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A new comprehensive method for detection of livestock-related pathogenic viruses using a target enrichment system

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ABSTRACT

We tested usefulness of a target enrichment system SureSelect, a comprehensive viral nucleic acid detection method, for rapid identification of viral pathogens in feces samples of cattle, pigs and goats. This system enriches nucleic acids of target viruses in clinical/field samples by using a library of biotinylated RNAs with sequences complementary to the target viruses. The enriched nucleic acids are amplified by PCR and subjected to next generation sequencing to identify the target viruses. In many samples, SureSelect target enrichment method increased efficiencies for detection of the viruses listed in the biotinylated RNA library. Furthermore, this method enabled us to determine nearly full-length genome sequence of porcine parainfluenza virus 1 and greatly increased Breadth, a value indicating the ratio of the mapping consensus length in the reference genome, in pig samples. Our data showed usefulness of SureSelect target enrichment system for comprehensive analysis of genomic information of various viruses in field samples.

1. Introduction

Proper control of infectious diseases greatly affects productivity of domestic animals. The data that infectious diarrhea causes death of more than 50% of the calf less than 1 month of age illuminate importance of control of infectious diseases for productivity of domestic animals [5,22]. Delayed fattening of pigs due to influenza A (H1N1) virus infection [2]; a decrease in the milk yield of cattle by bovine viral diarrhea virus, Akabane virus, and bovine coronavirus [1,7,18,19]; and a decline of reproductive performance of cattle under the influence of Akabane virus [18] also represent examples of ill effects of infectious diseases for productivity of domestic animals. The regulations of the International Epizootic Office limit the movement of domestic animals near farms that are affected by certain infectious diseases. Once an outbreak of an internationally important infectious disease, e.g., foot-and-mouth disease, occurs, the products of susceptible livestock are subject to export restrictions, causing severe economic damage in the affected county. Rapid detection of the infectious agents and prompt responding to the infection would minimize the economic losses.

Next-generation sequencing (NGS) has been widely used for comprehensive detection of viruses and several studies reported successful identification of many novel viruses in various animal samples [4,8,10,22]. Host-derived nucleic acids represent the majority of the nucleic acids in most of clinical/field samples and presence of low levels of nucleic acids of infectious agents often makes their detection and identification difficult. To efficiently

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detect viral genomes in various animal samples, the target enrichment method called SureSelect (Agilent Technologies) has been developed. SureSelect selectively captures the nucleic acids of target viruses by using streptavidin bead-bound biotinylated RNAs complementary to the target viruses. The captured viral nucleic acids are then amplified by PCR and subjected to next generation sequencing, leading to identification of target viruses in the sample. We tested usefulness of the SureSelect system for detection of various virus genomes present in field samples of cattle, pigs, and goats in Japan.

2. Materials and methods

2.1. Preparation of an analytical sample

2.1.1. Extraction of nucleic acids

2.1.1.1. Cattle and bovine herpesvirus 1 (BHV-1). The present study used seven samples of feces collected from calves in farms of Hokkaido, Ishikawa prefecture, and Kagoshima prefecture in Japan. BHV-1 served as a positive control. Feces were adjusted to a 10% emulsion with sterile PBS and centrifuged at 10,000 rpm for 10 min at 4 °C by using a microcentrifuge. RNA was extracted from the supernatant using the TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA) and treated with DNase I (TaKaRa Bio). Then, equal amounts of RNA obtained from each sample were mixed into 1 sample. For DNA extraction from the isolated strain of BHV-1, QIAamp DNA Mini kit (QIAGEN) was used.

2.1.1.2. Pig and goat samples. Feces of four piglets less than 3 weeks old collected from one farm in Japan and diarrheic feces obtained from two goats in Okinawa prefecture were analyzed. We previously reported the presence of a novel porcine rotavirus, astrovirus, posavirus, and circovirus in the sample group including these four samples [9,11–13,15]. Feces were adjusted to 10% emulsion with sterile PBS and centrifuged at 10,000 rpm for 10 min at 4 °C. RNA was extracted from the supernatant by using the ISOGEN LS Reagent (Nippon Gene Co., Ltd., Tokyo, Japan). DNA was extracted from the supernatant with the QIAamp Fast DNA stool kit (QIAGEN, Venlo, Netherlands).

