NOTE *Virology*

Detection of Bovine Group A Rotavirus Using Rapid Antigen Detection Kits, RT-PCR and Next-Generation DNA Sequencing

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ABSTRACT. We investigated the sensitivity of human rotavirus rapid antigen detection (RAD) kits, RT-PCR and next-generation DNA sequencing (NGS) for detection of bovine group A rotavirus (RVA). The Dipstick 'Eiken' Rota (Dipstick) showed the highest sensitivity out of the seven RAD kits against all selected strains in limited dilution analyses, which was consistent with the results for equine rotavirus previously reported. RT-PCR had 10^0–10^3-fold higher sensitivity than the Dipstick. NGS using thirteen RT-PCR-negative fecal samples revealed that all samples yielded RVA reads and especially that two of them covered all 11 genome segments. Moreover, mapping reads to reference sequences allowed genotyping. The NGS would be sensitive and useful for analysis of less dependent on specific primers and screening of genotypes.

KEY WORDS: antigen detection, next-generation DNA sequencing, rapid antigen detection kit, RT-PCR, species A bovine rotavirus.

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Group A rotavirus (RVA) is a causative agent of diarrhea in newborn animals [3, 14]. Bovine RVAs are distributed to young calves worldwide and are causes of an economic loss in the cattle industry [9, 13]. Since there are many infectious causes for neonatal calf diarrhea, laboratory assays are required of diagnosis of RVA infection [3]. RVA is a non-enveloped, triple-layered icosahedral virus possessing an 11-segmented double-stranded RNA genome that encodes six structural viral proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and six nonstructural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) [3]. VP4 and VP7 are the outer capsid proteins that independently elicit neutralizing antibodies, which mediate protective immunity [3]. Serotype is defined by a classification based on the neutralization of viral infectivity; however, the serotype of a rotavirus isolate can be predicted from the sequence of these regions [5]. Hence, genotyping is important for the clinician and diagnostician to obtain such information to interpret the results of vaccine studies and epidemiologic surveillance [6, 7].

Several rapid antigen detection (RAD) kits for human RVA are commercially available. Nemoto *et al.* [11] reported the usefulness of diagnosing with equine RVA of RAD kits; however, no paper has been reported for bovine RVA. The RT-PCR method of detecting VP7 with the primer pair Beg9/End9, which has been applied to each human serotype virus [4], is usually used in livestock hygiene service centers for detection bovine RVA in Japan. Recently, Zhu *et al.* demonstrated a one-step duplex RT-PCR for bovine RVA amplifying the VP6 gene. In this study, we compared the sensitivity of RAD kits and RT-PCR for the detection of bovine RVA. Furthermore, we evaluated the usefulness of next-generate DNA sequencing (NGS) for direct detection from fecal samples.

Three bovine RVA strains (IS1 (G6P [5]), NCDV (G6P [1]) and KK3 (G10P [11])) were subjected to study of sensitivity of detection. An established fetal rhesus monkey cell line (MA-104) was used for virus propagation, and culture fluids were used for tests. The titers of each virus stocks were $10^{7.0}$ $TCID_{50}$/m*l*. Thirteen fecal samples were taken twice

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Table 1. Detection limits of individual methods for seven bovine rotavirus strains

Rotavirus strains	Methods	Commercial kits or primers	Viral dilution								
			$\times10^0$	$\times10^1$	$\times10^2$	$\times10^3$	$\times10^4$	$\times10^5$	$\times10^6$	$\times10^7$	$\times10^8$
IS-1/96	Immunochromatographic assay	Dipstick 'Eiken' Rota	+	+	+	+					
		RapidTest Rota-Adeno	+	+	+						
		BD Rota/Adeno Examan stick	+	+							
		Rapid-SP "Rota"	+								
		ImmunoCard ST Rotavirus	+								
	Latex agglutination	Rotalex Dry	+	+							
		Rotascreen	+								
	RT-PCR	Beg9/End9	+	+	+	+	+	+	+		
		BRVF/BRVR	+	+	+	+	+	+	+		
NCDV	Immunochromatographic assay	Dipstick 'Eiken' Rota	+	+	+	+					
		RapidTest Rota-Adeno	+	+	+						
		BD Rota/Adeno Examan stick	+	+	+						
		Rapid-SP "Rota"	+								
		ImmunoCard ST Rotavirus	+	+							
	Latex agglutination	Rotalex Dry	+	+							
		Rotascreen	+								
	RT-PCR	Beg9/End9	+	+	+	+	+	+			
		BRVF/BRVR	+	+	+	+	+	+			
KK3	Immunochromatographic assay	Dipstick 'Eiken' Rota	+	+	+	+					
		RapidTest Rota-Adeno	+	+	+						
		BD Rota/Adeno Examan stick	+	+	+						
		Rapid-SP "Rota"	+	+							
		ImmunoCard ST Rotavirus	+	+							
	Latex agglutination	Rotalex Dry	+	+							
		Rotascreen	+								
	RT-PCR	Beg9/End9	+	+	+	+					
		BRVF/BRVR	+	+	+	+	+	+			

