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

Fujiko MINAMI-FUKUDA1)#, Makoto NAGAI2,3)#, Hikaru TAKAI1), Toshiaki MURAKAMI1), Shinobu TSUCHIKA2), Sachiko OKAZAKI2), Mami OBA2), Naomi NISHIURA2), Yukiko SASSA2), Tsutomu OMATSU2), Tetsuya FURUYA2,4), Satoshi KOYAMA5), Junsuke SHIRAI2,3), Hiroshi TSUNEMITSU6), Yoshiko FUJII7), Kazuhiko KATAYAMA7) and Tetsuya MIZUTANI2)*

1)Ishikawa Nanbu Livestock Hygiene Service Center, Saida, Kanazawa, Ishikawa 920–3101, Japan
2)Research and Education Center for Prevention of Global Infectious Diseases of Animal, Tokyo University of Agriculture and Technology, Saiwai, Fuchu, Tokyo 183–8509, Japan
3)Laboratory of Epizootiology, Department of Veterinary Medicine, Faculty and Agriculture, Tokyo University of Agriculture and Technology, Saiwai, Fuchu, Tokyo 183–8509, Japan
4)Laboratory of Microbiology, Department of Veterinary Medicine, Faculty and Agriculture, Tokyo University of Agriculture and Technology, Saiwai, Fuchu, Tokyo 183–8509, Japan
5)Laboratory of Ethology, Department of Veterinary Medicine, Faculty and Agriculture, Tokyo University of Agriculture and Technology, Saiwai, Fuchu, Tokyo 183–8509, Japan
6)National Agriculture and Food Research Organization, Toyohira, Sapporo, Hokkaido 062–0045, Japan
7)Department of Virology II, National Institute of Infectious Diseases, Gakuen, Musashi-murayama, Tokyo 208–0011, Japan

(Received 21 May 2013/Accepted 19 July 2013/Published online in J-STAGE 2 August 2013)

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 (RV A). 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 10^3–10^5-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 specific primers and screening of genotypes.

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

doi: 10.1292/jvms.13-0265; J. Vet. Med. Sci. 75(12): 1651–1655, 2013

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. 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 TCID50/ml. Thirteen fecal samples were taken twice
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 ImmunoCard 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 instructions. 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 100–103 and 102–103-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 101–102 and 101–103-fold higher sensitivity than the Dipstick, respectively (data not shown).

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

| Rotavirus strains | Methods | Commercial kits or primers | Viral dilution |
|-------------------|---------|-----------------------------|---------------|
|                   |         |                             | ×10⁰ | ×10¹ | ×10² | ×10³ | ×10⁴ | ×10⁵ | ×10⁶ | ×10⁷ | ×10⁸ |
| 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                  | +   | +   | +   | +   | +   | +   | +   | +   | +   |
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 ng 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 instructions. Briefly, RNA was fragmented, and then double-stranded cDNA was synthesized. The resulting double-stranded cDNA was end-repaired before ligating Illumina-specific adaptors and size selection of the resulting 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 all segments. 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 than that 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 10^5 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...
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.

ACKNOWLEDGMENT. This work was supported by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare, Japan (grant H24-shinkou-ippan-005).

REFERENCES

1. Ciarlet, M., Hyser, J. M. and Estes, M. K. 2002. Sequence analysis of the VP4, VP6, VP7, and NSP4 gene products of the bovine rotavirus WC3. Virus Genes 24: 107–118. [Medline] [CrossRef]
2. Djikeng, A., Halpin, R., Kuzmickas, R., DePasse, J., Feldblum, J., Sengamalay, N., Afonso, C., Zhang, X., Anderson, N. G., Ghedin, E. and Spiro, D. J. 2008. Viral genome sequencing by random primer methods. BMC Genomics 9: 5. [Medline] [CrossRef]
3. Estes, M. K. and Kapikian, A. Z. 2007. Rotaviruses. pp. 1917–1974. In: Fields Virology, 5th ed. (Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B. and Straus, S. E. eds.), Lippincott, Philadelphia.
4. Gouvea, V., Glass, R. I., Woods, P., Taniguchi, K., Clark, K. F., Forrester, B. and Fang, Z. 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J. Clin. Microbiol. 28: 276–282. [Medline] [CrossRef]
5. Green, K. Y., Sears, J. F., Taniguchi, K., Midthun, K., Hoshino, Y., Gorziglia, M., Nishikawa, K., Urasawa, S., Kapikian, A. Z., Chanock, R. M. and Flores, J. 1988. Prediction of human rotavirus serotype by nucleotide sequence analysis of the vp7 protein gene. J. Virol. 62: 1819–1823. [Medline]
6. Matthijnssens, J., Joelsson, D. B., Warakomski, D. J., Zhou, T., Mathis, P. K., van Maanen, M.H., Ranheim, T. S. and Cirrlet, M. 2010. Molecular and biological characterization of the 5 human-bovine rotavirus (WC3)-based reassortant strains of the pentavalent rotavirus vaccine, RotaTeq®. Virology 403: 111–127. [Medline] [CrossRef]
7. Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S. M., Palombo, E. A., Iturria-Gomara, M., Maes, P., Patton, J. T., Rahman, M. and Ranst, M. V. 2008. Full genome-based classification of rotaviruses reveals a common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J. Virol. 82: 3204–3219. [Medline] [CrossRef]
8. Matthijnssens, J., Potgieter, C. A., Ciarlet, M., Parreño, V., Martella, V., Bányaí, K., Garaicoechea, L., Palombo, E. A., Novo, L., Zeller, M., Arista, S., Gerna, G., Rahman, M. and Van Ranst, M. 2009. Are human P[14] rotavirus strains the result of interspecies transmissions from sheep or other ungulates that belong to the mammalian order Artiodactyla? J. Virol. 83: 2917–2929. [Medline] [CrossRef]
9. Mebus, C. A., Underdahl, N. R., Rhodes, M. B. and Twiehaus,
M. J. 1969. Further studies on neonatal calf diarrhea virus. Proc. Annu. Meet. U. S. Anim. Health Assoc. 73: 97–99. [Medline]

