Use of S1 nuclease in deep sequencing for detection of double-stranded RNA viruses

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ABSTRACT. Metagenomic approach using next-generation DNA sequencing has facilitated the detection of many pathogenic viruses from fecal samples. However, in many cases, majority of the detected sequences originate from the host genome and bacterial flora in the gut. Here, to improve efficiency of the detection of double-stranded (ds) RNA viruses from samples, we evaluated the applicability of S1 nuclease on deep sequencing. Treating total RNA with S1 nuclease resulted in 1.5–28.4- and 10.1–208.9-fold increases in sequence reads of group A rotavirus in fecal and viral culture samples, respectively. Moreover, increasing coverage of mapping to reference sequences allowed for sufficient genotyping using analytical software. These results suggest that library construction using S1 nuclease is useful for deep sequencing in the detection of dsRNA viruses.

KEYWORDS: deep sequencing, double-stranded RNA viruses, group A rotavirus, next-generation sequencer, S1 nuclease

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Non-Sanger-based next-generation sequencing (NGS) is a comprehensive technology for the characterization of nucleic acid sequences, without prior knowledge of genetic background [12, 14]. NGS has facilitated the discovery of many pathogenic viruses from a broad range of samples [7, 8]. However, numerous sequence reads originating from bacterial species and hosts are recovered when using conventional metagenomic approaches. Thus, to detect viral genome sequence reads from minor virus populations efficiently, it is important to exclude contaminating nucleic acids, including those of bacteria and hosts. S1 nuclease was first identified in Aspergillus oryzae as an endonuclease specific for single-stranded (ss) polynucleotides [1, 2]. S1 nuclease hydrolyzes ssRNA, ssDNA and ss regions of double-stranded (ds) polynucleotide, but it does not degrade dsRNA [3, 13, 15]. S1 nuclease was used in the present study, because it is inexpensive and easily inactivated by ethylenediaminetetraacetic acid.

Group A rotaviruses (RVAs) are major etiological agents of acute gastroenteritis, particularly in neonatal animals; RVAs can cause economic losses to the livestock industry [4, 11]. RVAs possess a dsRNA genome composed of 11 genome segments [5]. Whole genome analyses of RVA based on their nucleotide sequences are important for comprehensive understanding of the evolution of RVAs, which involves genetic re-assortment events and interspecies transmission [6, 8]. In a previous study, applying DNase I treatment to total RNA after RNA extraction markedly reduced the number of extra sequence reads in preliminary deep sequence analysis (data not shown). However, a considerable number of sequence reads from the RNA of bacterial species and hosts were detected in fecal and viral culture samples. In this study, to improve the efficiency of detection of dsRNA viruses from fecal and viral culture samples, we evaluated the applicability of S1 nuclease-treatment in deep sequencing.

Four fecal samples [2 from calves (calf no. 1: from 6 days old calf with diarrhea collected in 2013 in Japan and calf no. 2: from 26 days old calf with diarrhea collected in 2013 in Japan) and 2 from piglets (pig no.1: from healthy piglet collected in 2014 in Japan and pig no. 2: from piglet with diarrhea in 2014 in Japan)] and 3 viral culture samples were evaluated using the rapid antigen detection kit, Dipstick “Eiken” Rota immunochromatographic assay (Eiken Chemical Co., Ltd., Tokyo, Japan), for the presence of RVA. The results revealed that the samples from calf nos. 1 and 2 were weakly positive and positive, respectively, for RVA, while those from pig nos. 1 and 2 were strongly positive for RVA. The fecal samples were diluted (1:9 [v/v]) in sterile phosphate-buffered saline, centrifuged at 8,000 × g for 10 min...
using a Qubit® 2.0 Fluorometer (Invitrogen) and sequenced enriched by polymerase chain reaction (PCR), quantified steps were performed using the beads. The library was then in length after A-Tailing and adaptor ligation, 2 clean-up CA, U.S.A.). To select fragments of approximately 200-bp ing TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, U.S.A.). JPN/Miyamoto/1997/G4P[23]) were inoculated into MA- RV A (RV A/Pig-tc/JPN/BU9/2014/G9P[23] and RV A/Pig-tc/ (RV A/Cow-tc/JPN/Hori-No.14/1997/G6P[5]) were inoculated into MA-104 cells. Supernatants were collected at post-inoculation day 5 and stored at −80°C, without centrifugation, until further use. Total RNA was extracted from all samples using TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, U.S.A.), following which the RNA samples were treated with DNase I (0.5 U/µl; TaKaRa Bio Inc., Otsu, Japan). Each RNA sample was divided into 2 equal volumes, of which one was left untreated and the other was treated with S1 nuclease (27 U/µl; TaKaRa Bio Inc.) at 23°C for 15 min. After purification by ethanol precipitation, both the non-treated and S1 nuclease-treated samples were normalized to 50 ng/reaction using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, U.S.A.). A cDNA library was constructed using the NEB-Next® Ultra™ RNA Library Prep Kit to facilitate sequencing with Illumina version 2.0 (New England Biolabs, Ipswich, MA, U.S.A.), according to the manufacturer’s guidelines. Briefly, the RNA samples were fragmented and used to synthesize ds-cDNA, which was then purified using Agen- court® AMPure® XP Beads (Beckman Coulter, Pasadena, CA, U.S.A.). To select fragments of approximately 200-bp in length after A-Tailing and adaptor ligation, 2 clean-up steps were performed using the beads. The library was then enriched by polymerase chain reaction (PCR), quantified using a Qubit® 2.0 Fluorometer (Invitrogen) and sequenced using a MiSeq bench-top sequencer (Illumina, San Diego, CA, U.S.A.) with 51 single-end reads. The sequence data were analyzed using the MiSeq Reporter program (Illumina) to generate the reads in FASTQ format. Trimmed reads were assembled into contigs by de novo assembly with default parameters (automatic word and bubble size), using the CLC Genomics Workbench 6.0 (CLC; CLC bio, Aarhus, Denmark). Using the assembled contigs as references, consensus sequences for all the RVA segments were obtained. These consensus sequences were in turn used as references by the read mapper tool in CLC. The RVA sequence reads from the non-treated and S1 nuclease-treated samples were compared using mapping results of the read mapper tool with default mapping parameters (mismatch cost, 2; insertion cost, 3; deletion cost, 3; length function, 0.5; and similarity function, 0.8).

