



G and P genotype profiles of rotavirus a field strains circulating in a vaccinated bovine farm as parameters for assessing biosecurity level

Md. Amirul HASAN¹), Md. Humayun KABIR¹), Yu MIYAOKA¹),
Makiko YAMAGUCHI²) and Kazuaki TAKEHARA^{1,2})*

¹Laboratory of Animal Health, Cooperative Division of Veterinary Sciences, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan

²Laboratory of Animal Health, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan

ABSTRACT. After improvement of hygiene protocols on boots in a bovine operation (farm A) in Ibaraki, Japan in September 2017, mortality of calves and the detection of 4 viral pathogen indicators, including bovine rotavirus A (RVA), became significantly low for one year. Subsequently, in the present study, these indicators and mortality were monitored and confirmed all were still low, except for the detection rate of bovine RVA in calves less than 3 weeks old. The present study aimed to investigate G and P genotypic profiles of RVAs in farm A from 2018 to 2020. Molecular analysis using semi-nested multiplex RT-PCR of positive RVAs (n=122) and sequencing of selected samples revealed the presence of G6, G8, G10, P[1], P[5] and P[11] genotypes and the prevalence of G and/or P combination and mixed infections. The most common combination of G and P types was G10P[11] (41.8%), followed by mixed infection with G6+G10P[5] (11.5%). Phylogenetic analysis of RVAs showed clustering with bovine and other animal-derived RVA strains, suggesting the possibility of multiple reassortant events with strains of bovine and others animal origins. Noteworthy as well is that vaccinated cattle might fail to provide their offspring with maternal immunity against RVA infections, due to insufficient colostrum feeding. Our findings further highlight the importance of RVA surveillance in bovine populations, which may be useful to improving effective routine vaccination and hygiene practices on bovine farms.

KEYWORDS: biosecurity, bovine rotavirus A, genotyping, phylogenetic analysis, VP4 and VP7 genes

J. Vet. Med. Sci.

84(7): 929–937, 2022

doi: 10.1292/jvms.22-0026

Received: 18 January 2022

Accepted: 25 April 2022

Advanced Epub:

4 May 2022

Calf diarrhea is a commonly reported disease in young animals, and still a major cause of productivity and economic loss to cattle producers worldwide [9]. In Japan, the economic losses due to diarrhea in calves are estimated at approximately one billion yen per year according to the 2017 annual report from the Ministry of Agriculture, Forestry and Fisheries of Japan [31]. Rotavirus A (RVA) is the main pathogen associated with neonatal calf diarrhea (NCD), a syndrome that constitutes a major cause of mortality of calves before weaning and causes significant economic losses to the livestock industry [19, 35]. For humans, RVA also plays a major role as a cause of severe diarrhea in children worldwide, and is estimated to cause more than 215,000 deaths each year among children below 5 years of age [43]. RVA also causes diarrhea in a variety of mammals and birds worldwide.

RVAs are classified by a binary system of G and P genotypes for VP7 and VP4 gene respectively, determined by sequence analyses [14]. To date, RVAs have been classified into 41 G and 57 P genotypes [37]. Among them, G genotypes G6, G8 and G10, and P genotypes P[1], P[5] and P[11] are considered as major genotypes of bovine RVAs [29]. In addition, several G genotypes (G15, G18, G21, and G24) and P genotypes (P[14], P[17], P[29], and P[33]) have been detected sporadically in Japan [1, 32]. Certain animal RVA strains have antigenic similarities to some human strains and this may be an indication for animals playing a role as a source of RVA infections in humans [17].

Our previous study demonstrated that bovine RVA, bovine torovirus (BToV), bovine enterovirus (BEV) and bovine coronavirus (BCV) can be employed as viral pathogen indicators to monitor the biosecurity levels of bovine farms [44]. After improvement of hygiene protocols on boots by exchanging boots and appropriate usage of a footbath at the entrance of calf sheds in bovine farm A in Ibaraki prefecture in September 2017, mortality of calves and the detection of viral pathogen indicators became significantly

*Correspondence to: Takehara, K.: takehara@cc.tuat.ac.jp, Laboratory of Animal Health, Cooperative Division of Veterinary Sciences, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan (Supplementary material: refer to PMC <https://www.ncbi.nlm.nih.gov/pmc/journals/2350/>)

©2022 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

lower for one year [42].

Vaccination of cows at the end of pregnancy is one of the main health management strategies for the control and prophylaxis of bovine RVA infections. Therefore, the aims of the present study are to monitor mortality and viral pathogen indicators such as RVA, BToV, BEV and BCV following one year, to determine the frequency of RVA infections, as well as to conduct a genetic analysis of the circulating RVAs. The ultimate goals of this project are to improve vaccination programs and establish good hygiene practice at bovine farms.

MATERIALS AND METHODS

Hard information and Specimen collection

The study was conducted on bovine farm A in Japan [42] that has about 10,000 milking cows and produces around 120 calves of Japanese Black or F1 hybrids (Crossbred of Japanese black and Holstein) monthly. In farm A, the mother cows had been routinely vaccinated with a “Kyotobiken” Bovine diarrhea 5 combo inactivated vaccine containing RVAs serotypes Gunma 8701 strain G6P[1], Hyogo 9301 strain G6P[5] and Shimane 9501 strain G10P[11], as well as BCV, and calves were fed colostrum from their mothers for one day after birth. Calves are reared into individual calf hatches in calf sheds right after their birth and then raised in the same hatch until two months of age.

A total of 560 fecal samples were collected from calves at 1 to 8 weeks of age from December 2018 to February 2020, at intervals of two months. At each sampling, around 40 samples were collected from calves at three weeks of age or younger, as well as from calves older than three weeks of age. Fecal samples were kept cool during the shipment from the farm to the laboratory and stored at -20°C .

RNA extraction and detection of index viruses by one-step multiplex reverse-transcription polymerase chain reaction (RT-PCR)

Total viral RNA was extracted using ISOGEN-LS (Nippon Gene Co., Ltd., Tokyo, Japan) from supernatants of 10% fecal homogenates and tested for RVA, BToV, BEV and BCV genes by one-step multiplex RT-PCR using PrimeScript One Step RT-PCR Kit ver.2 (Takara Bio Inc., Kusatsu, Japan), as shown previously [42, 44].

