiency of the primer set N compared with that of the primer set C.

Discussion

NoVs are genetically diverse RNA viruses, and thousands of NoV sequences have emerged in recent years (Chhabra et al., 2019; de Graaf et al., 2016; Jia et al., 2020). In 2013, 8951

Table 2 Detection of oyster samples (*n* = 156) for GII noroviruses by the semi-nested RT-PCR

Primer	No. of the negative	No. of the positive						
		GII.12	GII.17	GII.2	GII.3	GII.3, GII.4 ^a	GII.4	Total
Set C	133	5	ND	1	7	1	9	23
Set N	125	5	2	3	11	1	9	31

ND not detected

^aDetected in a single oyster sample

NoV sequences were available from the NCBI database. At that time, NoVs were divided into at least six genogroups (GI–GVI) using phylogenetic clustering of the complete VP1 amino acid sequences (Kroneman et al., 2013). Soon afterward, the number of NoV sequences has increased dramatically due to the rapid evolution of the viral genome by genetic mutation and recombination (de Graaf et al., 2016). By 2019, the number of NoV sequences reached 45,542, of which, over 68.8% ($n = 31,343$) belonged to GII (available data from NCBI). The classification of NoVs was then updated into 10 genogroups (GI–GX), which were further divided into at least 49 capsid genotypes and 60 P-types, based on the diversity of VP1 and RdRP, respectively (Chhabra et al., 2019). Therefore, it is very urgent to update primers for the specific detection of NoVs in oysters.

In our previous study, we re-designed a new universal primer set of nested RT-PCR for the specific detection of GI NoVs in oyster samples (Guo et al., 2018). Our results indicate that the new nested primers (NGIOF/NGIOR) are much higher specific than the classic primers (GISKF/GISKR) (Guo et al., 2018; Kojima et al., 2002).

In this study, non-specific amplification was also observed when oyster samples were analyzed for GII NoV contamination with the presently widely used primer set C (Figure S1, Table S1). Accordingly, the new primer set N for the specific detection of GII NoVs was designed based on the conserved regions of all GII.4 sequences available in public databases (Fig. 1). Using *in silico* analysis, the new primer set N was found to specifically bind with all 28 GII genotypes. Compared with the classic primer set C, the primer set N possesses fewer mismatches with the reference sequences (Fig. 2). Moreover, the primer set N had minimal degeneracy and no degenerate sites near the 3' terminus of either primer, suggesting low amplification bias. Furthermore, unlike the primer set C, the primer set N gave no obvious non-specific amplicons with oyster samples (Fig. 3A). Several differences are notable between the two primer sets (Table 1). The primer set N has five degenerate bases versus seven in the primer set C. The reverse primer NG2OR (20 nt) is shorter than G2SKR (23 nt), uses the degenerate base “V” instead of “H” in G2SKR at the same position, and avoids the third degenerate base “R” at the 5' end of G2SKR (Table 1). In addition, the forward primer NG2OF avoids the second degenerate base “N” at the 5' end of G2SKF. These differences may be responsible for the observed higher binding specificity of the primer set N for GII NoVs, especially under a low viral load. When GII NoV templates were present in abundance, both primer sets were observed to preferentially bind with NoV cDNA and specifically amplify the target sequences, with no obvious non-specific amplifications (Fig. 3B).

For the artificially contaminated oyster samples, the amplification efficiency of the primer set N in detecting GII.4 and GII.3 NoVs may be lower than that of the primer set C (Fig. 3B). However, comprehensive assessment showed that the primer set N had a higher detection rate for genotypes GII.2, GII.3, and GII.17 than the primer set C (Table 2). Coincidentally, novel variants of two of these genotypes have recently emerged (GII.P17-GII.17 during the 2014–2015 season, and GII.P16-GII.2 during the 2016–2017 season), causing notable increases in AGE outbreaks worldwide (Ao et al., 2017; Gao et al., 2019; Lu et al., 2016; Rasmussen et al., 2016). This suggests that, as novel variants of NoVs continue to emerge, primers had to be updated regularly to ensure specific and sensitive detection.

Interestingly, in this study, the detection rate of NoVs is 19.87% (31/156; Table 2) for the 156 oysters collected from October 2020 to January 2021, which is slightly lower than that of the samples checked at other times. This probably results from the stringent policies regarding to the prevention and control of the epidemic of COVID-19 in China. Limitation of direct or indirect contact resulted in the significant decline of COVID-19 and simultaneously hampered the spreading of NoVs as well (Ahn et al., 2021; Eigner et al., 2021; Farah et al., 2021; Lennon et al., 2020).

