FULL PAPER *Virology*

Concise and Broadly Applicable Method for Determining the Genomic Sequences of North-American–Type Porcine Reproductive and Respiratory Syndrome Viruses in Various Clusters

Takeya MOROZUMI[1)], Hiroshi ISEKI[2)], Daisuke TOKI[1)], Michihiro TAKAGI[2)], Hiroshi TSUNEMITSU[2,5)] and Hirohide UENISHI[3,4)]*

[1)] *Animal Research Division, Institute of Japan Association for Techno-innovation in Agriculture, Forestry and Fisheries, 446–1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305–0854, Japan*
[2)] *Viral Disease and Epidemiology Research Division, National Institute of Animal Health, 3–1–5 Kannondai, Tsukuba, Ibaraki 305–0856, Japan*
[3)] *Animal Immune and Cell Biology Research Unit, Division of Animal Sciences, National Institute of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki 305–8602, Japan*
[4)] *Animal Genome Research Unit, Agrogenomics Research Center, National Institute of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki 305–8602, Japan*
[5)] *Present address: Dairy Hygiene Research Division, National Institute of Animal Health, 4 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062–0045, Japan*

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ABSTRACT. We developed a concise and broadly applicable method for accurate genomic sequencing of North American genotype (NA-type) porcine reproductive and respiratory syndrome viruses (PRRSVs) that overcomes high genetic variability of the viruses. The method, designated "combination of consensus oligonucleotide reverse transcription and multiple displacement amplification" (CORT-MDA), involves reverse-transcription of viral RNA followed by shotgun sequencing after amplification using only 11 degenerate oligonucleotide primers; these primers were designed against consensus regions within the open reading frames of the 124 NA-type PRRSV strains with reported full-length genomic sequences. Sequencing of the 192 shotgun clones generated per virus showed 80% to 94% coverage on the reported PRRSV genomic sequence, such that only 2 or 3 unread regions had to be resequenced after PCR amplification using custom primers. Direct sequencing of RT-PCR products confirmed absolute consistency between sequences determined by the CORT-MDA method and those from RT-PCR. These results suggest that our method is applicable to diverse NA-type viruses.
KEY WORDS: amplification, genome, PRRSV, shotgun sequencing

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Porcine reproductive and respiratory syndrome (PRRS) causes huge economic losses in the pig industry through reproductive failure in pregnant sows, preweaning mortality and respiratory distress in piglets. The etiologic agent of this syndrome, PRRS virus (PRRSV), is a member of the family *Arteriviridae* in the order *Nidovirales*. The viral genome is approximately 15 kb long and comprises 5′-capped, 3′-polyadenylated, positive-sense and single-stranded RNA. The PRRSV genome consists of 5′- and 3′-untranslated regions (UTRs) and at least eight open reading frames (ORFs). ORF1a and 1b encode polyproteins that are cleaved into nonstructural proteins (nsps). The other ORFs encode viral structural proteins [13].

Two distinct divergent PRRSV strains, namely the European (EU-type, type 1) and North American (NA-type, type 2) genotypes, were isolated nearly simultaneously and have since been designated Lelystad [25] and VR-2332 [3], respectively. Since the identification of the Lelystad and VR-2332 strains, other NA- and EU-type PRRSVs have been isolated, and the genomic sequences of these 2 viral types are known to be approximately 60% identical at the nucleotide level [1]. Furthermore, NA- and EU-type viruses show extremely high sequence variability, because of the extraordinary mutation rates in the viral genomes ($>10^{-2}$/site/year) [9]. NA-type viruses are classified mainly through genotyping of ORF5 [11, 20, 27, 28]. Moreover, the genomic sequence of nsp2, which has many nucleotide substitutions, insertions and deletions, is often used in PRRSV classification [12, 16, 19, 21, 29]. For example, the highly pathogenic PRRSV (HP-PRRSV) strains that have emerged in China are characterized by a 30-amino-acid deletion in nsp2. Although this deletion and the extraordinary virulence of HP-PRRSV are asssumed to be associated [12, 21], deletion of these 30 residues from nsp2 does not confer high pathogenicity on low-virulence PRRSV isolates [31]. This finding prompted us to determine the nucleotide sequences of various PRRSVs to clarify the relationship between the pathogenic and genomic sequence characteristics of the viruses—particularly in those for which the genetic background was unknown.

