NOTE *Virology*

Genetic and Serological Characterization of Novel Serotype G8 Bovine Group A Rotavirus Strains Isolated in Japan

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ABSTRACT. G8 bovine group A rotaviruses isolated in Japan were genetically and serologically characterized. The VP7 gene nucleotide and amino acid sequences revealed high identity with each other. All Japanese G8 strains were classified into the same lineage in the phylogenetic analysis based on VP7 gene sequences. Antisera to four Japanese G8 strains neutralized other G8 strains, but their neutralizing titers were between 8-fold lower and 2-fold higher than homologous strains. These results suggest that the VP7s of Japanese G8 strains have similar genetic and serologic characteristics. Observed differences in the neutralizing abilities of antisera f or each strain appear to depend on differences in the P serotypes/genotypes.
KEY WORDS: calf, diarrhea, rotavirus.

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Bovine group A rotavirus (BoRV-A) is the main pathogen of neonatal calf diarrhea [4, 6, 12, 14, 24–26]. BoRV-A is comprised of two independent outer capsid neutralization antigens, VP4 and VP7, which determine the P type (for protease-sensitive protein) and G type (for glycoprotein), respectively [3]. At present, it is possible to identify P and G types using not only serological methods, but also genetic methods [3]. Typing based on serological and genetic methods were termed P/G serotypes and P/G genotypes, respectively [3]. P genotypes have been numbered independently from P serotypes, and as a result P serotypes are denoted by an open number and P genotypes are indicated by closed brackets. In contrast, G serotypes were numbered dependently from G genotypes. Though at least three P (P6 [1], P7 [5] and P8 [11]) and eight G (G1, G2, G3, G6, G7, G8, G10 and G11) types have been reported among BoRV-A so far [1, 2, 6, 10, 16, 24], research on the distribution of these types has demonstrated that P7 [5], P8 [11], G6 and G10 are the most common [6, 12, 14, 24, 25]. Contrary to these findings, our research discovered a remarkable diversity of BoRV-A during our recently completed 2-year study of BoRV-A calf diarrhea in Kagoshima Prefecture, Japan [5]. Our studies were the first to describe G8 as the most predominant BoRV-A strain during the research period. Although our findings suggest that G8 BoRV-A should be investigated in detail, little investigation of G8 BoRV-A has been carried out to date because all G8 strains reported so far have been detected and/or isolated only sporadically [4, 19, 20, 22, 23, 26]. Even in Japan, there are few reports on the characterization of G8 BoRV-A [19, 23]. In this study, G8 BoRV-A strains recovered from five calves in Kagoshima Prefecture, Japan were genetically and serologically characterized by sequence analysis of the gene encoding the neutralization protein VP7 and virus neutralization studies.

Twenty G8 BoRV-A were isolated from diarrheal calf feces in Kagoshima Prefecture, Japan, as previously described [5]. Of these, the following five isolates from calves raised in five different herds were characterized in this study: KAG74, KAG75, KAG80, KAG87 and KAG91. BoRV-A NCDV (G6, P6 [1]) [17], UK (G6, P7 [5]) [28], BRV16 (G8, P6 [1]) [23], Tokushima9503 (G8, P [11]) [19], Niigata9801 (G8, P [14]-like) [19] and KK3 (G10, P8 [11]) [18] strains were used as reference strains. The isolated strains and reference strains were propagated and plaque-purified three times in MA-104 cells in the presence of trypsin [16]. Viral genomic double-stranded RNA (dsRNA) was extracted from plaque-purified virus using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Electrophoresis of the viral dsRNA on polyacrylamide gels and detection by silver nitrate staining was carried out as previously described [6]. Viral genomic dsRNA was used for first-strand cDNA synthesis by AMV-reverse transcription (TAKARA BIO, Shiga, Japan) and cDNA was amplified by PCR using Taq polymerase (TAKARA BIO) as described previously [6]. The primers employed in this study for cDNA synthesis and PCR amplification, sBeg9 and End9(UK), were synthesized based on the reports of Gouvea *et al.* [7, 8]. Amplified cDNA was subcloned using Blunt-ended PCR Cloning Kit/pMOS*Blue* Competent Cells (Amersham Biosciences, Piscataway, NJ, U.S.A.) following the manufacturer's instructions. The nucleotide (nt) sequences of the subcloned VP7 genes were determined using Thermo Sequenase Fluorescent-Labelled Primer Cycle Sequencing Kit (Amersham Biosciences) on a DSQ-2000L automated sequencer (Shimadzu, Kyoto, Japan) following the manufacturer's instructions. To avoid the introduction of Taq polymerase errors, three clones from each