In summary, the established semi-nested RT-PCR with updated primer sets bearing higher specificity and sensitivity is highly desirable and satisfies the urgent needs of surveying and genotyping of GII NoV contamination in oysters.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12560-022-09511-6>.

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Author Contributions YW conceived and designed this study. YY and TJ downloaded the sequences and designed the primers. TJ and LD performed the sampling and experiments. LD and YY wrote the manuscript. YW modified the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

- Ahmed, S. M., Hall, A. J., Robinson, A. E., Verhoef, L., Premkumar, P., Parashar, U. D., Koopmans, M., & Lopman, B. A. (2014). Global prevalence of norovirus in cases of gastroenteritis: A systematic review and meta-analysis. *The Lancet Infectious Diseases*, *14*(8), 725–730. [https://doi.org/10.1016/S1473-3099\(14\)70767-4](https://doi.org/10.1016/S1473-3099(14)70767-4)
- Ahn, S. Y., Park, J. Y., Lim, I. S., Chae, S. A., Yun, S. W., Lee, N. M., Kim, S. Y., Choi, B. S., & Yi, D. Y. (2021). Changes in the occurrence of gastrointestinal infections after COVID-19 in Korea. *Journal of Korean Medical Science*, *36*(24), e180. <https://doi.org/10.3346/jkms.2021.36.e180>
- Alfano-Sobsey, E., Sweat, D., Hall, A., Breedlove, F., Rodriguez, R., Greene, S., Pierce, A., Sobsey, M., Davies, M., & Ledford, S. L. (2012). Norovirus outbreak associated with undercooked oysters and secondary household transmission. *Epidemiology and Infection*, *140*(2), 276–282. <https://doi.org/10.1017/S0950268811000665>
- Anonymous, P. (2013). Microbiology of food and animal feed: Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR: Part 2: Method for qualitative detection (ISO/TS 15216-2:2013).
- Ao, Y., Wang, J., Ling, H., He, Y., Dong, X., Wang, X., Peng, J., Zhang, H., Jin, M., & Duan, Z. (2017). Norovirus GII.P16/GII.2-associated gastroenteritis, China, 2016. *Emerging Infectious Diseases*, *23*(7), 1172–1175. <https://doi.org/10.3201/eid2307.170034>
- Bhavanam, S., Freedman, S. B., Lee, B. E., Zhuo, R., Qiu, Y., Chui, L., Xie, J., Ali, S., Vanderkooi, O. G., Pang, X. L., The Alberta Provincial Pediatric Enteric Infection Team A. (2020). Differences in illness severity among circulating norovirus genotypes in a large pediatric cohort with acute gastroenteritis. *Microorganisms*, *8*(12), 1873. <https://doi.org/10.3390/microorganisms8121873>
- Bitler, E. J., Matthews, J. E., Dickey, B. W., Eisenberg, J. N. S., & Leon, J. S. (2013). Norovirus outbreaks: A systematic review of commonly implicated transmission routes and vehicles. *Epidemiology and Infection*, *141*(8), 1563–1571. <https://doi.org/10.1017/S095026881300006X>
- Cheng, P. K. C., Wong, D. K. K., Chung, T. W. H., & Lim, W. W. L. (2005). Norovirus contamination found in oysters worldwide. *Journal of Medical Virology*, *76*(4), 593–597.
- Chhabra, P., de Graaf, M., Parra, G. I., Chan, M. C., Green, K., Martella, V., Wang, Q., White, P. A., Katayama, K., Vennema, H., Koopmans, M. P. G., & Vinje, J. (2019). Updated classification of norovirus genogroups and genotypes. *Journal of General Virology*, *100*(10), 1393–1406. <https://doi.org/10.1099/jgv.0.001318>
- de Graaf, M., van Beek, J., & Koopmans, M. P. G. (2016). Human norovirus transmission and evolution in a changing world. *Nature Reviews Microbiology*, *14*(7), 421–433. <https://doi.org/10.1038/nrmicro.2016.48>
- Desdouts, M., Wacrenier, C., Ollivier, J., Schaeffer, J., & Guyader, F. S. L. (2020). A targeted metagenomics approach to study the diversity of norovirus GII in shellfish implicated in outbreaks. *Viruses*, *12*(9), 978. <https://doi.org/10.3390/v12090978>
- Eigner, U., Verstraeten, T., & Weil, J. (2021). Decrease in norovirus infections in Germany following COVID-19 containment measures. *The Journal of Infection*, *82*(6), 276–316. <https://doi.org/10.1016/j.jinf.2021.02.012>