Mortality of calves less than two months old

Total numbers of dead calves less than two months of age were recorded every month during the study period. Mortality was calculated as described previously [42].

G and P genotyping by semi-nested multiplex RT-PCR

RVA genotyping was performed by semi-nested multiplex RT-PCR according to the EuroRotaNet protocol (<http://www.eurota.net/docs.php>). In order to identify the G and P genotype, the first round conventional RT-PCR was performed, aiming to amplify the full length of the VP7 encoding gene (1,062 bp) and the VP8* region (876 bp) of VP4 encoding genes fragments, using the primer pairs detailed in [Supplementary Table 1](#) and previously described procedures [8, 16].

The second round semi-nested multiplex PCR assay was performed with the G-typing first round RT-PCR product as templates and primers VP7 forward Gra-5 primer and a pool of G type-specific reverse primers N168, H168, H500, HT8 and ET10, aiming to characterize the G6 (lineages III and IV), G8, and G10 genotypes, respectively ([Supplementary Table 1](#)), as described [4, 16, 21]. P-typing semi-nested multiplex PCR assay was performed using the reverse primer Con 2 as well as specific forward primers for genotypes P[1], P[5] and P[11], according to procedures described previously [16, 22]. When the multiple bands that suggested two or more G or P types were detected by semi-nested PCR, the simplex RT-PCR using each gene-specific single typing primer was performed for these samples, confirmed by sequencing, and considered a mixed infection.

Nucleotide sequencing and phylogenetic analysis

To get more insights about the strains of RVAs circulation, each detected genotype (G6: n=16, G8: n=3, G10: n=6, P[1]: n=5, P[5]: n=8, P[11]: n=11) was randomly selected from different sampling periods, and VP7 or VP4 partial coding genes were sequenced. For determination of sequences, the primers for VP7 and VP4 genes amplification are described elsewhere [2, 20] ([Supplementary Table 1](#)). PCR amplicons were purified using gel extraction and spin column purification with QIAquick Gel Extraction Kit (Qiagen Inc., Hilden, Germany). Purified cDNA was directly sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, CA, USA) and in an automated ABI 3500xL Genetic Analyzer (Applied Biosystem) as per protocol. The chromatogram files were inspected using Chromas 2.6.6 (Technelysium Pty Ltd., South Brisbane City, QLD, Australia).

Molecular Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) server on the GenBank database to retrieve previously collected sequences with high similarity. Sequences were aligned by ClustalW algorithm using BioEdit (version 7.0.9.0). Maximum likelihood phylogenetic tree with 1,000 bootstrap method was constructed by using MEGA-X (version 10.0.5) after finding the best nucleotide substitution model in ‘Find best DNA/ Protein Models (ML)’ program for each viral gene. Genotype and lineage of representative RVAs were identified according to the nomenclature proposed by the Rotavirus Classification Working Group (RCWG) [37].

Nucleotide sequence accession numbers

The VP7 and VP4 genes of the bovine RVAs described in this study are available in the GenBank database. The accession numbers for the G6 nt sequences are LC592879–LC592894, G8 nt sequences are LC594504–LC594506, G10 nt sequences are LC594507–LC594512, those for the P[1] nt sequences are LC594513–LC594517, P[5] nt sequences are LC594518–LC594525, and P[11] nt sequences are LC594526–LC594536.

Statistical analysis

Welch's *t*-test was performed using Excel 2013 to compare the virus detection rates before, after and following one year of implying of improved hygiene protocols on boots, which pertain to the age-related data. The mortality of calves less than 2 months old from November 2016 to October 2019 was also compared in reference to earlier data, after and following the one year improvement. A significant difference was considered at the level of $P < 0.05$ or $P < 0.01$.

RESULTS

Comparison of virus detection rates and mortality of calves before, after and following one year improvement of hygiene protocols on boots

In Table 1, age related dynamics of the targeted viruses was summarized for 3 years. In calves at three weeks of age or younger, before the improvement of hygiene protocols, RVA prevalence of 39.1% was the highest among the 4 targeted viruses, followed by BEV (28.9%), BCV (23.4%) and BToV (8.2%). After the improvement, all 4 viruses detection rates decreased significantly ($P < 0.01$). One year after the improvement, RVA (38.6%) and BToV (8.9%) prevalence increased but not significantly. On the contrary, in calves over three weeks of age, before the improvement, BEV prevalence of 56.6% was the highest, followed by BCV (33.2%), RVA (13.8%) and BToV (11.2%). After the improvement, all viruses detection rates decreased, except for BCV. One year after the improvement, prevalence of all viruses decreased significantly ($P < 0.01$), however, BToV prevalence increased though not significantly.

Table 2 shows mortality of calves less than 2 months of old before, after improvement and following one year improvement period—namely from November 2016 to October 2017, from November 2017 to October 2018 and November 2018 to October 2019, respectively— as compared chronologically. After the improvement mortality at each month was reduced and the average mortality was significantly reduced from 1.51% to 0.30%. Following one year improvement the average mortality was still significantly reduced, in that case from 1.51% to 0.70%.

Table 1. Comparison of virus detection rates (%) before and after improvement of hygiene protocols on boots, in reference to calves at different age groups

	Calves at three weeks of age or younger			Calves over three weeks of age		
	Before improvement of hygiene protocols on boots (n=256 samples) ^a	After improvement of hygiene protocols on boots (n=198 samples) ^b	Following one year improvement of hygiene protocols on boots (n=202 samples) ^c	Before improvement of hygiene protocols on boots (n=304 samples) ^a	After improvement of hygiene protocols on boots (n=202 samples) ^b	Following one year improvement of hygiene protocols on boots (n=198 samples) ^c
RVA	39.1	22.7**	38.6	13.8	10.4	2.0**
BToV	8.2	1.0**	8.9	11.2	4.0**	11.1
BEV	28.9	8.6**	13.9**	56.6	16.8**	12.6**
BCV	23.4	8.1**	12.9**	33.2	35.6	16.2**
Reference	[42]	[42]	This study	[42]	[42]	This study

The total number of samples of calves at three weeks of age or younger before, after and following one year of improvement of hygiene protocols on boots, was 256, 198 and 202 respectively. The total number of samples of calves over three weeks of age before, after and following one year improvement was 304, 202 and 198, respectively. ^a Sampling years from October 2016 to October 2017. ^b Sampling years from December 2017 to October 2018. ^c Sampling years from December 2018 to October 2019. **The significance of difference compared with the period preceding the improvement of hygiene protocols ($P < 0.01$).