The results of the comparison of total RVA sequence read counts and percentage of RVA sequence reads (RVA sequence reads/total reads) between the non-treated and S1 nuclease-treated samples are listed in Table 1. The number of RVA sequence reads in all the genome segments was higher in the S1 nuclease-treated samples than in the non-treated samples. The percentage of RVA sequence reads was also higher in the S1 nuclease-treated samples than in the non-treated samples, particularly in the viral culture samples. Moreover, S1-treatment did not show any strong bias in relative numbers homologous to viral segments (Table 2).

Furthermore, RVA genotyping was performed by mapping the reads of the samples to the RVA reference sequences by using CLC[9]. Figure 1 illustrates the mapping results of the bovine fecal (calf no. 1) and swine fecal (pig no. 1) samples, which represent the VP4 bovine (P[1], P[5], P[11] and P[14]; A) and VP7 swine genotypes (G2, G4, G5 and G9; B), respectively. Mapping sequence reads from the non-treated and S1 nuclease-treated samples to the reference sequences of representative strains revealed that the coverage of sequence reads of the S1 nuclease-treated-samples against the reference sequences (calf no.1: 98.1%, swine no.1: 97.7%) was higher than that of the non-treated samples (calf no.1: 37.0%, swine no.1: 39.1%).

In the present study, treating total RNA with S1 nuclease prior to NGS reduced the number of extra sequence reads and increased the number of reads of RVA obtained for the bovine fecal, swine fecal and viral culture samples. Furthermore, the increased number of sequence reads of RVA facilitated genotyping with mapping to reference sequences on the genome analysis software. These results suggest that S1 nuclease-treatment during preparation of viral RNA for NGS is more useful than DNase I treatment alone, for the detection of RVA and dsRNA viruses. S1 nuclease-treatment facilitates the detection of not only viruses belonging to the Reoviridae and Picobirnaviridae families, but also non-identified novel viruses.

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Table 2. Relative number of RVA sequence reads homologous to each segment