- Farah, Y., Syed Hasan, A., & Irfan, U. (2021). Norovirus outbreak amid COVID-19 in the United Kingdom; priorities for achieving control. *Journal of Medical Virology*. <https://doi.org/10.1002/jmv.27426>
- Gao, Z., Liu, B., Yan, H., Li, W., Jia, L., Tian, Y., Chen, Y., Wang, Q., & Pang, X. (2019). Norovirus outbreaks in Beijing, China, from 2014 to 2017. *The Journal of Infection*, *79*(2), 159–166. <https://doi.org/10.1016/j.jinf.2019.05.019>
- Guo, P., Yu, Y., Pan, Y., Yan, S., & Wang, Y. (2018). Design and evaluation of nested PCR primers for specific detection of genogroup I noroviruses in oysters. *Molecular and Cellular Probes*, *40*, 40–43. <https://doi.org/10.1016/j.mcp.2018.06.002>
- Imamura, S., Haruna, M., Goshima, T., Kanezashi, H., Okada, T., & Akimoto, K. (2016a). Application of next-generation sequencing to evaluate the profile of noroviruses in pre- and post-depurated oysters. *Foodborne Pathogens and Disease*, *13*(10), 559–565.
- Imamura, S., Haruna, M., Goshima, T., Kanezashi, H., Okada, T., & Akimoto, K. (2016b). Application of next-generation sequencing to investigation of norovirus diversity in shellfish collected from two coastal sites in Japan from 2013 to 2014. *The Japanese Journal of Veterinary Research*, *64*(2), 113–122.
- Iritani, N., Kaida, A., Abe, N., Kubo, H., Sekiguchi, J.-I., Yamamoto, S. P., Goto, K., Tanaka, T., & Noda, M. (2014). Detection and genetic characterization of human enteric viruses in oyster-associated gastroenteritis outbreaks between 2001 and 2012 in Osaka City, Japan. *Journal of Medical Virology*, *86*(12), 2019–2025. <https://doi.org/10.1002/jmv.23883>
- Iritani, N., Kaida, A., Kubo, H., Abe, N., Goto, K., Ogura, H., & Seto, Y. (2010). Molecular epidemiology of noroviruses detected in seasonal outbreaks of acute nonbacterial gastroenteritis in Osaka City, Japan, from 1996–1997 to 2008–2009. *Journal of Medical Virology*, *82*(12), 2097–2105. <https://doi.org/10.1002/jmv.21915>
- Ji, L., Hu, G., Xu, D., Wu, X., Fu, Y., & Chen, L. (2020). Molecular epidemiology and changes in genotype diversity of norovirus infections in acute gastroenteritis patients in Huzhou, China, 2018. *Journal of Medical Virology*. <https://doi.org/10.1002/jmv.26247>
- Jia, T., Yu, Y., & Wang, Y. (2020). A recombinase polymerase amplification-based lateral flow strip assay for rapid detection of genogroup II noroviruses in the field. *Archives of Virology*, *165*(12), 2767–2776. <https://doi.org/10.1007/s00705-020-04798-x>
- Jothikumar, N., Lowther, J. A., Henshilwood, K., Lees, D. N., Hill, V. R., & Vinjé, J. (2005). Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Applied and Environmental Microbiology*, *71*(4), 1870–1875.
- Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., Takeda, N., & Katayama, K. (2003). Broadly reactive and highly sensitive assay for norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology*, *41*(4), 1548–1557. <https://doi.org/10.1128/jcm.41.4.1548-1557.2003>
- Kojima, S., Kageyama, T., Fukushi, S., Hoshino, F. B., Shinohara, M., Uchida, K., Natori, K., Takeda, N., & Katayama, K. (2002). Genogroup-specific PCR primers for detection of Norwalk-like viruses. *Journal of Virological Methods*, *100*(1–2), 107–114.
- Kroneman, A., Vega, E., Vennema, H., Vinjé, J., White, P. A., Hansman, G., Green, K., Martella, V., Katayama, K., & Koopmans, M. (2013). Proposal for a unified norovirus nomenclature and genotyping. *Archives of Virology*, *158*(10), 2059–2068. <https://doi.org/10.1007/s00705-013-1708-5>
- Lennon, R. P., Griffin, C., Miller, E. L., Dong, H., Rabago, D., & Zgierska, A. E. (2020). Norovirus infections drop 49% in the United States with strict COVID-19 public health interventions. *Acta Medica Academica*, *49*(3), 278–280. <https://doi.org/10.5644/ama2006-124.317>
- Lu, J., Fang, L., Zheng, H., Lao, J., Yang, F., Sun, L., Xiao, J., Lin, J., Song, T., Ni, T., Raghwani, J., Ke, C., Faria, N. R., Bowden, T. A., Pybus, O. G., & Li, H. (2016). The evolution and transmission of epidemic GII.17 Noroviruses. *The Journal of Infectious Diseases*, *214*(4), 556–564. <https://doi.org/10.1093/infdis/jiw208>

