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Usefulness of the rapid determination system of viral genome sequences in human stool specimens

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A B S T R A C T

The rapid determination system of viral genome sequences (the RDV method) consists of detecting and determining the nucleotide sequences of viral genomes without using specific primers. To evaluate the usefulness of the RDV method, the detection of human norovirus (NV) genomes in stool specimens was investigated. In addition, the effect of nuclease treatment of the process was examined. A total of 23 human stool specimens were used, all of which were collected from patients with acute viral gastroenteritis, and were shown to contain NV genomes and also determined the cDNA copy numbers by the real-time reverse transcriptase-polymerase chain reaction. NV genomes were detected by the RDV method with nuclease treatment in nine specimens containing cDNA copies ranging between 6.2×10^9 and 9.8×10^{11}/g stool. In contrast, NV genome was found by the method in 15 specimens without nuclease treatment and the number of NV cDNA copies ranged between 1.2×10^6 and 9.8×10^{11}/g stool. These results suggest that the RDV method has potential for detecting viral genomes in stool specimens. The procedure without a step of nuclease treatment appears to be sensitive.

1. Introduction

The rapid determination system of viral genome sequences (the RDV method) consists of detecting and determining the viral genome sequences without using specific primers nor sub-cloning into plasmid vectors (Mizutani et al., 2007). The following are steps of the RDV method: (i) nuclease treatment to digest nucleic acids outside the viral particles, (ii) extraction of the viral genome, (iii) construction of the first complementary DNA (cDNA) library, (iv) fragmentation of the library, (v) construction of the second cDNA library using non-specific primer sets, and (vi) direct sequencing (Mizutani et al., 2007). This method has been used mainly for detecting viral genomes in virus-rich materials such as the culture supernatant of infected cells and the allantoic fluid of infected embryonated chicken eggs (Mizutani et al., 2007; Sakai et al., 2007). However, it has not been used in clinical samples such as stool specimens.

To detect viral genomes in stool specimens, the nuclease-treatment step prior to extraction of nucleic acids is used commonly to reduce the contamination of non-particle-protected-nucleic acids (Zhang et al., 2006; Victoria et al., 2009). However, the effect of nuclease treatment in the RDV method remains unclear.

In this study, the RDV method was evaluated for detecting viral genomes in stools of patients with acute viral gastroenteritis. Additionally, the effect of the nuclease-treatment step was investigated with respect to the sensitivity for detecting viral genomes.

2. Materials and methods

2.1. Stool specimens

A total of 23 human stool specimens were used, which were collected from patients with acute viral gastroenteritis in Hokkaido, Japan, from December 2005 to January 2007.

2.2. Genotyping and real-time RT-PCR

All specimens were positive for norovirus (NV) genomes by the conventional reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously (Miyoshi et al., 2010). The genogroup and genotype were identified based on the nucleotide sequences of N/S domain of capsid protein VP1 (Kageyama et al., 2004). The number of NV cDNA copies was also determined (Kageyama et al., 2003).

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2.3. RDV method

Scheme of the RDV method was shown in Fig. 1. After vigorous mixing of each stool specimen, 0.5 g of each sample was prepared as 10% emulsions with nuclease-free distilled water (Nippon Gene, Tokyo, Japan) and was centrifuged at 1600 × *g* for 20 min at 4 °C, and the supernatant was collected and further centrifuged at 9000 × *g* for 10 min at 4 °C. It was then filtered through a 0.22 μm filter (Millipore, Bedford, Massachusetts). The filtrate (100 μl) was incubated with a cocktail of 175 U Benzonase (Novagen, Madison, Wisconsin), 40 U RNase A (Nippon Gene), 10 U TURBO DNase (Ambion, Austin, Texas) with 1× TURBO DNase buffer for 2 h at 37 °C. The pH value of a reaction mixture was measured by pH-indicator strip (Merck, Darmstadt, Germany) in advance. The nuclease-treatment step was omitted in the non-nuclease-treated (untreated) specimens. RNA was extracted from each of the nuclease-treated or untreated filtrates using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). cDNA synthesis and construction of the first cDNA library were performed using random hexamers (Takara Bio, Otsu, Japan) by the method described previously (Mizutani et al., 2007). The first cDNA library was digested with 40 U of *Hae*III (Takara Bio) for 1 h at 37 °C. After digestion, the cDNA was purified using the UltraClean 15 DNA Purification Kit (MO BIO, Carlsbad, California), and a blunt *Eco*RI–*Not*I–*Bam*HI Adaptor (Takara Bio) was ligated for 1 h at 16 °C using the DNA Ligation Kit (Takara Bio), followed by purification using the MinElute PCR Purification Kit (QIAGEN). The second cDNA library was amplified by PCR with primer sets described elsewhere (Mizutani et al., 2007). In this study, the H9-11 primer was used as a forward and respective H9-1 to -16 (excluding H9-11) were used as reverse primers, respectively. PCR was performed using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, California). The reaction mixture was heated for 12 min at 95 °C, and then amplified by 75 cycles with denaturation for 30 s at 94 °C and with annealing and extension for 30 s at 71 °C. After electrophoresis of PCR products on agarose gels, each amplified fragment was excised and purified using the UltraClean 15 DNA Purification Kit (MO BIO), then was sequenced directly with the forward and/or reverse primers. Nucleotide sequences obtained from the amplified fragments were searched for the homologous sequences with the Basic Local Alignment Search Tool (BLAST) program through the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp). Viral genome sequences determined in this study were submitted to the DDBJ (GenBank ID: AB634856–AB634918).

