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Design and evaluation of nested PCR primers for specific detection of genogroup I noroviruses in oysters

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ABSTRACT

A pair of nested PCR universal primers (NGIOF and NGIOR) specific for genogroup I (GI) noroviruses was designed based on all GI sequences available in public databases. The primers were evaluated for their specificity, sensitivity and coverage, which demonstrate their reliable performance upon detection of GI noroviruses in oysters.

Norovirus, a member of the family *Caliciviridae*, is the major cause of acute non-bacterial gastroenteritis [3,5]. The small icosahedral virus has a single-stranded, positive-sense RNA genome of approximately 7700 nt in size, which encodes three open reading frames (ORFs) [1]. Presently, noroviruses are classified into seven genogroups (GI-GVII) and more than 30 genotypes [10]. Norovirus-related gastroenteritis outbreak is often associated with consumption of norovirus-contaminated oysters [11,14]. It has been shown that over 80% of human norovirus strains are present in oysters [15], including all GI genotypes [6]. Clearly, oysters play an important role in the environmental transmission of norovirus [2]. Hence, accurate detection of noroviruses in oysters is certainly crucial to monitor their spread in the environments as well as disease outbreaks.

Nested reverse transcription (RT)-PCR is widely used for detection of noroviruses. The classical primer pairs [COGIF (nucleotide position 5291–5310 refers to M87661)/GISKR (5671–5653 to M87661) for RT-PCR, GISKF (5342–5361 to M87661)/GISKR for nested PCR] specific for norovirus capsid protein gene (VP1) were designed for universal detection of the GI noroviruses in human samples [7,8,12]. When applying these primers to detect GI noroviruses in oysters, different sizes of non-specific PCR products including false positive amplicons were observed (Fig. 1A and B). To solve this problem, in this study, GI norovirus specific universal primers for nested PCR were re-designed based on all available GI sequences in public datasets and evaluated for their specificity, sensitivity and coverage.

A total of 2466 sequences of GI noroviruses, including whole-genomic and partial genomic sequences, were downloaded from the GenBank nucleotide database on December 31, 2015 and were classified into nine genotypes (GI.1-GI.9) based on the online Norovirus Genotyping Tool (http://www.rivm.nl/mpf/norovirus/typingtool) [9]. Representative sequences with similarity less than 90% from each of these nine genotypes were selected and aligned together (Geneious R9, Biomatters) to find conserved, primer binding regions (Fig. 2). As a result, a pair of primers, termed NGIOF (5′-TGATGGCGTCTAAGGACG-3'; nucleotide position 5362–5379 refers to M87661) and NGIOR (5′-TGAHTATCCAGGGGTCAAT-3'; 5529–5511 to M87661), for the nested PCR were obtained, and the theoretical PCR amplicon (part of the VP1 gene) is 168 bp in size (Fig. 2), which is sufficient for genotyping. In silico analysis showed that NGIOF/NGIOR are specific only to GI norovirus sequences available in GenBank.

In order to experimentally assess specificity of the newly designed primers NGIOF/NGIOR, total RNA that was extracted from either GI norovirus negative or positive oysters (confirmed based on PCR with the primers GISKF/GISKR and sequencing analysis) was used as the templates in nested RT-PCR assay.

Oyster samples (*Crassostrea gigas*, average body weight of 150 g) were collected from Luchaogang aquatic market, Shanghai, China. Their digestive tissues (DT, average weight of 350 mg) were immediately dissected on the day of sampling and homogenized by using FastPrep-24 (MP Biomedicals, USA) with the speed of 6 m/s for 30s. Total RNA was extracted from 100 μL of oyster DT homogenates with the QIAamp Viral RNA kit (QIAGEN, Germany) and stored at −80 °C