*CORRESPONDENCE TO: UENISHI, H., Animal Immune and Cell Biology Research Unit, Division of Animal Sciences, National Institute of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki 305–8602, Japan. e-mail: huenishi@affrc.go.jp

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Table 1. Oligonucleotide primers used in this study

Use	Primer name	Sequence (5′→3′)	Position (nt)
CORT-MDA	ORF1a_R1	CGTCCACCGGAGYGGCTCTTC	368 − 348
	ORF1a_R2	TGCCARCCRCARTTCCCKTC	1,507 − 1,488
	ORF1a_R3	GCGARTCAAACYCACAAGCA	4,152 − 4,133
	ORF1a_R4	GGCGTCCARGCATGYCCCAT	6,292 − 6,273
	ORF1a_R5	TCYTCRTARAACMCRTCACC	7,366 − 7,347
	ORF1b_R1	GCCATRGARTTCYTRGCVGGGTA	8,550 − 8,528
	ORF1b_R2	ATGTCAAADACRTARCAATG	10,407 − 10,388
	ORF1b_R3	TACACRTCYGTYARTGTRCA	11,475 − 11,456
	GP2_R	GCGGTACRTYCKRCGCGACACCAT	12,413 − 12,390
	GP5_R	TCBGCYGAAACTYTGGTTA	14,361 − 14,343
	M_R	TGCCACCCAACACGAGGC	14,839 − 14,822
RT-PCR	5′UTR_F	ATGWCGTATAGGTGTKGGCTCTATG	1 − 25
	3′UTR_R	ACGGYCVCCCTAATTGAATAGG	15,361 − 15,340

Primer positions are described according to the genomic sequence of the reference strain EDRD-1 (AB288356.1).

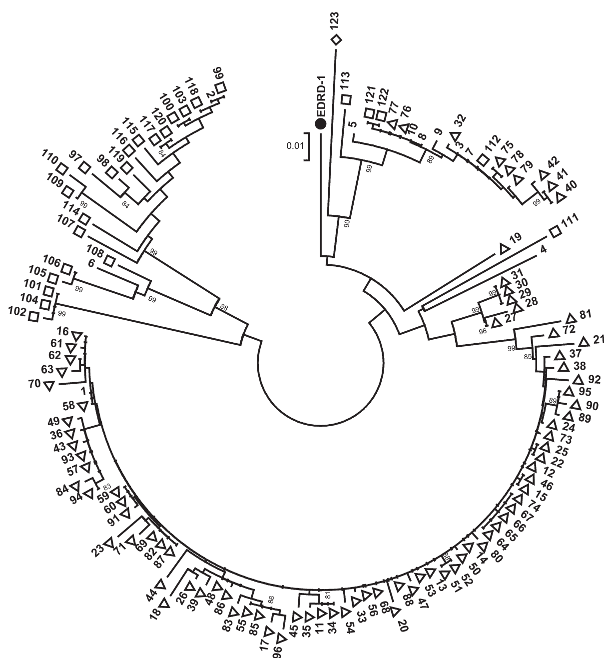

Fig. 1. Phylogenetic relationships of 124 NA-type PRRSV strains obtained from public nucleotide databases (DDBJ/EMBL/GenBank). The phylogenetic tree was constructed according to the neighbor-joining method after using ClustalW [2] to analyze the nucleotide sequences of ORF5 of these 124 strains. The accession numbers correspond to those in Table S1. Strains were isolated from Japan (closed circle), China (open triangles), the United States (open boxes) and South Korea (open diamond). Scales for lengths of branches are shown at the center. Bootstrap trials were conducted 1,000 times, and values exceeding 90% are shown at nodes.

Here, we report the development of a concise, broadly applicable method for determining the entire genomic sequences of NA-type PRRSV isolates by combination of a limited number of consensus primers for reverse transcription (RT) and amplification of the entire viral genome. We have designated this method "combination of consensus oligonucleotide RT and multiple displacement amplification" [4] (CORT-MDA).

MATERIALS AND METHODS

Design of primers for RT: We used ClustalW [2] to align the ORFs and UTRs of the 124 NA-type PRRSV strains for which complete genomic sequences were registered in public databases (DDBJ/EMBL/GenBank) (Table S1 and Fig. 1). We then designed degenerate consensus RT primers within the conserved regions thus identified (Table 1).