Table 2. Comparison of mortality (%) of calves less than 2 months-old before and after improvement of hygiene protocols

Period	Month												Average	Reference
	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.		
Before improvement	3.75	1.32	1.00	0.98	1.37	2.78	1.55	1.21	1.75	0.58	0.83	1.03	1.51	[42]
After improvement	0.36	0.74	0.37	0.00	0.32	0.00	0.00	0.44	0.00	0.33	0.68	0.33	0.30**	[42]
Following one year improvement	0.00	0.65	0.98	0.34	0.66	0.84	0.50	1.34	1.35	1.43	0.00	0.31	0.70**	This study

**The significance of difference of the average mortality compared with the period preceding the improvement ($P < 0.01$).

Distribution of G and P genotypes among RVA positive samples

A total of 560 calf fecal samples from farm A were tested during the study period (2018 to 2020), and 21.7% (122/560) were positive for bovine RVAs. Among the 122 RVAs positive samples, presence of the combinations of G and P genotypes are summarized in Table 3.

G genotyping revealed that G10 was the most prevalent genotype (45.9%), followed by G6 (25.4%) and G8 (4.1%). Mixed infection of G6 with G10 genotypes was detected at the rate of 20.5%, while G6 with G8 rated 4.1%. P genotyping indicated that P[11] was the dominant P type, with a prevalence of 56.6%, followed by P[5] (15.6%) and P[1] (8.2%). In mixed infection P[5+11] (10.6%) and P[1+11] (9.0%) were detected as well.

The genetic combination of G10P[11] was the predominant one (41.8%), followed by G6P[11] (10.7%), G6P[1] (4.9%), G6P[5] (4.1%) and G8P[1] (3.3%), respectively. The combination of mixed infection G6+G10P[5] (11.5%) was the most common one, more than any other combination. Overall, single infections of bovine RVAs comprising different genotypes were higher (64.8%) than mixed infections (35.2%).

Sequence and phylogenetic analysis of VP7 gene

The partial nucleotide sequences of VP7 gene from twenty five representative RVAs of G6, G8 and G10 in farm A were determined. A comparison of the nucleotide and deduced amino acid sequences of the VP7 gene was conducted, based on different geographical locations of animal and human origin of rotavirus reference strains available in NCBI GenBank database, using Blast server (Supplementary Table 2).

Sixteen RVAs showed high nucleotide 92.8–96.2% and deduced amino acid 94.3–96.9% identities within G6 strains, which include the bovine strains (DK1231C, V019, 1976-SC, LNA5, GBR, USA/UK, BRV105 and WC3) and the horse strain (OH-4). Phylogenetic analysis also confirmed that all G6 samples in the present study clustered together with strains from the G6-IV lineage (Fig. 1a). Three of the twenty-five bovine RVAs had 85.8–95.8% nucleotide and 95.2–98.0% deduced amino acid were found identical to the G8 RVAs, including the bovine (Cody I801 and Sun9), porcine (11–1), alpaca (880), human (PR/1300 and B12) and guanaco (Chubut) strains. Phylogenetically, representative RVAs in farm A are grouped with G8-I lineage of the bovine Cody I801 strain (Fig. 1b). The remaining six RVAs showed high nucleotide 95.5–97.2% and deduced amino acid 97.4–98.8% identities with G10 strains of bovine (GB20-25, 1929-PR, Azuk-7, TO1TR, Khorasan, DQ-75 and B223) and giraffe (GirRV-2). Phylogenetic analysis exhibited that all G10 RVAs clustered with those of lineage-IV (Fig. 1c).

Sequence and phylogenetic analysis of VP4 gene

The partial nucleotide sequences of VP4 gene from twenty four representative RVAs in farm A namely, P[1], P[5] and P[11] were analyzed and compared all with reference RVA strains of known animal and human origins available in NCBI GenBank database, using Blast server (Supplementary Table 3).

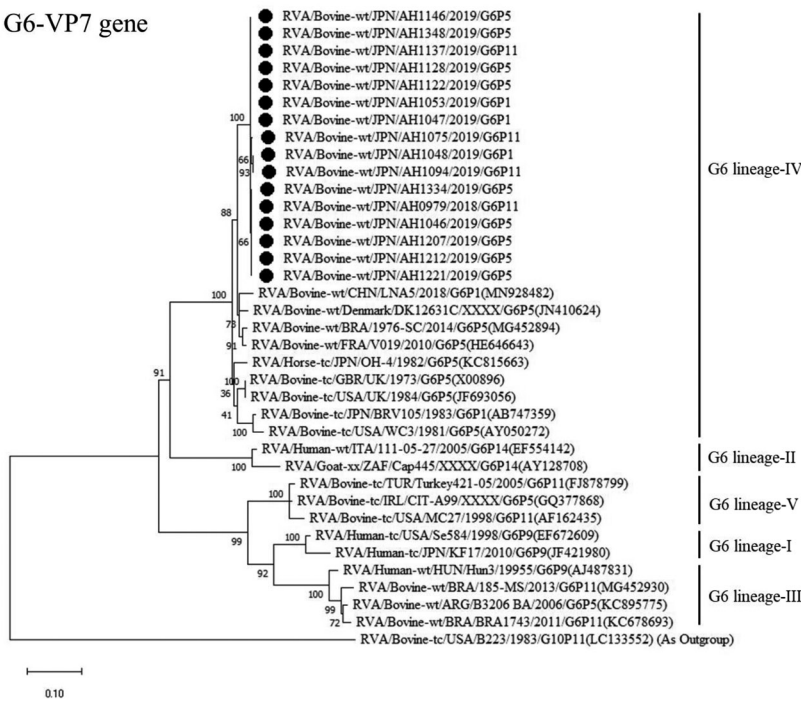
Among sequences of RVAs, five had high nucleotide 92.8–96.9% and deduced amino acid 97.3–100% identities with P[1] genotype strains, including the cervidae (14-02218), alpaca (1115), porcine (66-1), rhesus (PTRV), simian (SA11-30), bovine (NCDV, O Agent, HQ09, CR231, CR231 and BRV101), dog (88977) and goat (0040). Phylogenetic analyses of P[1] genotype with similar representative strains are illustrated in Fig. 2a. The sequences of the eight samples were most closely related to the human (CJN-M, RotaTeq SC2-9 and SSCRTV), bovine (VMRI and P4) and cat (FRV537) strains representing the P[5] genotype with 95.1–97.4% nucleotide and 96.1–98.0% deduced amino acid identities. Phylogenetically, these eight RVAs clustered with those of genotype P[5]-VIII lineage (Fig. 2b). The remaining eleven representative RVAs, shared high nucleotide 92.4–95.6% and deduced amino acid 95.7–97.2% identities to the P[11] genotype of bovine (GB20-25, 1929-PR, Azuk-7, TO1TR, Khorasan, DQ-75 and B223) and giraffe (GirRV-2) strains. Phylogenetic analysis of VP4 gene showed that those strains were grouped within the P[11]-lineage III (Fig. 2c).