- Mathijs, E., Stals, A., Baert, L., Botteldoorn, N., Denayer, S., Mauroy, A., Scipioni, A., Daube, G., Dierick, K., Herman, L., Van Coillie, E., Uyttendaele, M., & Thiry, E. (2012). A review of known and hypothetical transmission routes for noroviruses. *Food and Environmental Virology*, 4(4), 131–152. <https://doi.org/10.1007/s12560-012-9091-z>
- Mattison, C. P., Cardemil, C. V., & Hall, A. J. (2018). Progress on norovirus vaccine research: Public health considerations and future directions. *Expert Review of Vaccines*, 17(9), 773–784. <https://doi.org/10.1080/14760584.2018.1510327>
- McIntyre, L., Galanis, E., Mattison, K., Mykytczuk, O., Buenaventura, E., Wong, J., Prystajek, N., Ritson, M., Stone, J., Moreau, D., & Youssef, A. (2012). Multiple clusters of norovirus among shellfish consumers linked to symptomatic oyster harvesters. *Journal of Food Protection*, 75(9), 1715–1720. <https://doi.org/10.4315/0362-028X.JFP-12-113>
- Medici, M. C., Martinelli, M., Ruggeri, F. M., Abelli, L. A., Bosco, S., Arcangeletti, M. C., Pinardi, F., De Conto, F., Calderaro, A., Chezzi, C., & Dettori, G. (2005). Broadly reactive nested reverse transcription-PCR using an internal RNA standard control for detection of noroviruses in stool samples. *Journal of Clinical Microbiology*, 43(8), 3772–3778.
- Meghnath, K., Hasselback, P., McCormick, R., Prystajek, N., Taylor, M., McIntyre, L., Man, S., Whitfield, Y., Warshawsky, B., McKinley, M., Bitzikos, O., Hexemer, A., & Galanis, E. (2019). Outbreaks of norovirus and acute gastroenteritis associated with British Columbia oysters, 2016–2017. *Food and Environmental Virology*, 11(2), 138–148. <https://doi.org/10.1007/s12560-019-09374-4>
- Nenonen, N. P., Hannoun, C., Olsson, M. B., & Bergström, T. (2009). Molecular analysis of an oyster-related norovirus outbreak. *Journal of Clinical Virology*, 45(2), 105–108. <https://doi.org/10.1016/j.jcv.2009.04.011>
- Nishida, T., Kimura, H., Saitoh, M., Shinohara, M., Kato, M., Fukuda, S., Munemura, T., Mikami, T., Kawamoto, A., Akiyama, M., Kato, Y., Nishi, K., Kozawa, K., & Nishio, O. (2003). Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. *Applied and Environmental Microbiology*, 69(10), 5782–5786. <https://doi.org/10.1128/aem.69.10.5782-5786.2003>
- Parrón, I., Álvarez, J., Jané, M., Cornejo Sánchez, T., Razquin, E., Guix, S., Camps, G., Pérez, C., & Domínguez, Á. (2019). A food-borne norovirus outbreak in a nursing home and spread to staff and their household contacts. *Epidemiology and Infection*, 147, e225. <https://doi.org/10.1017/S0950268819001146>
- Pu, J., Miura, T., Kazama, S., Kōta, Y., Azraini, N. D., Ito, E., Ito, H., Omura, T., & Watanabe, T. (2018). Weekly variations in norovirus genogroup II genotypes in Japanese oysters. *International Journal of Food Microbiology*, 284, 48–55. <https://doi.org/10.1016/j.ijfoodmicro.2018.06.027>
- Qin, S. W., Chan, T. C., Cai, J., Zhao, N., Miao, Z. P., Chen, Y. J., & Liu, S. L. (2017). Genotypic and epidemiological trends of acute gastroenteritis associated with noroviruses in China from 2006 to 2016. *International Journal of Environmental Research and Public Health*, 14(11), 1341. <https://doi.org/10.3390/ijerph14111341>