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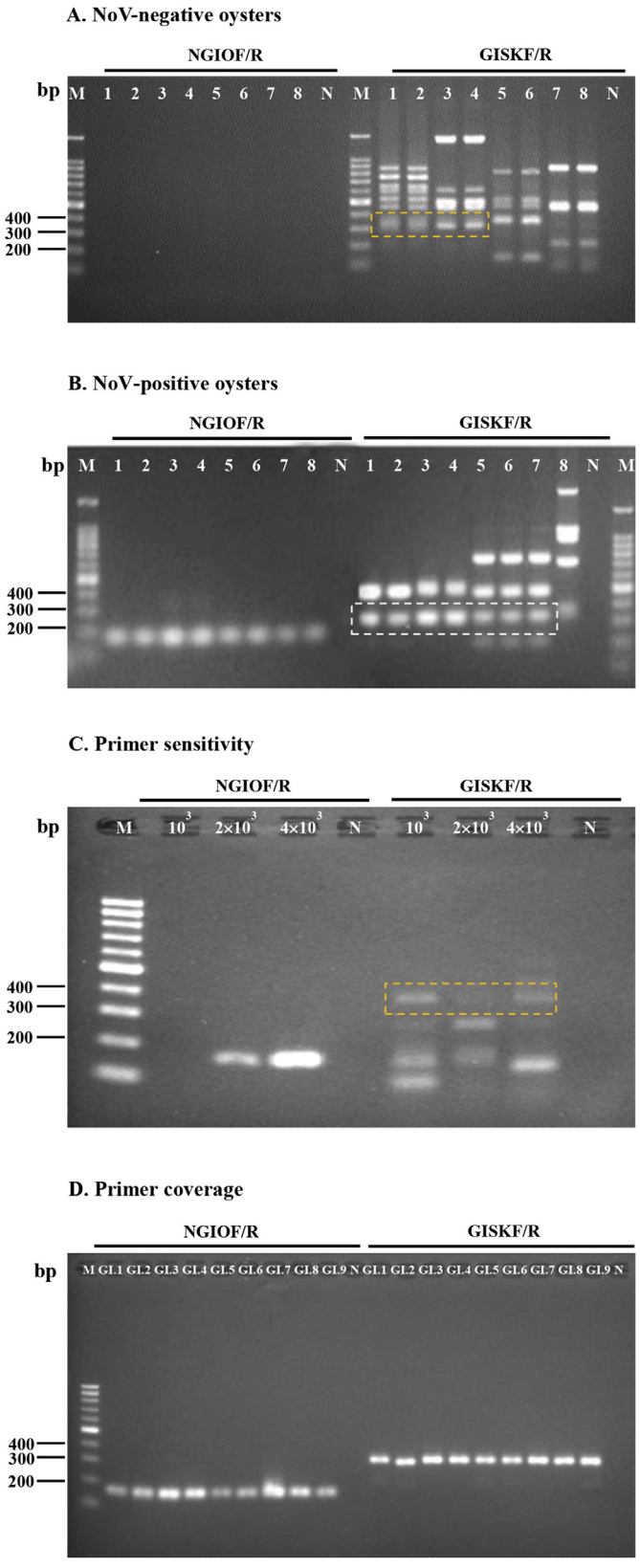

Fig. 1. Experimental evaluation of the primers. (A) Specificity. 1–8: eight representative norovirus-negative oyster samples; (B) Specificity. 1–8: eight representative norovirus-positive oyster samples; (C) Sensitivity. 10^3, 2×10^3, 4×10^3: the number of GI.6 viral copies added to oyster samples; (D) Coverage. M: 100 bp DNA ladder. N: negative control; White dashed box: specific positive PCR products; Yellow dashed box: false positive PCR products. Different primers are indicated above black line.

until use.