Table 3. Distribution and relative frequencies of G and P genotype combinations due to mixed infections of different rotavirus A (RVA)

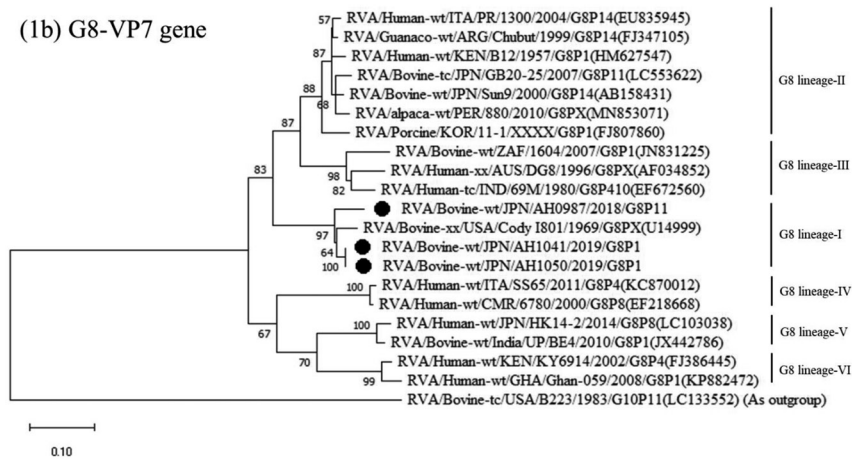
Genotype	P[1]	P[5]	P[11]	P[1+5] ^a	P[1+11] ^a	P[5+11] ^a	Total
G6	6 (4.9%)	5 (4.1%)	13 (10.7%)	0 (0%)	1 (0.8%)	6 (4.9%)	31 (25.4%)
G8	4 (3.3%)	0 (0%)	0 (0%)	0 (0%)	1 (0.8%)	0 (0%)	5 (4.1%)
G10	0 (0%)	0 (0%)	51 (41.8%)	0 (0%)	5 (4.1%)	0 (0%)	56 (45.9%)
G6+G8 ^a	0 (0%)	0 (0%)	1 (0.8%)	0 (0%)	4 (3.3%)	0 (0%)	5 (4.1%)
G6+G10 ^a	0 (0%)	14 (11.5%)	4 (3.3%)	0 (0%)	0 (0%)	7 (5.7%)	25 (20.5%)
Total	10 (8.2%)	19 (15.6%)	69 (56.6%)	0 (0%)	11 (9.0%)	13 (10.6%)	
		79 (64.8%)			43 (35.2%) ^a		122 (100%)

^a Mixed infection.

(1a) G6-VP7 gene



(1b) G8-VP7 gene



(1c) G10-VP7 gene

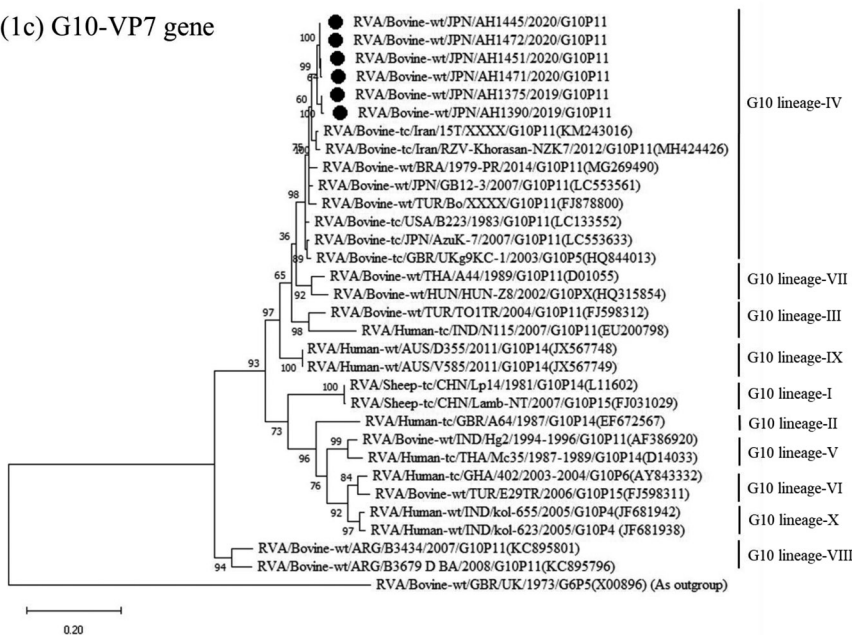


Fig. 1. Phylogenetic tree of (1a) G6, (1b) G8 and (1c) G10 genotype constructed using MEGA-X based on nucleotide sequences of the VP7 encoding genes for representative Japanese bovine rotaviruses A (closed circle). The GeneBank accession numbers of the strains are shown, and lineages are indicated. For the construction of the phylogenetic trees, the evolutionary history was inferred using the maximum-likelihood method based on the general time reversible model with discrete gamma distribution and invariant sites by 1,000 bootstrap replicates.