2.1.2. Synthesis of double-stranded (ds) cDNA

For constructing libraries by the target enrichment method, ds cDNA was synthesized from the extracted RNA from the feces samples using the PrimeScript Double strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan). The synthesized dsDNA was purified with phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich Japan, Tokyo, Japan).

2.1.3. Sample preparation for library construction

The mixed ds cDNA were synthesized from a mixture of seven cattle fecal RNA samples. DNA of BHV-1, which was added as a positive control, was prepared for a bovine-related virus capture library. The ds cDNA from the mixture of seven bovine fecal RNA samples and the BHV1 DNA was mixed so that the ratio of ds cDNA to DNA was 1:7.

Extracted nucleic acids from feces of pigs and goats were prepared for the capture library of bovine-swine-caprine-related viruses. The extracted DNAs and the ds cDNAs synthesized from the extracted RNAs were mixed in equal amounts. The concentration of each sample after mixing was measured on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific K.K. Yokohama, Japan). Samples with concentrations below the measurement limit were excluded from analysis (data not shown).

2.1.4. Custom capture library

The SureSelect XT custom capture library, which was synthesized based on a selected virus genome sequence by Agilent Technologies Co., Ltd., was used.

2.1.5. Construction of a sample library by the target enrichment method

An analytical sample was constructed for the library using the SureSelect QXT Reagent kit (Agilent Technologies, Tokyo, Japan). The overview of each step is shown in Fig. 1. First, we generated the DNA library of obtained ds DNA samples by randomly fragmenting the dsDNA, and subsequent addition of adapter sequence, to which the index primer anneals. Then, the DNA samples were purified using Agencourt AMPure XP (Beckman Coulter, Inc., Brea, CA, USA). Next, the adapter-attached library was amplified using the SureSelect QXT Primer Mix. The PCR was conducted as follows: 68 °C for 2 min, at 98 °C for 2 min, followed by 8 cycles of 98 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 5 min. Immediately after the reaction, the DNA sample was purified using Agencourt AMPure XP. The adapter-attached DNA library was hybridized to the virus capture library designed for this study, and nucleic acids of the target viruses were enriched. Specifically, a sample was mixed with the SureSelect QXT Fast Blocker Mix and was incubated at 95 °C for 5 min and 65 °C for 10 min using a thermal cycler. After holding the sample at 65 °C for 1 min in the thermal cycler, we added SureSelect QXT Fast Hybridization Buffer to the sample and performed 60 cycles of incubation at 65 °C for 1 min and 37 °C for 3 s. Immediately after the reaction, SureSelect RNase Block solution was added to the sample, and the captured DNA was purified by using Dynabeads MyOne Streptavidin T1 beads (Thermo Fisher Scientific K.K. Yokohama, Japan). Then, the DNA library, which was attached to streptavidin beads, was amplified by PCR after addition of the index primer, dNTP mix, Herculase II Reaction Buffer, and Herculase 2 Fusion DNA polymerase. The PCR cycling conditions were as follows: an initial denaturation at 98 °C for 2 min; followed by 20 cycles of 98 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. After PCR, streptavidin beads were removed from the sample by using a magnet stand, and the PCR products, which were not associated with the beads, were further purified with Agencourt AMPure XP. The obtained purified product was subjected to NGS analysis. We call the NGS data analysis of the library on the basis on this method postcapture sequencing.

2.1.6. Library preparation by the conventional method

As a conventional method, libraries were constructed from ds cDNA using the Nextera DNA Library Preparation Kit (Illumina, San Diego, Calif., USA). Specifically, ds cDNA was randomly fragmented and was mixed with an adapter and was attached to an index. After the reaction, size selection was performed using Agencourt AMPure XP. The NGS data analysis of the library constructed by the conventional method was called precapture sequencing.