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isolate were sequenced. Vector-specific primers were used to determine the complete nt sequences of both strands of the VP7 genes. Genetic characterization was performed for the nt sequences (nt 22–1038) excepted primer sequences. The nt and deduced amino acid (aa) sequences of the VP7 genes of each isolate were compared with previously published, corresponding group A rotavirus (RV-A) gene sequences. Phylogenetic trees were constructed by the neighbor-joining method [21] using Clustal W [27], and the bootstrap probabilities of each node were calculated using 1,000 replications. The VP7 nt sequences for each of the five isolates and BRV16 (newly determined in this study) were deposited under accession numbers AB077053 to AB077058. Antisera were raised using purified KAG75, BRV16, NCDV and KK3 with the aid of Freund's adjuvant in guinea pigs, which were determined to be free of BoRV-A neutralizing antibodies by fluorescent focus neutralization (FFN) assay performed as previously described [15]. The P genotypes of five G8 isolates have not been identified yet. The results of P genotyping on these five isolates following the methods of Gouvea *et al.* [9] and Isegawa *et al.* [13] were not in agreement. Therefore, only VP7 genes of these isolates were characterized in this study. The P genotypes of these five isolates are now being analyzed.

All isolates were identified as long-genome electropherotypes, and were classified into five different electropherotypes (data not shown). Representative isolates of each electropherotype were selected in this study and were analyzed genetically and serologically.

The VP7 gene of each isolate was 1062 base pairs in length and contained an open reading frame (ORF) of 981 base pairs (positions 49 to 1029) that encoded a 326 aa protein, as observed in other RV-A VP7 genes [3] (data not shown). The nt and aa sequences of the VP7 genes from each of the isolates showed high sequence identity with all G8 strains (Fig. 1). The nt and aa identities for all the G8 strains ranged from 81.9 to 96.2% and 89.6 to 97.2%, respectively. In particular, nt and aa sequences showed high identity (from 93.9 to 96.2% and 92.9 to 97.2%, respectively) with BRV16, Tokushima9503 and Niigata9801, which were isolated in Japan. The nt and aa sequence identities among isolates ranged from 95.5 to 97.8% and 92.3 to 96.0%, respectively. These results suggest that these isolates, and Japanese G8 BoRV-A strains are genetically similar to each other. Although the nt and aa sequences of isolates exhibited 64.2 to 75.5% and 56.7 to 82.8% identities, respectively, to those of other G types strains Wa, S2, HO5, HOCHI, OSU, NCDV, UK, PO-13, 116E, KK3, YM, L26, L338 and CH3 (DDBJ/EMBL/GenBank accession numbers K02033, M11164, AB046464, AB012078, X04613, M12394, D82979, L14072, D01056, M23194, M58290, D13549 and D25229, respectively), sequence analysis of the VP7 genes of the isolates and reference strains showed that the isolates were correctly identified as G8.

The phylogenetic relationships among the isolates and reference strains based on nt sequences of the VP7 genes were analyzed by the neighbor-joining method (Fig. 1). G8 strains formed four lineages in the phylogenetic analysis (tentatively identified as G8a, G8b, G8c and G8d). The majority of BoRV-A strains were placed in the G8a lineage, only Cody-I801 placed with the G8b lineage, and the majority of human RV-A strains were classified as either G8c or G8d lineages. Okada *et al.* reported that Tokushima9503 and Niigata9801 clustered together and formed a new group in the phylogenetic analysis of the VP7 gene sequences [19]. In this study, the five isolates placed in the G8a lineage, together with BRV16, Tokushima9503 and Niigata9801. Along with the homology analysis, these results suggest that these isolates and Japanese G8 BoRV-A strains are genetically similar to each other. In contrast, other G8 BoRV-A strains, namely 678, Cody-I801 and A5, were classified into a distinct lineage (Fig. 1).