RT-PCR was performed in a 25 μL reaction following the protocols described in the PrimeScript One Step RT-PCR Kit Ver.2 (Takara, Japan). It contained 0.5 μM of each primer of COGIF/GISKR and 30 ng of RNA extracts. The RT-PCR thermal cycling program was as follows: RT at 50 °C for 30 min; initial denaturation at 94 °C for 2 min, then 40 cycles of 94 °C for 60s, 50 °C for 30s and 72 °C for 1 min. Nested PCR reaction (25 μL) contained 1 μL of the RT-PCR amplicons, 12.5 μL of 2 × *Taqman* Master mix (Takara, Japan), 0.5 μM of each primer (GISKF/GISKR or NGIOF/NGIOR) and sterile water. The thermal cycling program was achieved by initial denaturation at 94 °C for 4 min, then 35 cycles of 94 °C for 60s, 50 °C for 30s and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

No bands were observed on 2% agarose gel by using NGIOF/NGIOR for all eight representative negative samples (Fig. 1A). In contrast, the classical primers GISKF/GISKR amplified different sizes of PCR products including 330 bp of the expected size (Fig. 1A). After cloned to pUCm-T vector (Sangon Biotech, China), sequenced (Sangon Biotech, China) and analyzed for sequence similarity based on BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi), these amplicons were demonstrated to be amplified from oysters and their gut microbiota instead of noroviruses, which indicates the non-specific false positive amplification. For the GI norovirus positive samples, although specific PCR products were obtained for both NGIOF/NGIOR and GISKF/GISKR (Fig. 1B) and further confirmed upon sequencing analysis, the number eight positive sample was negative with the primers GISKF/GISKR. It suggests that NGIOF/NGIOR appear to be more sensitive than GISKF/GISKR upon detection of oyster samples. Like the GI norovirus negative samples, different non-specific PCR products were also obtained with GISKF/GISKR but not NGIOF/NGIOR upon detection of GI norovirus positive samples (Fig. 1B). These non-specific PCR products were sequenced and confirmed to be amplified from oysters and their gut microbiota as well based on BLAST analysis. Hence, although they are widely used to detect GI noroviruses in human stool specimens and show desirable specificity, the GISKF/GISKR turn out to be non-specific when oyster samples, albeit prepared by using different RNA extraction methods, instead of human samples are detected. Additionally, no bands were observed with NGIOF/NGIOR for both rotavirus A (RVA) and hepatitis A virus (HAV) (data not shown), which were kindly provided by Chinese Center for Disease Control and Prevention (CDC, Beijing, China). Taken together, these results clearly demonstrate that the new nested primers are highly specific.

To understand sensitivity of the primers NGIOF/NGIOR upon detection of GI noroviruses in oyster samples, the nested RT-PCR was performed by using viral RNA that was extracted from the norovirus negative oyster DT seeded with different amounts of GI.6 norovirus-contaminated human stool samples.

The number of GI.6 norovirus in human stool samples was quantified based on prepared GI RNA standard with one-step real-time RT-PCR (TaqMan RNA-to-CT 1-Step Kit, Applied Biosystems, USA). Q-PCR primers and probe were referred to [7]. To generate GI RNA standard, GI.6 norovirus sequence (part of the VP1 gene) that contains the targets of the RT-PCR primers COGIF/GISKR was amplified (5′-ATATCACTGC TGGGTGAGGC-3′ and 5′-CCAACCCAACCATTRTACA-3′) with the PrimeScript One Step RT-PCR Kit Ver.2 (TaKaRa, Japan) according to the manufacturer's instructions, and then ligated to the pGM-T vector (TIANGEN, China). Subsequently, RNA standard based on the cloned GI.6 sequence was transcribed as described previously [4]. After quantification assay, 1.4×10^2 copies of norovirus per mg of stool specimen were determined. The norovirus negative oyster DT (50 mg) was then mixed with three different amounts (10^3, 2×10^3, 4×10^3 norovirus copies) of GI.6-contaminated human stool samples, which were subjected to RNA isolation and the nested RT-PCR detection as described above.

As shown in Fig. 1C, the primers NGIOF/NGIOR were capable of specifically detecting of 2×10^3 and 4×10^3 norovirus copies, which,