|        | Calf No.1 | Calf No.2 | Pig No.1 | Pig No.2 |
|--------|-----------|-----------|----------|----------|
|        | non-treated | S1 nuclease treated | non-treated | S1 nuclease treated | non-treated | S1 nuclease treated | non-treated | S1 nuclease treated |
| VP1    | 402       | 18.4      | 12,357    | 19.8     | 5,641      | 20.4      | 29,769    | 21.2     | 7,662      | 24.9      | 92,178    | 22.9     | 73,770    | 20.1     | 108,149   | 20.0     |
| VP2    | 367       | 16.8      | 10,527    | 16.9     | 4,076      | 15.3      | 26,013    | 18.5     | 5,480      | 17.8      | 71,964    | 17.9     | 67,888    | 18.5     | 106,540   | 19.7     |
| VP3    | 424       | 19.4      | 12,568    | 20.2     | 3,791      | 15.3      | 23,040    | 16.4     | 4,333      | 14.1      | 58,400    | 14.5     | 60,925    | 16.6     | 82,565    | 15.3     |
| VP4    | 76        | 3.5       | 3,226     | 5.2      | 1,015      | 4.1       | 7,899     | 5.6      | 3,325      | 10.8      | 45,288    | 11.3     | 29,090    | 7.9      | 57,786    | 10.7     |
| VP6    | 108       | 4.9       | 2,832     | 4.5      | 1,459      | 5.9       | 5,720     | 4.1      | 778        | 2.5       | 15,830    | 3.9      | 13,229    | 3.6      | 22,394    | 4.1      |
| VP7    | 92        | 4.2       | 1,742     | 2.8      | 1,674      | 6.8       | 6,250     | 4.5      | 1,263      | 4.1       | 20,731    | 5.2      | 14,146    | 3.9      | 21,563    | 4.0      |
| NSP1   | 324       | 14.8      | 5,453     | 8.8      | 3,001      | 12.1      | 13,421    | 9.6      | 2,126      | 6.9       | 26,172    | 6.5      | 27,043    | 7.4      | 35,535    | 6.5      |
| NSP2   | 149       | 6.8       | 5,140     | 8.3      | 1,227      | 5.0       | 10,132    | 7.2      | 1,986      | 6.5       | 23,917    | 6.0      | 28,073    | 7.7      | 36,870    | 6.8      |
| NSP3   | 130       | 5.9       | 6,153     | 9.9      | 2,327      | 9.4       | 11,510    | 8.2      | 2,791      | 9.1       | 34,164    | 8.5      | 33,953    | 9.3      | 44,243    | 8.2      |
| NSP4   | 85        | 3.9       | 1,198     | 1.9      | 742        | 3.0       | 3,557     | 2.5      | 814        | 2.6       | 10,751    | 2.7      | 9,188     | 2.5      | 12,441    | 2.3      |
| NSP5   | 33        | 1.5       | 1,065     | 1.7      | 386        | 1.6       | 3,090     | 2.2      | 201        | 0.7       | 2,465     | 0.6      | 8,764     | 2.4      | 12,060    | 2.2      |
| total  | 2,190     | 100       | 62,261    | 100      | 24,730     | 100       | 140,401   | 100      | 30,759     | 100       | 401,860   | 100      | 368,339   | 100      | 539,964   | 100      |

| RVA/Cow-tc/JPN/Hori-No.14/1997/G6P[5] | RVA/Pig-tc/JPN/BU9/2014/G9P[23] | RVA/Pig-tc/JPN/Miyamoto/1997/G4P[23] |
|--------------------------------------|----------------------------------|--------------------------------------|
| non-treated | S1 nuclease treated | non-treated | S1 nuclease treated | non-treated | S1 nuclease treated |
| VP1        | 470      | 24.7      | 97,019     | 24.3     | 192        | 19.7      | 37,413     | 20.6     | 7,037      | 20.4      | 73,527     | 21.2     |
| VP2        | 415      | 21.8      | 79,098     | 19.8     | 154        | 15.8      | 34,893     | 19.2     | 6,418      | 18.6      | 63,481     | 18.3     |
| VP3        | 364      | 19.1      | 88,677     | 22.2     | 118        | 12.1      | 27,585     | 15.2     | 4,958      | 14.4      | 56,224     | 16.2     |
| VP4        | 58       | 3.1       | 17,853     | 4.5      | 134        | 13.7      | 21,674     | 11.9     | 4,475      | 13.0      | 40,859     | 11.8     |
| VP6        | 32       | 1.7       | 8,742      | 2.2      | 32         | 3.3       | 6,263      | 3.4      | 715        | 2.1       | 8,704      | 2.5      |
| VP7        | 43       | 2.3       | 8,810      | 2.2      | 19         | 1.9       | 4,174      | 2.3      | 1,779      | 5.2       | 18,166     | 5.2      |
| NSP1       | 108      | 5.7       | 27,380     | 6.9      | 85         | 8.7       | 16,127     | 8.9      | 2,112      | 6.1       | 21,856     | 6.3      |
| NSP2       | 182      | 9.6       | 30,286     | 7.6      | 67         | 6.9       | 10,641     | 5.9      | 2,275      | 6.6       | 23,188     | 6.7      |
| NSP3       | 155      | 8.2       | 28,768     | 7.2      | 114        | 11.7      | 13,912     | 7.6      | 3,625      | 10.5      | 31,816     | 9.2      |
| NSP4       | 55       | 2.9       | 8,621      | 2.2      | 37         | 3.8       | 5,451      | 3.0      | 925        | 2.7       | 8,117      | 2.3      |
| NSP5       | 19       | 1.0       | 3,596      | 0.9      | 23         | 2.4       | 3,737      | 2.1      | 180        | 0.5       | 1,540      | 0.4      |
| total      | 1,901    | 100       | 398,850    | 100      | 975        | 100       | 181,870    | 100      | 34,499     | 100       | 347,478    | 100      |

a) RVA sequence reads of each viral segment/total RVA sequence reads of the sample.
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