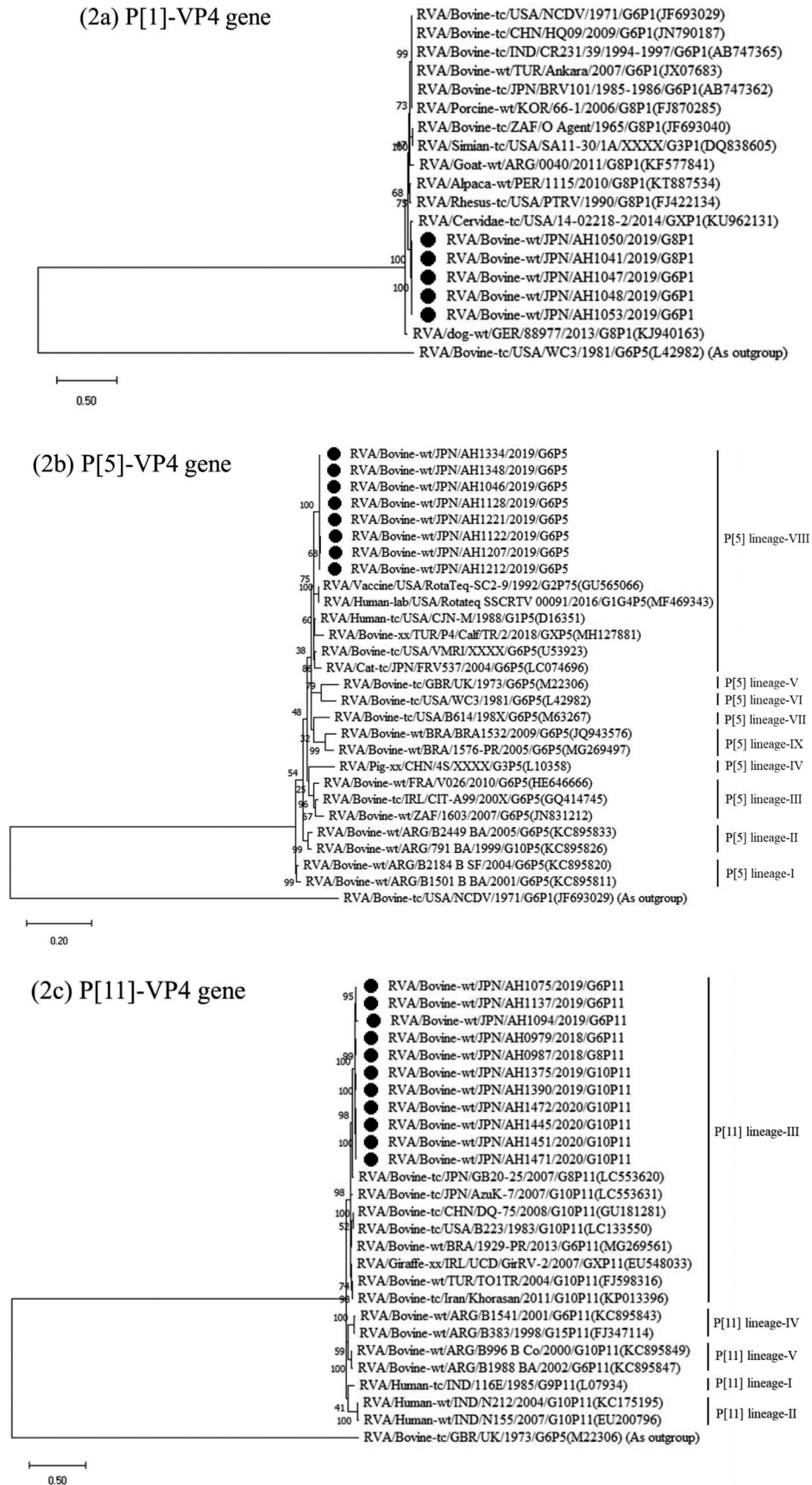


Fig. 2. Phylogenetic tree of (2a) P[1], (2b) P[5] and (2c) P[11] genotype constructed using MEGA-X based on nucleotide sequences of the VP4 encoding genes for representative Japanese bovine rotaviruses A (closed circle). The GeneBank accession numbers of the strains are shown, and lineages are indicated. For the construction of the phylogenetic trees, the evolutionary history was inferred using the maximum-likelihood method based on the general time reversible model with discrete gamma distribution and invariant sites by 1,000 bootstrap replicates.

DISCUSSION

The viral etiology of diarrhea, which causes considerable economic loss for bovine farms, includes RVA, BToV, BEV and BCV; hence, those viruses were selected as candidate “viral pathogen indicators” [44]. After improvement of hygiene protocols on boots in farm A, mortality of calves and the levels of viral pathogen indicators became significantly lower for one year [42], but reincreased after December 2018. As shown in Table 1, detection rates of RVA increased in the younger calves, comparing the others indicator viral pathogens, thereby suggesting the importance of RVA infection control in this bovine farm (and at large). Not only the detection levels of RVAs, but also their genotyping, seem to be important for RVA infection control.

For the current molecular investigation, we analyzed G and P genotypic profiles of bovine RVAs in farm A in Ibaraki, Japan, during 2018–2020. Semi-nested multiplex RT-PCR based methods has been successfully used for genotyping of bovine RVA and proved as a reliable epidemiological tool, enabling the detection of genomic combinations and mixed genotypes infections [4, 16, 36]. The present study revealed that the combination of G and P genotypes, G10P[11] (41.8%) was the predominant bovine RVA combination. In addition, respectively G6P[1], G6P[5], G6P[11], G8P[1] and G8P[11] have been detected. A continuous surveillance of bovine RVAs using diarrheic samples from calves collected in Kagoshima prefecture during 1995–1998 showed that bovine RVAs with G10P[11], G8P[X], and G6P[5] were predominantly common in 1995, 1996, and both 1997 and 1998, respectively [15]. On the other hand, a systematic surveillance of bovine RVAs in diarrheic samples of calves collected from 29 dairy and Japanese beef farms in 11 prefectures from 1987 to 2000 indicated the existence of bovine RVAs with multiple genotypes, namely, G6P[5] (37%), G10P[11] (30%), G6P[1] (11%), G6P[11] (11%), G10P[5] (9%), and G8P[11] (1%) [34]. Compared with previous studies, our data demonstrated that bovine RVAs with G6P[1], G6P[5], G6P[11] and G10P[11] genotypes have been maintained in cattle herds for over 30 years, and bovine RVAs with G8P[1] genotype have been recently widespread in cattle herds. Moreover, bovine RVAs mixed genotype infections occurred at high frequency during the present study (35.2%), as observed in several studies showing multiple G and/or P genotypes within a single stool sample [6, 40]. Unusual mixed combinations (G6+G10P[5], G6+G10P[11], G6+G8P[11], G6P[5+11], G10P[1+11] and G8P[1+11]) were also detected. This is likely due to farming practices, whereby calves are housed closely in pens, as observed in the current study, rather than being dispersed in a field [16, 39, 40]. The occurrence of mixed genotype infections makes natural reassortment between strains feasible, and could result in natural diversity of rotaviruses [5, 27].