2.1.7. Deep sequencing and sequencing data analysis

Deep sequencing was performed on a MiSeq benchtop sequencer (Illumina, San Diego, CA, USA). The constructed library was analyzed as a read of a 76-bp paired end by means of the MiSeq Reagent Kit v3 (150 cycles) (Illumina, San Diego, CA, USA). The sequence of each obtained read was output in FASTAQ format, using a MiSeq reporter, and analyzed in CLC Genomic Workbench 6.5.1 (CLC bio, Aarhus, Denmark). Each read was processed by a quality trim command to trim low-quality sequences, and contigs were obtained via the *de novo* assembly command. A BLAST search was conducted on all contigs using a virus database obtained from NCBI.

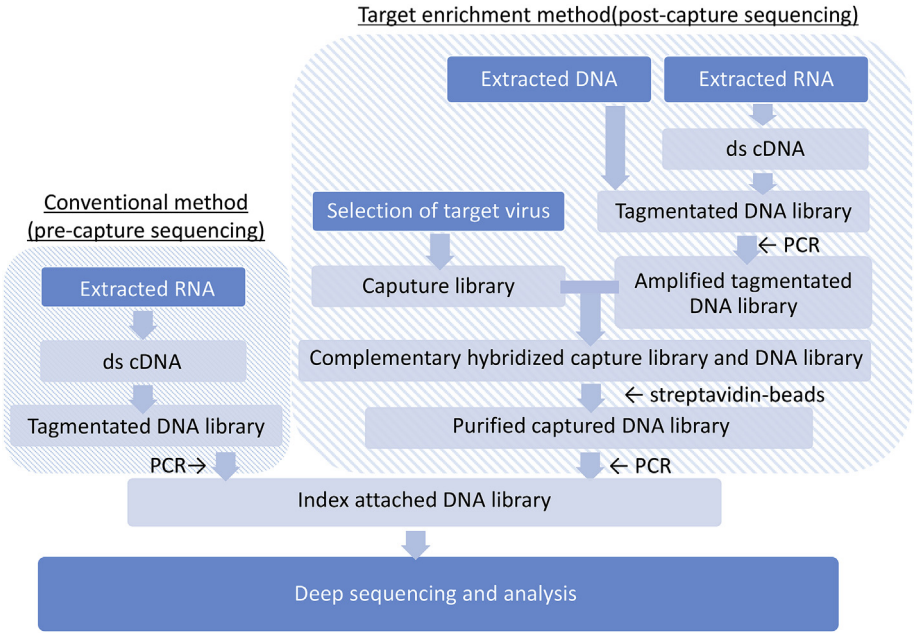


Fig. 1. Overview of the sample library construction by the conventional method (precapture sequencing), and by the target enrichment method (postcapture sequencing). For the verification of cattle samples, only RNA extracted from the sample was used. In pig and goat samples, both DNA and RNA from samples were used.

3. Results

3.1. The design of the SureSelect enrichment system

We designed two sets of capture libraries, one for bovine-related viruses and the other for bovine-swine-caprine–related viruses. Both library sets took into consideration of various viral pathogens of domestic animals in Japan. Based on the literature search on PubMed.gov and the master list from International Virus Classification Committee (ICTV), we added other viruses that are at risk of disease outbreaks. Unclassified viruses that are not listed in the ICTV classification table were also selected from the NCBI Virus database. Viruses possibly related to cattle, pigs and goats were also added from Virus-Host-DB (http://www.genome.jp/virushostdb/). The capture library set of the bovine-related viruses included four species of single-stranded (ss) DNA viruses, 28 species of dsDNA viruses, 17 species of dsRNA viruses, 35 species of ssRNA (−) viruses, 36 species of ssRNA (+) viruses, and three species of ssRNA viruses encoding a reverse transcriptase. The capture library set of bovine-swine-caprine–related viruses included 48 species of ssDNA viruses, 81 species of dsDNA viruses, 416 species of dsRNA viruses, 156 species of ssRNA (−) viruses, 135 species of ssRNA (+) viruses, 14 species of ssRNA viruses encoding a reverse transcriptase, and 15 species of unclassified viruses. The entire regions of these viral genomes were used to capture target viral nucleic acids, except for herpesvirus, for which we screened the samples for the polymerase gene and eight functionally conserved genes; these genes were used for phylogenetic analysis of the α, β, γ Herpesvirinae (HEP1–12) [3]. The virus genomes used for the capture libraries are shown in Supplementary Material.