daily from seven beef farms located in Ishikawa Prefecture, Japan, that had an epidemic history of RVA. These specimens were collected between 2012 and 2013 from calves (aged<1 year) that showed watery or sticky diarrhea that was white, yellow or greenish brown.

Five immunochromatographic assays, the Dipstick 'Eiken' Rota (Eiken Chemical Co., Ltd., Tokyo, Japan), RapidTest Rota Adeno (Sekisui Medical Co., Ltd., Tokyo, Japan), BD Rota/Adeno Examan stick (Becton, Dickinson and Franklin Lakes, NJ, U.S.A.), Rapid-SP "Rota" (DS Pharma Biomedical Co., Ltd., Osaka, Japan) and Immuno-Card ST Rotavirus (TFB, Inc., Tokyo, Japan), and two latex agglutination assays (Rotalex Dry (Sekisui Medical Co., Ltd.) and Rotascreen (Denka Seiken Co., Ltd., Tokyo, Japan) were conducted in this study. Serial 10-fold dilutions of IS1, NCDV and KK3 were prepared in phosphate buffer saline (PBS), and 100 μl of viral dilutions were mixed with the extraction solution provided with each kit. The procedures were conducted according to the manufacturer's instructions for each kit. For RT-PCR, 100 μl was used to extract viral RNA from each sample using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instrutions. All extracted RNAs were denatured at 97°C for 3 min and immediately placed on ice. Then, one-step RT-PCR was performed with PrimeScript One Step RT-PCR Kit Ver. 2 (Takara, Otsu, Japan) using the extracted RNA described above. The primer pair Beg9/End9 [4] was used for amplification of the full-length VP7 under the following conditions: 50°C for 30 min and 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1 min with a final extension of 72°C for 10 min. The primer pair BRVF/BRVR described by Zhu *et al.* (2010) [21] was used for amplification of partial VP6 gene under the following conditions: 50°C for 30 min and 94°C for 5 min, followed by 35 cycles of 94°C for 50 sec, 55°C for 50 sec and 72°C for 1 min with a final elongation of 72°C for 10 min. The RT-PCR products were electrophoresed on 2% agarose gel.

The results regarding the detection limits of RAD kits and RT-PCR for the three bovine RVA strains are shown in Table 1. Limited dilution analyses showed that the Dipstick exhibited the highest sensitivity to all strains of bovine RVA of the seven RAD kits. RT-PCR using primer pairs Beg9/End9 and BRVF/BRVR had 10^0–10^3 and 10^2–10^3-fold higher sensitivity than the Dipstick, respectively. Furthermore, limited dilution analyses using two fecal samples were performed and revealed that RT-PCR using primer pairs Beg9/End9 and BRVF/BRVR had 10^1–10^2 and 10^1–10^3-fold higher sensitivity than the Dipstick, respectively (data not shown).

Table 2. Read numbers and coverages of bovine group a rotavirus for each segment obtained from next-generation DNA sequencing