Phylogenetic investigations of both partial VP7 genes and the deduced amino acid sequences revealed that the Japanese G6 strains could be grouped into lineage-IV. The cluster includes sixteen G6 strains together with bovine strains those from Denmark, France, Brazil, China and UK, plus an equine G6 strain from Japan [11, 18, 24]. The Japanese bovine G8 RVAs identified in this study appeared closely related to the bovine Cody (I-801) strain. Here, we report that the G8-I lineage possesses an infrequent genotype for the first time within the RVA strains identified by Chang *et al.* [7, 8]. The G10 genotype of Japanese bovine RVA sequences occurred more frequently in the G10-IV lineage, and appeared to be closely related to the bovine GB12-3 Japanese strain and other strains from Iran, Turkey, Brazil and UK [2, 11, 28].

Phylogenetic analyses of partial VP4 gene of nucleotide and deduced amino acid sequences of P[1] have been reported, indicating the prevalence of P types of different reassortants in animal host species in Japan, United States, South America and most of countries of Europe [3, 12, 13, 25]. The P[5] genotype of bovine RVA sequences also clustered with the P[5]-VIII lineage, and clustered with bovine-derived human CJN-M and RotaTeq vaccine strains [30, 33]. Moreover, phylogenetic analysis of the Japanese bovine P[11] strain revealed close similarity to other P[11]-III lineage strains detected in bovine and grouped in a cluster with the well-known B223, GB12-3 Japanese strains plus other strains from Iran, Turkey, Brazil and UK [2, 11, 26, 28].

When compared at the nucleotide sequence level, the identified bovine RVAs clustered with other animal-derived RVA strains. Unusual equine RVA strain OH-4 was found to be more closely related to those of bovine and bovine-like human RVAs than to those of other RVAs, providing the first conclusive evidence for artiodactyl (likely bovine)-to-equine interspecies transmission events [18]. The incidental transmission of RVAs from animals to humans may not be clinically relevant, but it does provide an opportunity for reassortment, and thereby the generation of novel strains which may be pathogenic to humans [23, 41].

The findings in the present study are in good agreement with the surveillance of calves from regularly vaccinated caws, which demonstrated that vaccines may fail to provide good protection against heterologous and also homologous infections. The amount and the timing of the feeding of lactogenic antibodies and the dose, virulence and serotype of the virus strain will affect the apparent susceptibility of calves to the infection [10]. Lactogenic immunity only works during the suckling period, and more specifically when the environmental viral loads are lower than the virus-neutralizing antibody levels contained in the colostrum and milk. However, continuous colostrum feeding as all or part of the diet for 10–14 days is essential for effective clinical protection [38]. On farm A, calves were fed colostrum from their mothers for one day after birth, then fed commercial milk replacer. We hypothesized that because milk from the mother cow was fed for as short as one day instead of for a longer period of time, antibodies were not supplied to the gastrointestinal tract and the calves were not protected against RVA infections.

Overall compared with previous studies, the present data helps to understand the RVAs diversity in Japanese cattle, and that further research is needed to determine their relationship to vaccines and other strains circulating worldwide, as well as to assess their zoonotic potential. These findings suggest the importance of controlling prevalence of pathogens on bovine farms. It is possible to increase the level of biosecurity at bovine farms through the farm HACCP approach by proper vaccination, changing boots and clothing, the appropriate use of disinfectants, or limiting access to the farms [44]. In such cases, the presence and detection of the prevalent indicator viruses will be decreased.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENTS. The authors are extremely grateful to Farm A for providing us with valuable specimens. The authors would like to express their appreciation to Dr. Dany Shoham, Bar Ilan University, Israel, for the grammatical review of the manuscript. This study was supported in part by the JRA Livestock Industry Promotion Project 2019–2021.