Table 1 shows the number of reads and contigs obtained from analysis of each precapture sequencing and postcapture sequencing. Contigs of each sample were subjected to a local BLAST search using a virus database obtained from NCBI. Contigs with E value* <1E-100 were reused for BLASTn search on NCBI, and the results with the highest score are shown in Table 2. (*E value means "Expected value" where a hit between an entry and a query sequence happens by chance.). We defined E values less than 1E-100 as positive. The viral genomes that were positive in the post-capture sequencing are shown in Tables 2A and 3. Table 3 also shows the names, accession numbers and lengths of genomes used for the mapping. The number of reads mapped to each reference sequence is shown as a total read count. The ratio of the consensus length of the mapped reads to the total length of the reference sequence is indicated as Breadth (%) in Table 3. High Breadth means a value indicating the ratio of the mapping consensus length in the reference genome.

Table 1
Comparison between pre- and post-capture sequencing of read count and number of contig.

	Pre-caputure sequencing		Post-capture sequencing	
	Total reads	Contigs	Total reads	Contigs
Cattle *A	6,317,276	570	50,967,228	264
Pig 1 *B	629,140	579	3,074,544	71
Pig 2 *C	957,684	370	3,798,954	232
Goat 1 *D	3,280,164	1387	2,546,868	190
Goat 2 *E	3,390,671	73	1,044,452	122

*A to *E in this table correspond to A to E in Table 2 respectively.
Pig sample 3 and pig sample 4 were excluded from the analysis because the concentration of the sample prepared for library construction was below the measurement limit (data not shown).

3.2. Verification on the cattle samples

Bovine hungarovirus 1 (BHuV-1) was detected only in the postcapture sequencing, while the contigs homologous to bovine astrovirus (BastV), enterovirus F, BHV-1, bovine picornavirus (BPV), RVA, BtoV, bovine calicivirus (BECV), bovine kobu virus (BKV), and stealth virus 1 (STV-1) were obtained from both precapture and postcapture sequences (Table 2). *Cryptosporidium parvum* virus (CPV) was detected only in precapture sequencing because CPV was not included in the capture library. Reads were mapped to each viral genome sequence (Table 2).

Breadth of three viruses, BhuV-1, BEV and BAstV, in postcapture

Table 2
Captured viruses in pre-capture sequencing and post-capture sequencing.

Name of virus	Pre-capture sequencing	Post-capture sequencing
A Cattle		
Bovine astrovirus	+	+
Bovine calicivirus	+	+
Bovine enterovirus	+	+
Bovine hungarovirus 1	−	+
Bovine herpesvirus 1	+	+
Bovine kobuvirus	+	+
Bovine picornavirus	+	+
Bovine torovirus	+	+
Cryptosporidium parvum virus 1	+	−
Dromedary picobirnavirus	+	−
Bovine rotavirus A	+	+
Stealth virus 1	+	+
B Pig 1		
Porcine astrovirus 3	+	+
Porcine astrovirus 4	+	+
Porcine endogenous retrovirus	−	+
Porcine kobuvirus	+	+
Porcine parainfluenza virus 1	−	+
Porcine stool-associated circular virus 3	−	+
Enterovirus	+	+
Human picobirnavirus	+	−
Rotavirus A	+	+
C Pig 2		
Porcine astrovirus 3	+	+
Porcine astrovirus 4	+	+
Porcine endogeous retrovirus	−	+
Porcine kobuvirus	+	+
Porcine parainfluenzavirus 1	+	+
Porcine stool-associated circular virus 3	−	+
Enterovirus	+	+
Human picobirnavirus	+	+
Rotavirus A	+	+
D Goat 1		
Goat enterovirus	+	+
E Goat 2		
Not applicable		