Preparation of genomic RNA and cDNA of viruses: Viruses sequenced in the current study were strains EDRD-1 (an NA-type reference strain in Japan [15, 29], cluster III), Nagasaki 11-14 (isolated in 2011, cluster I), Jam2 (Aomori 00; isolated in 2000, cluster II), Yamagata 10-7 (isolated in 2010, cluster III) and Aomori 10-5 (isolated in 2010, cluster III) (Fig. 2). Viral genomic RNA was prepared as described previously [29]. Briefly, porcine alveolar macrophages and the monkey kidney cell line MARC-145 were used for virus propagation. Propagated PRRSV strains in supernatants of the infected cell cultures were concentrated by sucrose density-gradient centrifugation [23], and viral genomic RNA was extracted with TRIzol reagent (Life Technologies, Tokyo, Japan). Approximately 1 μg of the resulting RNA was reverse-transcribed into first-strand cDNA by using the consensus oligonucleotide primers (Table 1) and SuperScript III kit (Life Technologies) according to the manufacturer's instructions.

Amplification and fragmentation of viral cDNA: A 4-μl aliquot of the first-strand viral cDNA underwent multiple displacement amplification [4] by using a REPLI-g UltraFast Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. A 10-μl aliquot of the 20 μl amplified DNA product was digested with 1 unit S1 nuclease (Promega, Tokyo, Japan) in 50 μl of 1× S1 nuclease buffer at 37°C for 30 min. The reaction was terminated by add-