Description of sample				Number of bovine rotavirus group A reads (% of RNA segment covered)										
Calf No.	Farm	Breed	Days after birth	VP1	VP2	VP3	VP4	VP6	VP7	NSP1	NSP2	NSP3	NSP4	NSP5
1	A	Holstein	3	3 (4.6)	0	1 (1.9)	0	0	0	0	0	1 (4.7)	0	0
2	B	Holstein	121	8 (10.9)	3 (5.6)	7 (8.9)	0	2 (7.5)	0	0	0	4 (15.5)	1 (6.8)	0
3	B	Holstein	2	24 (30.6)	17 (24.4)	29 (37.8)	20 (33.1)	13 (40.2)	10 (31.5)	3 (9.5)	7 (25.1)	10 (38.1)	5 (26.9)	6 (33.1)
4	C	Japanese Black	83	0	0	0	0	0	0	0	0	1 (4.7)	0	0
5	D	Japanese Black	10	0	0	0	1 (2.2)	0	0	1 (3.2)	0	2 (9.5)	0	0
6	E	Japanese Black	13	0	0	2 (3.8)	0	1 (3.8)	1 (4.8)	0	1 (4.8)	2 (9.5)	1 (6.8)	0
7	F	Japanese Black	95	0	0	0	0	0	0	0	1 (4.8)	0	1 (6.8)	0
8	F	Japanese Black	156	59 (54.5)	19 (27.4)	94 (66.4)	29 (45.7)	76 (85.5)	73 (75.0)	60 (57.0)	116 (97.2)	191 (88.6)	38 (51.8)	72 (72.3)
9	G	Japanese Black	158	0	0	2 (3.9)	0	0	0	1 (3.2)	0	1 (4.7)	0	0
10	H	Japanese Black	178	2 (1.5)	0	1 (2.0)	0	0	0	0	0	3 (14.2)	0	0
11	H	Japanese Black	177	4 (6.1)	2 (3.8)	5 (8.1)	3 (6.5)	4 (15.0)	2 (9.4)	2 (3.5)	1 (4.8)	6 (21.0)	1 (6.8)	0
12	H	Japanese Black	209	5 (7.7)	13 (21.0)	13 (21.7)	2 (4.3)	5 (11.6)	1 (4.8)	1 (3.2)	3 (14.4)	14 (43.9)	1 (6.7)	0
13	I	Japanese Black	362	3 (3.3)	0	8 (14.5)	1 (2.2)	0	0	2 (4.1)	2 (9.6)	1 (4.7)	0	1 (7.6)

For NGS, we performed RNA-seq using commercial kits. First, total RNA was prepared from fecal samples diluted 1:9 (W/V) in sterile PBS using ISGEN LS (Nippon Gene, Tokyo, Japan), and this was followed by DNase I treatment (Takara, Otsu, Japan). Quantification of the RNA samples was performed using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, U.S.A.). RNA samples were normalized to 10–100 *n*g of RNA, and RNA library preparation was performed using a NEBNext® Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, U.S.A.) according to the manufacturer's intruction. Briefly, RNA was fragmented, and then double-stranded cDNA was synthesized. The resulting double-stranded cDNA was end-repaired before ligation of Illumina-specific adaptors and size selection of the libraries with approximately 200 bp inserts and was finally PCR enriched. After assessing the library quality on a Bioanalyzer® (Agilent Technologies, Santa Clara, CA, U.S.A.), sequencing was carried out on a MiSeq sequencer (Illumina, San Diego, CA, U.S.A.) using 51 single-end reads. Data analysis was performed using the MiSeq Reporter Software (Illumina) to generate sequence data in FASTQ format. To obtain consensus sequences for 11 segments of bovine RVA, respectively, reads were aligned with the sequences of all 11 segments (G6-P [5]-I2-R2-C2-M2-A3-N2-T6-E2-H3) of the bovine RVA strain WC3 [1, 15] as reference sequences using CLC Genomics Workbench (CLC bio, Cambridge, MA, U.S.A). For VP4, VP7 and NSP1, the sequences of NCDV Lincoln (P [1]), B223 (P [11]) [17], A5 (G8) [16], 61A (G11) [15] and B383 (A13) [8] were also used as references.

Thirteen fecal samples, which were negative for the Dipstick and RT-PCR, were subjected to next-generation DNA sequencing. Bovine RVA reads were recovered from all samples (Table 2). The two of them yielded 155 and 828 reads covering 11 segments (24 and 59 VP1, 17 and 19 VP2, 29 and 94 VP3, 20 and 30 VP4, 13 and 76 VP6, 21 and 73 VP7, 3 and 60 NSP1, 7 and 117 NSP2, 10 and 191 NSP3, 5 and 38 NSP4 and 6 and 72 NSP5 sequences). Mapping the 50-mer short reads of two samples to the reference VP4 and VP7 sequences allowed genotyping of G and P. Almost all of the reads of No. 3 and No. 8 were aligned with G10P [11] and G6P [5] reference sequences, respectively (Fig. 1).