REFERENCES

1. Abe, M., Ito, N., Masatani, T., Nakagawa, K., Yamaoka, S., Kanamaru, Y., Suzuki, H., Shibano, K., Arashi, Y. and Sugiyama, M. 2011. Whole genome characterization of new bovine rotavirus G21P[29] and G24P[33] strains provides evidence for interspecies transmission. *J. Gen. Virol.* **92**: 952–960. [Medline] [CrossRef]
2. Abe, M., Ito, N., Morikawa, S., Takasu, M., Murase, T., Kawashima, T., Kawai, Y., Kohara, J. and Sugiyama, M. 2009. Molecular epidemiology of rotaviruses among healthy calves in Japan: isolation of a novel bovine rotavirus bearing new P and G genotypes. *Virus Res.* **144**: 250–257. [Medline] [CrossRef]
3. Anbalagan, S. and Peterson, J. 2016. Detection and whole-genome characterization of a G8P[1] group A rotavirus strain from deer. *Genome Announc.* **4**: 59659. [Medline] [CrossRef]
4. Badaracco, A., Garaicoechea, L., Rodríguez, D., Uriarte, E. L., Odeón, A., Bilbao, G., Galarza, R., Abdala, A., Fernandez, F. and Parreño, V. 2012. Bovine rotavirus strains circulating in beef and dairy herds in Argentina from 2004 to 2010. *Vet. Microbiol.* **158**: 394–399. [Medline] [CrossRef]
5. Bwogi, J., Jere, K. C., Karamagi, C., Byarugaba, D. K., Namuwulya, P., Baliraine, F. N., Desselberger, U. and Iturriza-Gomara, M. 2017. Whole genome analysis of selected human and animal rotaviruses identified in Uganda from 2012 to 2014 reveals complex genome reassortment events between human, bovine, caprine and porcine strains. *PLoS One* **12**: e0178855. [Medline] [CrossRef]
6. Cashman, O., Lennon, G., Sleator, R. D., Power, E., Fanning, S. and O’Shea, H. 2010. Changing profile of the bovine rotavirus G6 population in the south of Ireland from 2002 to 2009. *Vet. Microbiol.* **146**: 238–244. [Medline] [CrossRef]
7. Chang, K. O., Parwani, A. V. and Saif, L. J. 1995. Comparative nucleotide and deduced amino acid sequence analysis of VP7 gene of the NCDV Cody (I-801) strain of group A bovine rotavirus. *Arch. Virol.* **140**: 1279–1283. [Medline] [CrossRef]
8. Chang, K. O., Parwani, A. V. and Saif, L. J. 1996. The characterization of VP7 (G type) and VP4 (P type) genes of bovine group A rotaviruses from field samples using RT-PCR and RFLP analysis. *Arch. Virol.* **141**: 1727–1739. [Medline] [CrossRef]
9. Cho, Y. I. and Yoon, K. J. 2014. An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. *J. Vet. Sci.* **15**: 1–17. [Medline] [CrossRef]
10. Crouch, C. F. 1985. Vaccination against enteric rota and coronaviruses in cattle and pigs: enhancement of lactogenic immunity. *Vaccine* **3**: 284–291. [Medline] [CrossRef]
11. da Silva Medeiros, T. N., Lorenzetti, E., Alfieri, A. F. and Alfieri, A. A. 2019. G and P genotype profiles of rotavirus A field strains circulating in beef and dairy cattle herds in Brazil, 2006–2015. *Comp. Immunol. Microbiol. Infect. Dis.* **64**: 90–98. [Medline] [CrossRef]
12. da Silva Medeiros, T. N., Lorenzetti, E., Alfieri, A. F. and Alfieri, A. A. 2015. Phylogenetic analysis of a G6P[5] bovine rotavirus strain isolated in a neonatal diarrhea outbreak in a beef cattle herd vaccinated with G6P[1] and G10P[11] genotypes. *Arch. Virol.* **160**: 447–451. [Medline] [CrossRef]
13. Doan, Y. H., Nakagomi, T., Aboudy, Y., Silberstein, I., Behar-Novat, E., Nakagomi, O. and Shulman, L. M. 2013. Identification by full-genome analysis of a bovine rotavirus transmitted directly to and causing diarrhea in a human child. *J. Clin. Microbiol.* **51**: 182–189. [Medline] [CrossRef]
14. Estes, M. K. and Greenberg, H. B. 2013. Rotaviruses. In: Knipe DM, Howley PM, editors-in-chief, editors. *Field’s virology*. 6th. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins. **2**: 1347–1401.
15. Fukai, K., Maeda, Y., Fujimoto, K., Itou, T. and Sakai, T. 2002. Changes in the prevalence of rotavirus G and P types in diarrheic calves from the Kagoshima prefecture in Japan. *Vet. Microbiol.* **86**: 343–349. [Medline] [CrossRef]
16. Garaicoechea, L., Bok, K., Jones, L. R., Combessies, G., Odeón, A., Fernandez, F. and Parreño, V. 2006. Molecular characterization of bovine rotavirus circulating in beef and dairy herds in Argentina during a 10-year period (1994–2003). *Vet. Microbiol.* **118**: 1–11. [Medline] [CrossRef]
17. Geletu, U. S., Usmael, M. A. and Bari, F. D. 2021. Rotavirus in calves and its zoonotic importance. *Vet. Med. Int.* **2021**: 6639701. [Medline] [CrossRef]
18. Ghosh, S., Taniguchi, K., Aida, S., Ganesh, B. and Kobayashi, N. 2013. Whole genomic analyses of equine group A rotaviruses from Japan: evidence for bovine-to-equine interspecies transmission and reassortment events. *Vet. Microbiol.* **166**: 474–485. [Medline] [CrossRef]
19. Gomez, D. E. and Weese, J. S. 2017. Viral enteritis in calves. *Can. Vet. J.* **58**: 1267–1274. [Medline]
20. Gouvea, V., Glass, R. I., Woods, P., Taniguchi, K., Clark, H. F., Forrester, B. and Fang, Z. Y. 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol.* **28**: 276–282. [Medline] [CrossRef]
21. Gouvea, V., Santos, N. and Timenetsky, M. C. 1994. Identification of bovine and porcine rotavirus G types by PCR. *J. Clin. Microbiol.* **32**: 1338–1340. [Medline] [CrossRef]
22. Gouvea, V., Santos, N. and Timenetsky, M. C. 1994. VP4 typing of bovine and porcine group A rotaviruses by PCR. *J. Clin. Microbiol.* **32**: 1333–1337. [Medline] [CrossRef]
23. Hoa-Tran, T. N., Nakagomi, T., Vu, H. M., Nguyen, T. T. T., Takemura, T., Hasebe, F., Dao, A. T. H., Anh, P. H. Q., Nguyen, A. T., Dang, A. D. and Nakagomi, O. 2020. Detection of three independently-generated DS-1-like G9P[8] reassortant rotavirus A strains during the G9P[8] dominance in Vietnam, 2016–2018. *Infect. Genet. Evol.* **80**: 104194. [Medline] [CrossRef]
24. Kaplon, J., Fremy, C., Bernard, S., Rehby, L., Aho, S., Pothier, P. and Ambert-Balay, K. 2013. Impact of rotavirus vaccine on rotavirus genotypes and caliciviruses circulating in French cattle. *Vaccine* **31**: 2433–2440. [Medline] [CrossRef]
25. Komoto, S., Adah, M. I., Ide, T., Yoshikawa, T. and Taniguchi, K. 2016. Whole genomic analysis of human and bovine G8P[1] rotavirus strains isolated in Nigeria provides evidence for direct bovine-to-human interspecies transmission. *Infect. Genet. Evol.* **43**: 424–433. [Medline] [CrossRef]
26. Komoto, S., Pongsuwanna, Y., Tacharoenuang, R., Guntapong, R., Ide, T., Higo-Moriguchi, K., Tsuji, T., Yoshikawa, T. and Taniguchi, K. 2016. Whole genomic analysis of bovine group A rotavirus strains A5-10 and A5-13 provides evidence for close evolutionary relationship with human rotaviruses. *Vet. Microbiol.* **195**: 37–57. [Medline] [CrossRef]
27. Komoto, S., Tacharoenuang, R., Guntapong, R., Ide, T., Tsuji, T., Yoshikawa, T., Tharmaphornpilas, P., Sangkitporn, S. and Taniguchi, K. 2016. Reassortment of human and animal rotavirus gene segments in emerging DS-1-like G1P[8] rotavirus strains. *PLoS One* **11**: e0148416. [Medline] [CrossRef]