- Rasmussen, L. D., Schultz, A. C., Uhrbrand, K., Jensen, T., & Fischer, T. K. (2016). Molecular evidence of oysters as vehicle of norovirus GII.P17-GII.17. *Emerging Infectious Diseases*, 22(11), 2024–2025. <https://doi.org/10.3201/eid2211.161171>
- Robilotti, E., Deresinski, S., & Pinsky, B. A. (2015). Norovirus. *Clinical Microbiology Reviews*, 28(1), 134–164. <https://doi.org/10.1128/CMR.00075-14>
- Sarmento, S. K., Guerra, C. R., Malta, F. C., Coutinho, R., Miagostovich, M. P., & Fumian, T. M. (2020). Human norovirus detection in bivalve shellfish in Brazil and evaluation of viral infectivity using PMA treatment. *Marine Pollution Bulletin*, 157, 111315. <https://doi.org/10.1016/j.marpolbul.2020.111315>
- Strubbia, S., Schaeffer, J., Besnard, A., Wacrenier, C., Le Menec, C., Garry, P., Desdoutis, M., & Le Guyader, F. S. (2020). Metagenomic to evaluate norovirus genomic diversity in oysters: Impact on hexamer selection and targeted capture-based enrichment. *International Journal of Food Microbiology*, 323, 108588. <https://doi.org/10.1016/j.ijfoodmicro.2020.108588>
- Tan, D. M., Lyu, S. L., Liu, W., Zeng, X. Y., Lan, L., Qu, C., Zhuge, S. Y., Zhong, Y. X., Xie, Y. H., & Li, X. G. (2018). Utility of droplet digital PCR assay for quantitative detection of norovirus in shellfish, from production to consumption in Guangxi, China. *Biomedical and Environmental Sciences*, 31(10), 713–720. <https://doi.org/10.3967/bes2018.096>
- Ueki, Y., Sano, D., Watanabe, T., Akiyama, K., & Omura, T. (2005). Norovirus pathway in water environment estimated by genetic analysis of strains from patients of gastroenteritis, sewage, treated wastewater, river water and oysters. *Water Research*, 39(18), 4271–4280. <https://doi.org/10.1016/j.watres.2005.06.035>
- Vinje, J. (2015). Advances in laboratory methods for detection and typing of norovirus. *Journal of Clinical Microbiology*, 53(2), 373–381. <https://doi.org/10.1128/JCM.01535-14>
- Yu, Y., Cai, H., Hu, L., Lei, R., Pan, Y., Yan, S., & Wang, Y. (2015). Molecular epidemiology of oyster-related human noroviruses and their global genetic diversity and temporal-geographical distribution from 1983 to 2014. *Applied and Environmental Microbiology*, 81(21), 7615–7624. <https://doi.org/10.1128/AEM.01729-15>
- Yu, Y., Yan, S., Li, B., Pan, Y., & Wang, Y. (2014). Genetic diversity and distribution of human norovirus in China (1999–2011). *BioMed Research International*, 2014, 1–13. <https://doi.org/10.1155/2014/196169>
- Zhang, X. F., Chen, J. R., Song, C. L., Xie, D. J., Tan, M., Wang, L., Koroma, M. M., Hou, Y. Z., Dong, Z. P., Yu, J. R., Duan, W. T., Zhao, D. D., Du, J. R., Zhu, L., & Dai, Y. C. (2020). Characterization of a hospital-based gastroenteritis outbreak caused by GII6 norovirus in Jinshan, China. *Epidemiology and Infection*, 148, e289. <https://doi.org/10.1017/S0950268820002538>
- Zhou, Z., Tian, Z., Li, Q., Tian, P., Wu, Q., Wang, D., & Shi, X. (2017). Capture RT-qPCR: A new simple and sensitive method to detect human norovirus in oysters. *Frontiers in Microbiology*, 8, 554. <https://doi.org/10.3389/fmicb.2017.00554>

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