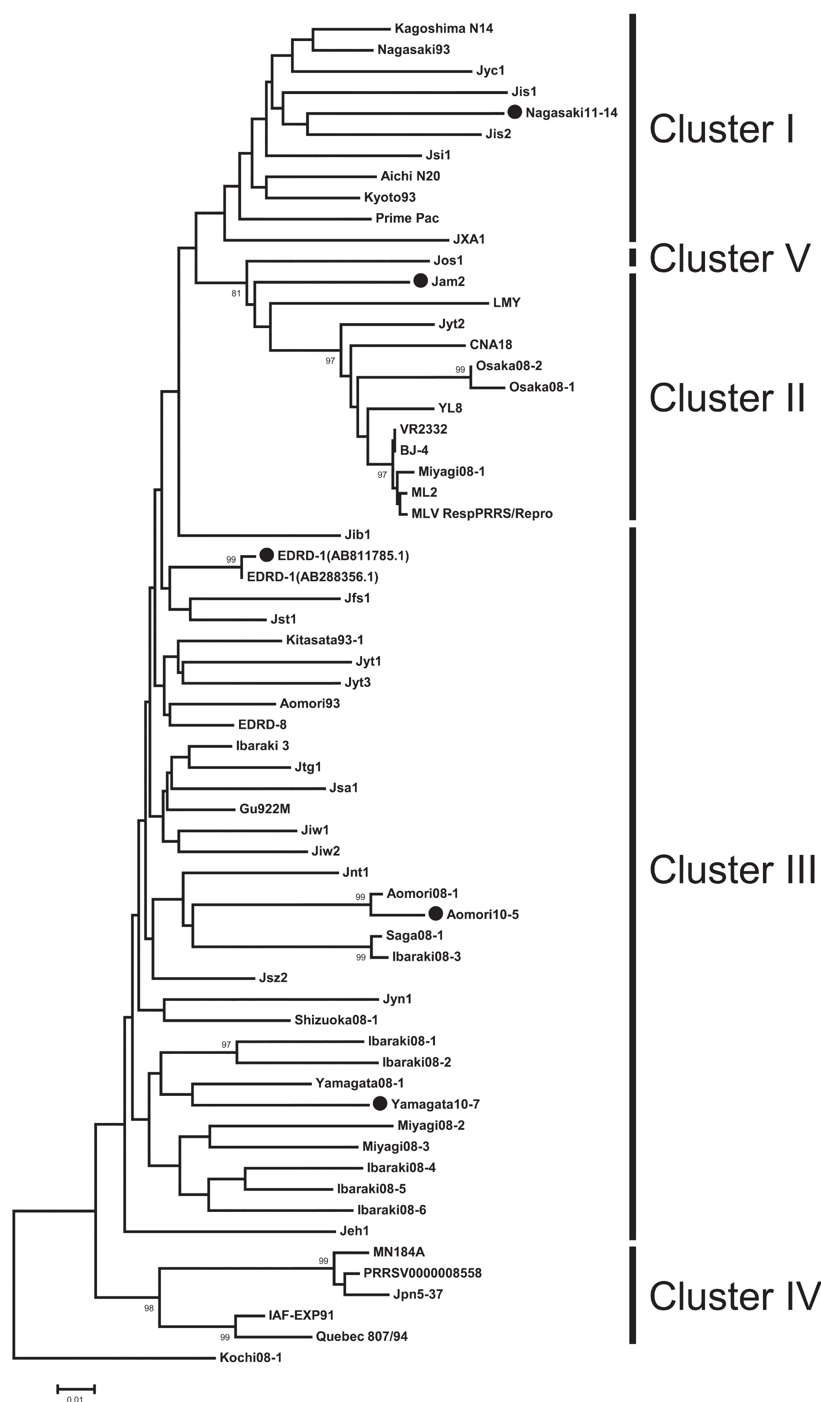

Fig. 2. Phylogenetic relationships among Japanese isolates of NA-type PRRSVs. The phylogenetic analysis was performed according to the neighbor-joining method by using the nucleotide sequences of ORF5 [11] determined according to ClustalW [2]. Scale for lengths of branches is shown at the bottom. Bootstrap trials were conducted 1,000 times, and values exceeding 80% are shown at nodes. Closed circles indicate the strains sequenced by the CORT-MDA method in this study.

ing 1 μl 0.3 M Tris–0.05 M EDTA (pH 8.0) and incubating for 10 min at 70°C. The resultant DNA was fragmented in a sonicator (duty cycle, constant; output control, 4; Sonifier 250, Branson Ultrasonics, Danbury, CT, U.S.A.) for 5 sec.

Table 2. Summary of the assemblies of shotgun sequencing reads generated by the CORT-MDA method

Strain	Number of reads	Length (bp)	Position (nt)*
EDRD-1	257	13,331	1,831 − 15,161
Nagasaki 11-14	87	7,081	524 − 7,605
	121	6,417	8,712 − 15,128
Jam2	233	12,358	2,319 − 14,677
Yamagata 10-7	277	14,458	597 − 15,054
Aomori 10-5	184	9,400	964 − 10,370
	27	2,988	10,616 − 13,606

*Positions of the contigs are indicated relative to the reference sequence EDRD-1 (AB288356.1).

The fragmented DNA solution then was desalted (Amicon Ultra-0.5 m*l* 50K, Merck Millipore, Billerica, MA, U.S.A.).

The ends of the fragmented DNA were repaired and blunted by using 5 μl 10× T4 DNA polymerase buffer (Life Technologies), 12.5 μl 2 mM dNTP mixture and 10 units *Escherichia coli* DNA polymerase (Life Technologies) at room temperature for 1 hr, followed by treatment with 5 units of T4 DNA polymerase (Life Technologies) at 37°C for 5 min and at 75°C for 20 min. A 25-μl aliquot of the blunted DNA was treated with 1 unit of calf intestine alkaline phosphatase (Promega) and 1 unit of shrimp alkaline phosphatase (Promega) at 37°C for 1 hr. Approximately 1 to 2 kb of the dephosphorylated fragments were size-selected by using 1% SeaKem GTG agarose (Cambrex, Rockland, ME, U.S.A.) and then extracted from the gel by using UltraFree DA (Merck Millipore). The recovered DNA was purified by phenol–chloroform extraction, precipitated with ethanol [17] and resuspended in 10 μl of nuclease-free H_2O.

Shotgun sequencing: Purified DNA fragments were cloned into pCR4 Blunt-TOPO (Life Technologies) according to the manufacturer's instructions, and 1 μl of the resulting plasmid was introduced into DH10B (Life Technologies). Details regarding electroporation conditions, plasmid extraction, sequencing conditions and the sequence assembly method have been described previously [14, 22]. Briefly, plasmid DNA from 192 clones cultured in Terrific Broth [17] containing ampicillin was extracted and sequenced by using M13-40 (5′- GTTTTCCCAGTCACGACGTTG −3′) and M13-reverse (5′- GGAAACAGCTATGACCATG −3′) primers. The chromatograms were basecalled by using Phred [5, 6] and assembled by using Cap3 [10].