3. Results

There were three genogroup I (GI/2, G/4, and GI/8) and nine genogroup II (GII/1, GII/2, GII/3, GII/4, GII/5, GII/6, GII/7, GII/9, and GII/10) of NV in the 23 stool specimens, respectively. The

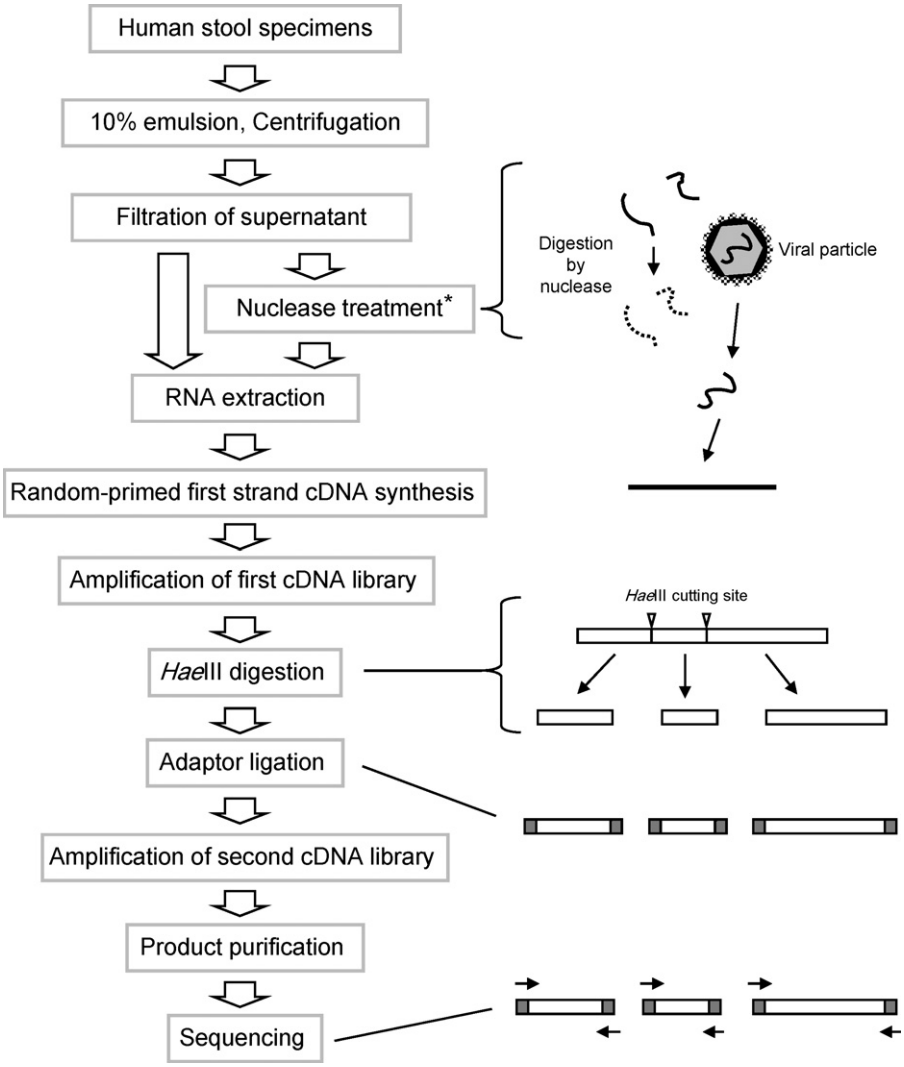

Fig. 1. Process diagram of the RDV method. *Untreated specimens were omitted this nuclease-treatment step.