Evalue <1 E−100 was defined as positive.

sequencing was more than 20% better than that in precapture sequencing (BhuV-1: pre- 40.9%, post- 72.1%; BEV: pre- 34.8%, post- 56.6%; BAstV: pre-19.5%, post-40.8%). Breadth of BHV-1, BPV, BtoV, BECV, and STV-1 in pre- and postcapture sequencing were equally high. RVA/Human-wt/IND/N36/2003/G10P [11] segment 9 had higher Breadth in precapture sequencing than in postcapture sequencing (pre- 95.1%, post- 80.8%). Further study is needed to clarify this reason.

3.3. Detection of viruses in pigs and goats

We analyzed two pig and two goat samples. In pig sample 1, only postcapture sequencing detected porcine parainfluenza virus 1 and porcine stool-associated circular virus 3, while only precapture sequencing detected human picobirnavirus. In pig sample 2, only postcapture sequencing detected porcine stool-associated circular virus and porcine endogenous retrovirus, and there was no virus that was detected only by precapture sequencing. A clearly large increase in Breadth (%) was seen in the two pig samples (Fig. 2). The number of reads mapped to porcine parainfluenza virus strain 1438-1, partial genome (KT749882.1), increased from 19 to 4989 for pig sample 1 and from 237 to 20,581 for pig sample 2. Also Breadth rose from 8.5% to 98.9% and from 81.7% to 99.8% in pig sample 1 and pig sample 2, respectively (Fig. 2). Both pre- and postcapture sequencing detected goat enterovirus [21] in goat sample 1, whereas no virus was detected in goat sample 2.

4. Discussion

Whole genome sequencing of viruses usually requires virus amplification in cultured cells or eggs, whereas many recent studies determined whole virus genome sequences by metagenomic analysis of nucleic acids directly extracted from clinical or field samples [4,16,23]. Because the availability of reads of the target virus is depended on the relative amount of the target viral genome to other nucleic acids of host and other agents in a given sample, sample pretreatments that eliminate the host genome have been carried out to obtain the target viral sequence [13,17]. Without these pretreatment, determining the whole or partial genome sequence of the target virus would be difficult. Hence, the target enrichment system represents an important method for detection of target viral genomes in a mixture of nucleic acids derived from both host and infectious agents. The target enrichment system also has been widely applied for all exosome sequencing, particularly in large-scale cohort research for taxonomic identification and for identification of causative genes of specific diseases such as cancer [6,14,20].

The present study examined the usefulness of a target enrichment method, SureSelect, for efficient concentration of genomes of various viruses of domestic animals. By using this enrichment method, we were able to efficiently detect the sequence of the target viruses, assemble longer contigs and directly obtain the genome data, including sequences of nearly full-length of viral