RESULTS

Evaluation of accuracy of the CORT-MDA method: We first evaluated the CORT-MDA method by sequencing EDRD-1 [15, 29]. We obtained a contiguous sequence of 13,331 bp in length, comprising 257 shotgun reads and covering more than 86% of the entire reported EDRD-1 genomic sequence (GenBank accession number, AB288356.1). Because the sequence thus obtained lacked the 5′- and 3′-ends of the viral genome, we conducted PCR amplification using the consensus RT primers and EDRD-1-specific primers (Table S2) and sequenced the amplification products. Combining the data obtained by CORT-MDA shotgun sequencing with those from the PCR analysis yielded 15,311 bp of genomic sequence (excluding nucleotides corresponding to the consensus RT primers at both ends of the genomic sequence) (AB811785.1). The sequence of EDRD-1 obtained by our CORT-MDA method was perfectly identical with that obtained by RT-PCR, which required 43 primer pairs (Table S3). The EDRD-1 virus sequenced in this study had 33 nucleotide substitutions in comparison with the sequence registered in DDBJ/EMBL/GenBank (AB288356.1), because of mutations acquired during *in vitro* passage (Table 3). In addition, we found 2 subpopulations that had A and G, respectively, at nucleotide position 13,850 (AB811785.1) in the EDRD-1 virus sequenced here. The mutated locations and nucleotides, including position 13,850, were identical between those obtained by the CORT-MDA method and RT-PCR (data not shown), demonstrating the accuracy of the sequence by the CORT-MDA method and its potential application to detecting ongoing mutations in the viral genome.

Applicability of the CORT-MDA method to various PRRSV strains: Next, we evaluated the applicability of the CORT-MDA method by sequencing 4 PRRSV field strains belonging to various clusters classified according to their ORF5 sequences [11, 28]. Viruses Nagasaki 11-14 (isolated in 2011, cluster I), Jam2 (Aomori 00 isolated in 2000, cluster II), Yamagata 10-7 (isolated in 2010, cluster III) and Aomori 10-5 (isolated in 2010, cluster III) underwent sequencing by the CORT-MDA method (Fig. 2). More than 78% of the 384 shotgun sequencing reads were available for assembly (data not shown); this assembly generated either 1 (Jam2 and Yamagata 10-7) or 2 (Aomori 10-5 and Nagasaki 11-14) major contigs for each isolate and covered an estimated 80% to 94% of the entire PRRSV genome (Table 2). Sequencing of gaps between contigs and unread regions in the 5′- and 3′-UTRs by PCR provided contiguous genomic sequences of 15,313 bp for Nagasaki 11-14 (AB811786.1), 15,306 bp for Jam2 (AB811787.1), 15,308 bp for Yamagata 10-7 (AB811788.1) and 15,286 bp for Aomori 10-5 (AB811789.1) (excluding PCR primer sequences at both ends of the genomic sequences). Dot-plot analyses by PipMaker [18] of the 4 viral genome sequences thus obtained against the EDRD-1 genome indicated that there were no inversions or low-similarity regions between the compared sequences (Fig. S1). The data shown here demonstrate the robustness and applicability of the method for sequencing of NA-type PRRSVs in various clusters.

DISCUSSION

The CORT-MDA method for determining the genomic sequences of NA-type PRRSV isolates had several advantages in comparison with other sequencing methods. The CORT-MDA method requires far fewer oligonucleotide primers for amplification and sequencing of the PRRSV genomes. Previous methods require numerous strain-specific primers for step-by-step primer walking [16, 19, 29]. The CORT-MDA method provides a simplified sequencing procedure

Table 3. Mutations revealed through CORT-MDA sequencing of the EDRD-1 strain relative to the reference sequence (AB288356.1) registered in public databases (DDBJ/EMBL/GenBank)

Nucleotide			Region	Amino acid	
Position	Reference	CORT-MDA		Reference	CORT-MDA
158	T	C	5′-UTR	−	−
248	G	A	ORF1a	E	E
856	A	C	ORF1a	D	A
864	C	T	ORF1a	H	Y
1,866	C	T	ORF1a	L	L
1,879	T	C	ORF1a	V	A
2,165	T	C	ORF1a	A	A
2,682	G	A	ORF1a	E	K
3,246	T	C	ORF1a	S	P
3,850	C	T	ORF1a	S	F
3,994	C	A	ORF1a	S	Y
4,998	G	A	ORF1a	G	S
5,822	C	T	ORF1a	D	D
6,043	T	G	ORF1a	L	R
6,983	T	C	ORF1a	L	L
7,897	T	C	ORF1b	S	S
7,962	A	T	ORF1b	H	L
8,033	G	A	ORF1b	V	I
8,368	G	A	ORF1b	K	K
8,650	C	T	ORF1b	V	V
9,886	C	T	ORF1b	G	G
11,104	T	C	ORF1b	H	H
11,305	G	A	ORF1b	A	A
11,308	C	T	ORF1b	S	S
12,594	T	A	ORF2	Y	N
13,368*	C	T	ORF3	V	V
			ORF4	S	F
13,746	C	T	ORF4	F	F
13,837	C	T	ORF5	I	I
13,874	G	A	ORF5	D	N
13,996	G	A	ORF5	L	L
14,598	G	A	ORF6	V	V
15,059	T	G	ORF7	Y	D