There are many assays, such as those using electron microscopy, virus isolation and gel electrophoresis of genomes, for the detection of rotavirus in specimens [12, 18, 20]. Though these methods are gold standards for diagnosis of bovine RVA, they require expensive equipment and complicated techniques. In addition, it is difficult to isolate RVA using cell lines [18]. In the clinical field, clinical veterinarians need rapid and sensitive detection methods that are routinely available in daily clinical practice. Of the seven commercial kits in this study, the Dipstick showed the highest sensitivity to the three bovine RVA strains, and these results were consistent with those obtained with equine RVA [11]. Although the sensitivity of the Dipstick was lower that than with RT-PCR, the Dipstick would be useful for field diagnosis because of its simple, easy and rapid procedure. RT-PCR has been employed as a useful method for the detection of pathogens. In Japan, the primer pair Beg9/End9, which has been applied to each human serotype virus [4], is usually used in livestock hygiene service centers for the detection and genotyping of VP7 of bovine RVA. Genotyping VP7 using this primer pair is useful. In a limited dilution study, the primer pair Beg9/End9 amplified VP7 of KK3 up to a 10^3 dilution; on the other hand, the primer pair BRVF/BRVR amplified VP6 of KK3 up to a 10^5 dilution. The reason for this may be the variability of the VP7 reverse primer region, and this indicates that amplification of VP6 is better for detection of bovine RVA.

Finally, we demonstrated the detection of RVA from fecal samples using a next-generation DNA sequencer. Exhaustive investigation is useful for directly detecting pathogenic viruses without advance genetic information [2, 10, 19]. In this study, all samples yielded reads of the RVA genome in spite of being negative for RT-PCR. Moreover, the RVA reads from two samples covered all 11 segments of RVA, and the VP4 and VP7 reads could be divided into genotypes using CLC Genomics Workbench by mapping to reference sequences of representative strains of VP4 and VP7 genotypes. Although

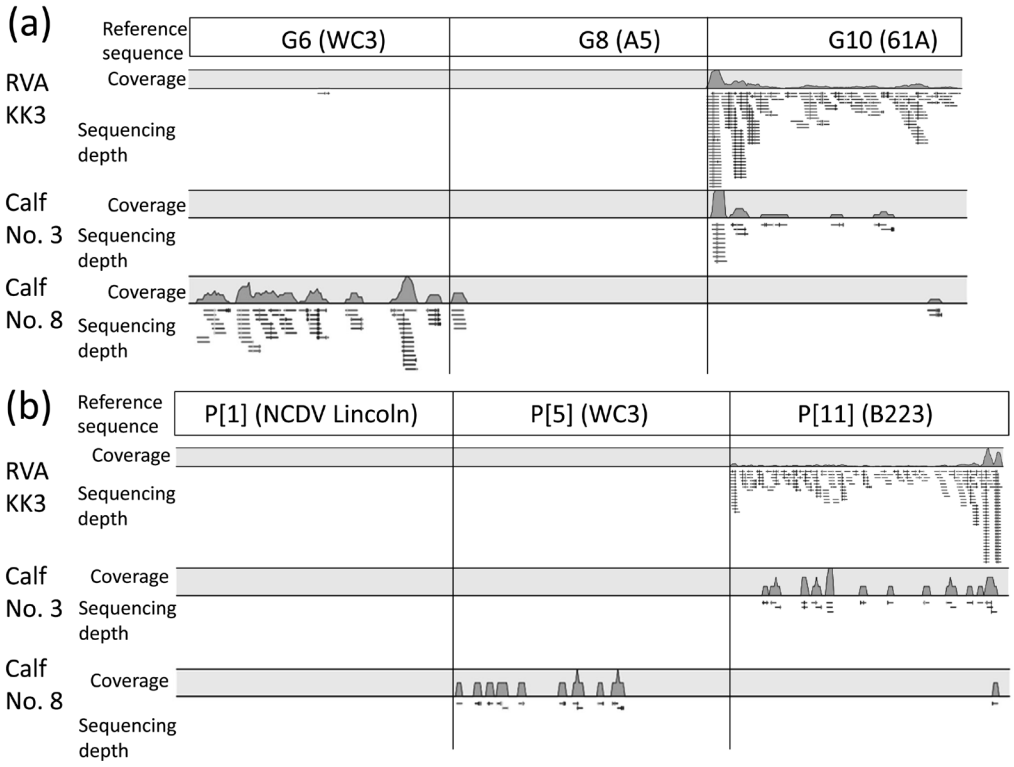

Fig. 1. Mapping of rotavirus VP4- and VP7-specific reads of calf No. 3 and 8 against bovine reference sequences of (a) VP7 and (b) VP4. Positional sequence coverage and sequencing depth of VP7 (G6, G8 and G10) and VP4 (P [1], P [5] and P [11]) of calf No. 3 and 8 based on reference assembly to WC3 (AY050272), A5 (D01054), 61A (X53403), NCDV Lincoln (AB119636), WC3 (AY05071) and B223 (D13394).

the cost and expensive equipment make it unlikely that NGS will soon be the diagnostic standard worldwide, the present investigation by RNA-seq is useful for less sequence-depend detection of bovine RVA from fecal samples and screening of the genotypes.

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