The antigenic relationships between isolates and reference strains were analyzed by FFN assay (Table 1). Hoshino and Kapikian established G and P serotypes based on the criterion of a 20-fold or greater difference between homologous and heterologous reciprocal neutralizing antibody titers [11]. However, antiserum to BRV16 (G8, P6 [1]) neutralized the VP7- and VP4-heterologous UK (G6, P7 [5]) to 16-fold lower titer than homologous strain in FFN assay performed in this study. Therefore, a 16-fold or greater difference was used to distinguish between G and P serotypes in this study.

Antisera to KAG75, BRV16, Tokushima9503 and Niigata9801 neutralized VP4-heterologous G8 strains to between 8-fold lower and 2-fold higher titers than homologous strains. BRV16 has been identified as G serotype 8 [23]. The isolates (KAG74, KAG80, KAG87 and KAG91) were identified as G serotype 8 based on the two-way antigenic relationship found between BRV16 and the isolates and the one-way antigenic relationship between serologically determined G8 strains and the isolates. Antiserum to KAG75 neutralized VP7-heterotypic UK and KK3 to 4- and 8-fold lower titers than the homologous strain. In contrast, antiserum to KK3 did not neutralized KAG75. These results suggest that KAG75 VP4 is slightly related serologically to P serotypes 7 and 8; however, the P serotype of KAG75 could not be identified in this study. Further analysis of the VP4 of KAG75 is needed.

Antiserum to Tokushima9503 neutralized homologous strain and VP7-heterotypic KK3 to the same titer. Antiserum to KK3 neutralized Tokushima9503 to an 8-fold lower titer than the homologous strain. The P serotype of Tokushima9503 was identified as P8 in this study. Antiserum to NCDV neutralized VP7-heterotypic KAG74 to a 4-fold lower titer than the homologous strain. This result suggests that KAG74 VP4 is related serologically to P serotype 6 VP4.

In conclusion, isolates recovered from Kagoshima Prefecture, Japan between 1995 and 1996 were genetically and serologically similar to each other and G8 reference strains, especially Japanese G8 BoRV-A BRV16, Tokushima9503 and Niigata9801. Differences in the neutralizing antibody