Positions are indicated according to those in the reference sequence. *Position 13,368 belongs to both ORF3 and ORF4; the corresponding respective amino acids are indicated.

involving only 11 consensus primers, which can be used for strains from multiple clusters. If unread regions remain after sequencing by the CORT-MDA method, only a few additional strain-specific primers are required to obtain the entire genomic sequence. Laborious iteration of sequencing is not required.

The PRRSV genomic sequence that we obtained by using the CORT-MDA method was highly accurate. Our method precisely detected mutations in the EDRD-1 genome that were generated during passage, and these mutations were confirmed by the conventional PCR-based sequencing method. In this regard, our CORT-MDA method was able to detect ongoing mutations in the PRRSV genome, indicating that the method can be applicable to tracing PRRSV genomes, which are unstable and accumulate numerous mutations during a limited number of generations [8, 26, 30].

Most importantly, the CORT-MDA method is robust and versatile regardless of the genetic background of the NA-type PRRSV to be sequenced. Coding sequences of the PRRSV genomes determined by the CORT-MDA method in this study showed identities from 84.0% (Jam2 vs. Yamagata 10-7) to 88.8% (EDRD-1 vs. Aomori 10-5), demonstrating that the method is applicable for sequencing viral genomes that are more than 10% divergent at the nucleotide level. The consensus primers for our method were designed by using the 124 full-length genomic sequences of NA-type viruses, including isolates derived from the United States, China, Korea and Japan and several strains known to be HP-PRRSVs. Therefore, our method likely can be used to determine the genomic sequence of any NA-type PRRSV virus strain, regardless of its country of origin and even if the strain is newly emerged. Our rapid, convenient and robust method for the sequencing of PRRSV genomes will help greatly to elucidate the genetic factors that affect the pathogenicity

of PRRSVs by enabling detailed comparisons between the genomic sequences and virulence of different strains.

The CORT-MDA method presented here includes a step for purification by ultracentrifugation through a sucrose gradient. However, if the ultracentrifugation step is dispensable, the method will obtain broader applicability, particularly in the case of samples derived from the field. Samples collected in the field or from piggeries under epidemic conditions of PRRS—such as viremic plasma from pigs reared in conventional environments—are often contaminated with RNA derived from other microorganisms and host animals. In the CORT-MDA method, the PRRSV genome is converted into cDNA, which is much more stable during transportation to laboratories than is the RNA genome and can be amplified to the amount appropriate for shotgun sequencing. Furthermore, because of selective amplification of viral cDNA by using PRRSV-specific consensus primers, the CORT-MDA method can be modified for RNA samples that are not highly purified. Further improvement of the CORT-MDA method may reduce the need for complicated sample-handling measures at the often insufficiently equipped facilities near epidemic areas.

We conducted shotgun sequencing of the amplified viral cDNAs according to the Sanger method. The method thus presented can be conducted with traditional sequencers and computers without high specification. However, it is worth considering the use of next-generation sequencing technology, which is becoming popular and is potentially more cost-effective than is our current method. In addition, establishment of a CORT-MDA–based sequencing method for EU-type PRRSV strains is worth considering. A random PCR cloning approach that generated partial genomic sequences for an EU-type PRRSV strain with an unknown genetic background was reported recently [24]. However, the genomic sequence (before filling of gaps) obtained by using the random PCR cloning method was less than half of the entire PRRSV genome, and the method's robustness in regard to viruses derived from various clusters has not been well confirmed. Development of the CORT-MDA-based sequencing method for EU-type strains will be beneficial for dissecting PRRS outbreaks, because NA- and EU-type strains cause analogous clinical signs [7] and can be epidemic simultaneously in the same location. Regardless of the type or cluster to which the strain belongs, CORT-MDA–based methodology will facilitate rapid identification of the prevailing PRRSV isolate on an affected farm.

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