10. Nakamura, S., Yang, C. S., Sakon, N., Ueda, M., Tougan, T., Yamashita, A., Goto, N., Takahashi, K., Yasunaga, T., Ikuta, K., Mizutani, T., Okamoto, Y., Tagami, M., Morita, R., Maeda, N., Kawai, J., Hayashizaki, Y., Nagai, Y., Horii, T., Iida, T. and Nakaya, T. 2009. Direct metagenomic detection of viral pathogens in nasal and fecal specimens using an unbiased high-throughput sequencing approach. PLoS ONE 4: e4219. [Medline] [CrossRef]

11. Nemoto, M., Hata, H., Higuchi, T., Imagawa, H., Yamanaka, T., Niwa, H., Bannai, H., Tsujimura, K., Kono, T. and Matsumura, T. 2010. Evaluation of rapid antigen detection kits for diagnosis of equine rotavirus infection. J. Vet. Med. Sci. 72: 1247–1250. [Medline] [CrossRef]

12. Rodger, S. M. and Holmes, I. H. 1979. Comparison of the genome of simian, bovine, and human rotavirus by gel electrophoresis and detection of genomic variation among bovine isolates. J. Virol. 30: 839–846. [Medline]

13. Saif, L. J. and Jiang, B. 1994. Nongroup A rotaviruses of humans and animals. Curr. Top. Microbiol. Immunol. 185: 339–371. [Medline] [CrossRef]

14. Saif, L. J., Rosen, B. I. and Parwani, A. V. 1994. Animal rotavirus. pp 279–367. In: Viral infections of the gastrointestinal tract (Kapikian, A. Z. ed.), Marcel Dekker, Inc., New York.

15. Taniguchi, K., Urasawa, T. and Urasawa, S. 1993. Independent segregation of the VP4 and the VP7 genes in bovine rotaviruses as confirmed by VP4 sequence analysis of G8 and G10 bovine rotavirus strains. J. Gen. Virol. 74: 1215–1221. [Medline] [CrossRef]

16. Taniguchi, K., Pongsuwanna, Y., Choonthanom, M. and Urasawa, S. 1990. Nucleotide sequence of the VP7 gene of a bovine rotavirus (strain 61A) with different serotype specificity from serotype 6. Nucleic Acids Res. 18: 4613. [Medline] [CrossRef]

17. Taniguchi, K., Urasawa, T., Pongsuwanna, Y., Choonthanom, M., Jayavasu, C. and Urasawa, S. 1991. Molecular and antigenic analyses of serotypes 8 and 10 of bovine rotaviruses in Thailand. J. Gen. Virol. 72: 2929–2937. [Medline] [CrossRef]

18. Urasawa, T., Urasawa, S. and Taniguchi, K. 1981. Sequential passages of human rotavirus in MA-104 cells. Microbiol. Immunol. 25: 1025–1035. [Medline]

19. Watanabe, S., Mizutani, M., Sakai, K., Kato, K., Towya, Y., Fukushi, S., Saijo, M., Yoshikawa, Y., Kurane, I., Morikawa, S. and Akashi, H. 2008. Ligation-mediated amplification for effective rapid determination of viral RNA sequences (RDV). J. Clin. Virol. 43: 56–59. [Medline] [CrossRef]

20. Yolken, R. and Wilde, J. 1994. Assays for detecting human rotavirus. pp. 251–278. In: Viral Infections of the Gastrointestinal Tract, 2nd ed. (Kapikian, A. Z. ed.), Marcel Dekker, New York.

21. Zhu, W., Dong, J., Haga, T., Goto, Y. and Sueyoshi, M. 2011. Rapid and sensitive detection of bovine coronavirus and group A bovine rotavirus from fecal samples by using one-step duplex RT-PCR assay. J. Vet. Med. Sci. 73: 531–534. [Medline] [CrossRef]