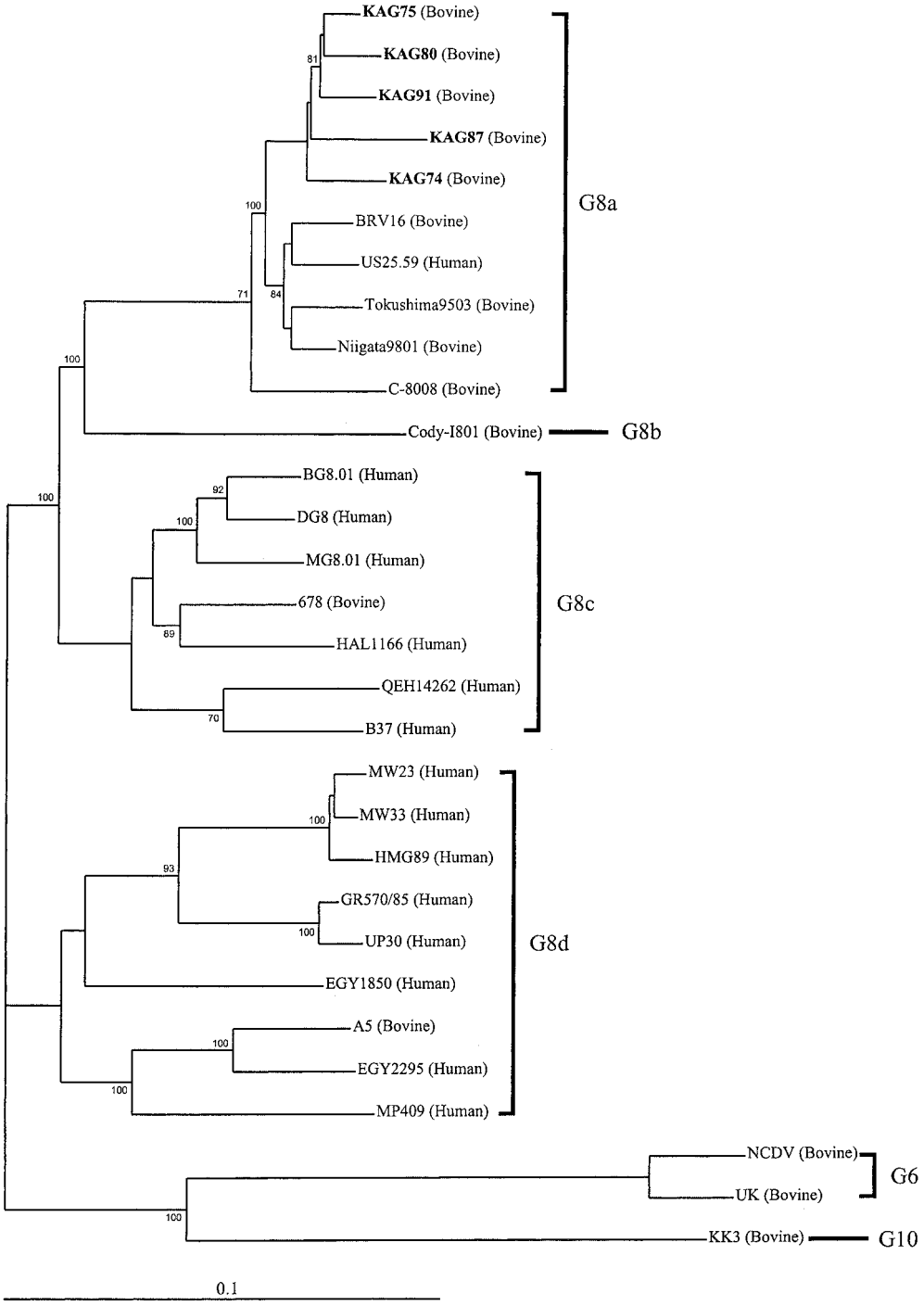

Fig. 1. Phylogenetic tree was constructed by the neighbor-joining method using the nt sequences of the VP7 genes of G8 reference strains and five isolates (indicated in boldface type). Bootstrap values above 70% are shown at branch nodes. Grouping and G types are marked separately. The scale bar represents 10% nucleotide difference. Host species are indicated in parentheses. The accession numbers of the VP7 gene nt sequences are Tokushima9503, AB044294; Niigata9801, AB044293; C-8008, U14998; 678, L20883; Cody-I801, U14999; A5, D01054; MW23, AJ278254; HMG89, X98918; MP409, AF141918; MW333, AJ278257; EGY1850, AF104102; EGY2295, AF104104; BG8.01, AF207060; MG8.01, AF207061; DG8, AF034852; GR570/85, AF143688; US25.96, AF039524; UP30, AF143690; QEH14262, AF143689; HAL1166, L20882; B37, J04334; NCDV, M12394; UK, X00896; KK3, D01056.

Table 1. Serological characterization of bovine group A rotavirus strains by fluorescent focus neutralization assay

Strain	G type	P type	Reciprocal of neutralization titer of antiserum to					
			KAG75	BRV16	Tokushima9503	Niigata9801	NCDV	KK3
KAG74	G8	unidentified	1,600	1,600	3,200	1,600	800	<100
KAG75	G8	unidentified	**1,600***	1,600	3,200	800	100	<100
KAG80	G8	unidentified	800	800	800	800	<100	<100
KAG87	G8	unidentified	1,600	1,600	1,600	800	100	<100
KAG91	G8	unidentified	3,200	1,600	3,200	1,600	100	<100
BRV16	G8	P6[1]	200	**3,200**	1,600	400	800	<100
Tokushima9503	G8	P[11]	800	800	**6,400**	800	100	400
Niigata9801	G8	P[14]-like	400	800	800	**1,600**	<100	<100
NCDV	G6	P6[1]	<100	1,600	200	<100	**3,200**	<100
UK	G6	P7[5]	400	200	<100	<100	800	<100
KK3	G10	P8[11]	200	<100	6,400	<100	<100	**3,200**

*: Homologous values are shown in boldface type.

titers of antisera to each strain in serological analysis among G8 strains appeared to be dependent on the differences in the P serotypes/genotypes of each strain. Further analysis of these isolates should be performed in order to establish preventive strategies against novel G8 BoRV-A diarrhea.

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