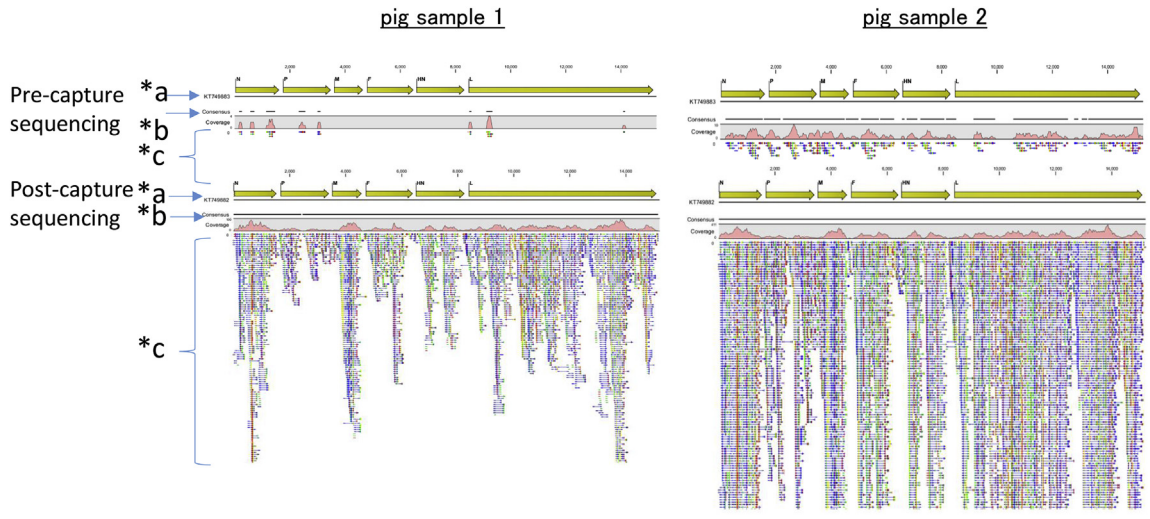
Table 3
Comparison of results of mapping reads obtained pre- and post-capture sequencing.

Accession No.	Sequence name of registered on Genbank	Reference length	Pre-capture sequensing			Post-capture sequencing		
			Consensus length	Breadth(%)	Read count	Consensus length	Breadth(%)	Read count
LC047787.1	Bovine astrovirus genomic RNA, nearly complete genome	6287	1224	**19.5**	186	2565	**40.8**	3037
AB117797	Calicivirus isolate TCG genomic RNA, complete genome, isolate: TCG 14	7453	6279	90.3	1234	7281	97.7	728,260
DQ092794.1	Enterovirus F strain PS87/Belfast polyprotein gene, complete cds	7394	2573	**34.8**	149	4185	**56.6**	13,828
JQ941880	Bovine hungarovirus 1 strain BHUV1/2008/HUN, complete genome	7583	3103	**40.9**	394	5464	**72.1**	15,134
KU198480.1	Bovine herpesvirus 1 strain Cooper, complete genome	3744	3692	98.6	483	3744	100.0	502,980
LC055960.1	Kobuvirus cattle/Kagoshima-2-24-KoV/2015/JPN genomic RNA	8496	8250	**97.1**	2552	5730	**67.4**	14,671
LC036582.1	Bovine picornavirus genomic RNA, complete genome	7635	7602	**99.6**	11,084	5327	**69.8**	390
LC088095.1	Bovine torovirus genomic RNA, complete genome	28,308	28,256	99.8	89,433	27,456	97.0	5,202,269
EU183403.1	Cryptosporidium dsRNA virus RNA-dependent RNA polymerase (RDRP) gene	1783	1762	**98.8**	332	456	**25.6**	25
KM573802.1	Dromedary picobirnavirus isolate c4566	1623	1287	**79.3**	361	549	**33.8**	24
JF693026	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 1	3267	3267	100.0	509,480	3267	100.0	8,105,770
JF693035	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 10	528	528	100.0	20,751	528	100.0	157,753
JF693036	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 11	597	597	100.0	12,604	597	100.0	871,968
JF693027	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 2	2643	2643	100.0	193,068	2643	100.0	6,389,174
JF693028	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 3	2508	2503	100.0	926,149	2508	100.0	3,428,327
JF693029	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 4	2331	723	31.0	152	640	27.5	37
JF693030	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 5	1476	1475	99.9	197,905	1476	100.0	941,606
JF693031	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 6	1194	1194	100.0	33,151	1194	100.0	2,006,621
JF693032	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 7	954	954	100.0	76,801	954	100.0	519,469
JF693033	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 8	942	942	100.0	133,442	942	100.0	3,742,643
JF693034	Bovine rotavirus A isolate bovine-tc/USA/NCDV/1971/G6P[1] segment 9	981	981	100.0	35,204	981	100.0	532,493
KC175118	Rotavirus A strain RVA/Human-wt/IND/N160/2003/G10P[11] segment 4	2301	2301	100.0	91,147	2246	97.6	1117
KC174871	Rotavirus A strain RVA/Human-wt/IND/N36/2003/G10P[11] segment 9	1025	975	95.1	14,652	828	80.8	869
AF191073.1	Stealth virus 1 clone 3B43, genomic sequence	3620	2953	81.6	644,124	2949	81.5	3,774,005