28. Madadgar, O., Nazaktabar, A., Keivanfar, H., Zahraei Salehi, T. and Lotfollah Zadeh, S. 2015. Genotyping and determining the distribution of prevalent G and P types of group A bovine rotaviruses between 2010 and 2012 in Iran. *Vet. Microbiol.* **179**: 190–196. [[Medline](#)] [[CrossRef](#)]
29. Matthijnssens, J., Ciarlet, M., McDonald, S. M., Attoui, H., Bányai, K., Brister, J. R., Buesa, J., Esona, M. D., Estes, M. K., Gentsch, J. R., Iturriza-Gómara, M., John, R., Kirkwood, C. D., Martella, V., Mertens, P. P. C., Nakagomi, O., Parreño, V., Rahman, M., Ruggeri, F. M., Saif, L. J., Santos, N., Steyer, A., Taniguchi, K., Patton, J. T., Desselberger, U. and Van Ranst, M. 2011. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). *Arch. Virol.* **156**: 1397–1413. [[Medline](#)] [[CrossRef](#)]
30. Matthijnssens, J., Joëlsson, D. B., Warakowski, D. J., Zhou, T., Mathis, P. K., van Maanen, M. H., Ranheim, T. S. and Ciarlet, 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](#)]
31. Ministry of Agriculture Forestry and Fisheries of Japan, report. 2020 Annual case report for livestock disease from the ministry of agriculture, forestry and fisheries of Japan. https://www.maff.go.jp/j/tokei/kouhyou/katiku_kyosai/ [accessed on November 28, 2020].
32. Mitake, H., Ito, N., Okadera, K., Okada, K., Nakagawa, K., Tanaka, T., Katsuragi, K., Kasahara, K., Nihongi, T., Tsunemitsu, H. and Sugiyama, M. 2015. Detection of avian-like rotavirus A VP4 from a calf in Japan. *J. Vet. Med. Sci.* **77**: 221–224. [[Medline](#)] [[CrossRef](#)]
33. Nakagomi, O., Isegawa, Y., Ward, R. L., Knowlton, D. R., Kaga, E., Nakagomi, T. and Ueda, S. 1994. Naturally occurring dual infection with human and bovine rotaviruses as suggested by the recovery of G1P8 and G1P5 rotaviruses from a single patient. *Arch. Virol.* **137**: 381–388. [[Medline](#)] [[CrossRef](#)]
34. Okada, N. and Matsumoto, Y. 2002. Bovine rotavirus G and P types and sequence analysis of the VP7 gene of two G8 bovine rotaviruses from Japan. *Vet. Microbiol.* **84**: 297–305. [[Medline](#)] [[CrossRef](#)]
35. Papp, H., László, B., Jakab, F., Ganesh, B., De Grazia, S., Matthijnssens, J., Ciarlet, M., Martella, V. and Bányai, K. 2013. Review of group A rotavirus strains reported in swine and cattle. *Vet. Microbiol.* **165**: 190–199. [[Medline](#)] [[CrossRef](#)]
36. Phan, T. G., Khamrin, P., Trinh, D. Q., Dey, S. K., Yagyu, F., Okitsu, S., Nishio, O. and Ushijima, H. 2007. Genetic characterization of group A rotavirus strains circulating among children with acute gastroenteritis in Japan in 2004–2005. *Infect. Genet. Evol.* **7**: 247–253. [[Medline](#)] [[CrossRef](#)]
37. RCWG Rotavirus Classification Working Group. List of accepted genotypes. Laboratory of Viral Metagenomics. <https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg> [accessed on July 2, 2021].
38. Saif, L. J., Redman, D. R., Smith, K. L. and Theil, K. W. 1983. Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from immunized or nonimmunized cows. *Infect. Immun.* **41**: 1118–1131. [[Medline](#)] [[CrossRef](#)]
39. Suzuki, Y., Sanekata, T., Sato, M., Tajima, K., Matsuda, Y. and Nakagomi, O. 1993. Relative frequencies of G (VP7) and P (VP4) serotypes determined by polymerase chain reaction assays among Japanese bovine rotaviruses isolated in cell culture. *J. Clin. Microbiol.* **31**: 3046–3049. [[Medline](#)] [[CrossRef](#)]
40. Swiatek, D. L., Palombo, E. A., Lee, A., Coventry, M. J., Britz, M. L. and Kirkwood, C. D. 2010. Detection and analysis of bovine rotavirus strains circulating in Australian calves during 2004 and 2005. *Vet. Microbiol.* **140**: 56–62. [[Medline](#)] [[CrossRef](#)]
41. Tacharoenmuang, R., Komoto, S., Guntapong, R., Ide, T., Haga, K., Katayama, K., Kato, T., Ouchi, Y., Kurahashi, H., Tsuji, T., Sangkitporn, S. and Taniguchi, K. 2015. Whole genomic analysis of an unusual human G6P[14] rotavirus strain isolated from a child with diarrhea in Thailand: Evidence for bovine-to-human interspecies transmission and reassortment events. *PLoS One* **10**: e0139381. [[Medline](#)] [[CrossRef](#)]
42. Takahashi, S., Hasan, M. A., Ito, M., Komura, M., Daio, C., Ono, M., Yamaguchi, M., Alam, M. S., Kabir, M. H., Miyaoka, Y., Shoham, D. and Takehara, K. 2020. Regression of viral pathogen indicators due to improvement of hygiene protocols on boots in a bovine farm. *J. Vet. Med. Sci.* **82**: 1793–1797. [[Medline](#)] [[CrossRef](#)]
43. Tate, J. E., Burton, A. H., Boschi-Pinto, C., Steele, A. D., Duque, J., Parashar U. D., WHO-coordinated Global Rotavirus Surveillance Network. 2012. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *Lancet Infect. Dis.* **12**: 136–141. [[Medline](#)] [[CrossRef](#)]
44. Yamada, M., Kubota, K., Takahashi, S., Toyofuku, C., Hakim, H., Alam, M. S., Hasan, M. A., Shoham, D. and Takehara, K. 2020. Longitudinal and cross-sectional detection of four bovine enteric viruses by multiplex- reverse transcription polymerase chain reaction: Identification of possible indicator viruses to assess biosecurity level at bovine farms. *J. Vet. Med. Sci.* **82**: 314–319. [[Medline](#)] [[CrossRef](#)]