The numbers in shaded showed that Breadth increased by more than 20% in post-capture sequense than pre-cupture sequense. The numbers in bold letters indicated that Breadth was lower than pre-cupture sequense by 30% or more in post-capture sequense.

genome, from animal feces. The result of BLAST analysis of contigs obtained from SureSelect enrichment method for bovine-related viruses was slightly inferior to that of the conventional method for BPV and BKV, whereas the target enrichment method was better than conventional method for contigs of BhuV-1, BEV and BAstV. The results of comparison using BLAST against the BPV TCH 6 strain (KM 589358), which was included in the bovine-related capture library, and the contig obtained from the postcapture sequencing analysis showed that homology to BPV manifested 86% Query cover and 68% Ident. Similar analysis of BKV revealed 83% query cover and 69% Ident. These results indicated that SureSelect target enrichment method could detect viruses that had over 69% homology with the reference. CPV and DPV were detectable only in precapture sequencing because those viruses were not included in the bovine-related capture library, whereas only postcapture sequencing analysis detected BHnV-1, which was present in the bovine-related capture library. These results indicate that SureSelect target enrichment system can detect the viral genomes present in the capture library more efficiently.

Analyses of the samples obtained from pigs and goats also illuminated strength and limitation of SureSelct method. Almost all viruses detected by precapture sequencing were also detected by postcapture sequencing in both pig samples. One exception was human picobirnavirus in pig sample 1; precapture sequencing, but not postcapture sequencing, detected this virus. We included four strains of picobirnavirus in the capture library for bovine-swine-caprine–related viruses. A BLAST search showed that one contig in precapture sequencing had homology to human picobirnavirus, whereas this contig showed little homology to any of four strains of picobirnavirus in the capture library; those showing the most homology were in agreement on only 20 bases. As SureSelect could detect sequences showing homology over 69% to the reference (see above), SureSelect is suitable to detect many emerging mutated viruses. However, picobirnavirus is known for its diversity of sequences [18]; hence, for successful detection of virus species with high diverse sequences, inclusion of sequences of as many strains as possible in the capture library would be helpful. The SureSelect target enrichment system substantially increased the number of reads mapped to porcine parainfluenza virus strain 1438-1, allowing to reveal 98.9% and 99.8% of the whole genome sequence (KT749882.1) of the virus, in pig sample 1 and 2, respectively (Fig. 2). Thus, SureSelect target enrichment system determined



	pig sample 1			pig sample 2		
	number of mapped reads	mapping concensus length	Breadth(%)	number of mapped reads	mapping concensus length	Breadth(%)
pre−caputure sequencing	19	1285	8.5%	237	12,423	81.7%
post−capture sequencing	4989	15,039	98.9%	20,581	151,881	99.8%

Fig. 2. A comparison of mapping read numbers and consensus lengths for pre- and postcapture sequencing. "*a" indicates a reference sequence. "*b" indicates a consensus sequence by reads mapped to the reference sequence. "*C" indicated reads mapped to the reference sequence. In other words, the results showed that reads mapped to porcine parainfluenza virus strain 1438-1, partial genome (KT749882.1), were clearly more pronounced in postcapture sequencing than in precapture sequencing.

nearly full-length of the viral genome without virus isolation. Although the porcine parainfluenza virus genome was detected in feces, this virus had been reported as a cause of porcine respiratory disease (ref); biological significance of porcine parainfluenza virus in feces is currently unclear.

In summary, our data imply that SureSelect-based target enrichment system has an excellent potential for identification of viruses without incubating and amplifying viruses.

Conflict of interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.12.017.

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