Whole genome characterization of new bovine rotavirus G21P[29] and G24P[33] strains provides evidence for interspecies transmission

Masako Abe,¹ Naoto Ito,¹,² Tatsunori Masatani,¹ Keisuke Nakagawa,¹ Satoko Yamaoka,¹ Yoshihiro Kanamaru,³ Hiroto Suzuki,⁴ Ken-ichi Shibano,⁵ Yasuhiro Arashi¹,⁵ and Makoto Sugiyama¹,²

¹The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
²Laboratory of Zoonotic Diseases, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
³Department of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan
⁴Gujo City Veterinary Clinic Center, Gujo, Gifu 501-4297, Japan
⁵Hyogo Prefectural Federation of Agricultural Mutual Aid Association, 3405-1 Kaibara, Kaibara, Tanba, Hyogo 669-3309, Japan

We have reported a novel bovine rotavirus, the AzuK-1 (G21P[29]) strain, isolated from an asymptomatic calf. We isolated another bovine rotavirus, the Dai-10 strain, bearing new G24P[33] genotypes, assigned by the Rotavirus Classification Working Group (RCWG), from an asymptomatic cow in Hyogo Prefecture, Japan in 2007. To gain an insight into the origins and evolution of these strains, we determined the complete ORF sequences of all 11 genes of the two strains. The NSP3 genes of both strains were confirmed to belong to a new NSP3 genotype, T9, by the RCWG. Genotype determination of AzuK-1 and Dai-10 strains revealed that eight gene segments of both strains possessed genotypes typically observed in bovine rotaviruses, with the exception of VP4, VP7 and NSP3 gene segments. Unexpectedly, phylogenetic analyses showed that VP6 and NSP2 gene segments of the AzuK-1 and Dai-10 strains were clustered with those of simian or canine/feline rotaviruses, rather than with those of bovine rotaviruses. These findings indicate the possibility that both strains originated by interspecies transmission and multiple reassortment events involving bovine, simian and canine/feline rotaviruses, resulting in the introduction of some genes into the genetic background of bovine rotaviruses.

INTRODUCTION

Group A rotaviruses are the major pathogens causing acute gastroenteritis in infants and a wide range of animals, including birds. Rotavirus-induced diarrhoea is a serious public health problem worldwide, responsible for more than 600 000 child deaths each year (Parashar et al., 2006). Likewise, in domestic animals, rotavirus-induced diarrhoea is a major problem causing significant economic losses (Dhama et al., 2009; Martella et al., 2010).

Rotaviruses are members of the family Reoviridae. Rotaviruses possess a genome of 11 segments of dsRNA, which encode six viral structural proteins (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6). Because of the segmented nature of the genome, a reassortment event can occur in cells co-infected with two or more different strains (Estes & Kapikian, 2007; Palombo, 2002; Ramig, 1997). The rotavirus virion is a triple-layered icosahedral particle. The outer capsid is composed of VP7 and VP4. They elicit neutralizing antibodies independently. In a dual classification system, rotaviruses are classified into 24 G genotypes and 32 P genotypes based on the nucleotide sequences of VP7 and VP4 genes, respectively (Collins et al., 2010; Esona et al., 2010; Matthijnssens et al., 2006, 2008a; Schumann et al., 2009; Solberg et al., 2009; Ursu et al., 2009). Recently, a new classification system has been established using nucleotide sequences of all of the 11 genomic RNA segments by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2008b). In this system, the
notations Gx-Px-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx are used for representing the genotypes of the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5-encoding gene segments, respectively.

Analysing full genome constellations of rotaviruses is a good method for investigating how the virus strains have evolved. For example, Matthijnssens et al. (2008a) demonstrated that Wa-like human rotaviruses and porcine rotaviruses have a common origin and that DS-1-like human rotaviruses and bovine rotaviruses have a common origin. More recently, determination of the complete genome sequences of simian rotaviruses indicated that simian rotaviruses are of diverse ancestry that originated largely by interspecies transmission and reassortment with non-human animal rotaviruses (Matthijnssens et al., 2010b). Furthermore, several studies have provided evidence for naturally occurring reassortants between humans and animals by analysis of whole genomes of rotavirus strains (Bányai et al., 2009; Esona et al., 2009; Ghosh et al., 2010; Matthijnssens et al., 2008c, 2009, 2010a; Mukherjee et al., 2009; Tsugawa & Hoshino, 2008).

Recently, we isolated a novel bovine rotavirus, the AzuK-1 strain, belonging to G21P[5] genotypes from an asymptomatic calf (Abe et al., 2009). In addition, our data indicated that G21P[5] rotaviruses can be considered to be endemic in the cattle population in Japan over the past decade and would have escaped detection due to their low pathogenicity in cattle. Among bovine rotaviruses, three G genotypes (G6, G8 and G10) and three P genotypes (P[1], P[5] and P[11]) are the most common (Estes & Kapikian, 2007). Bovine rotavirus strains with additional unusual G genotypes (G1, G2, G3, G5, G15 and G18) and P genotypes (P[3], P[14] and P[17]) have also been described (Blackhall et al., 1992; El-Attar et al., 2002; Estes & Kapikian, 2007; Fukai et al., 2004; Ghosh et al., 2007; Hussein et al., 1993; Isegawa et al., 1994; Martella et al., 2010; Park et al., 2006; Rao et al., 2000). However, the relationships among most of these unusual strains including G21P[5] genotypes and other rotavirus strains are unclear because information on the genetic background of these unusual strains is limited.

In this study, we determined the complete ORF sequences of all 11 genes of the AzuK-1 strain and evaluated the origin and evolution of G21P[5] rotaviruses. Furthermore, we isolated another novel bovine rotavirus, the Dai-10 strain, bearing G24P[33] genotypes from an asymptomatic cow. Thus, the Dai-10 strain was also included in this study. The results suggest that the two strains have originated by reassortment of simian, canine/feline or other animal rotavirus genes into the genetic background of bovine rotaviruses.

RESULTS

Dai-10 strain belongs to new G and P genotypes

The Dai-10 strain was isolated from an asymptomatic cow in January 2007. Partial sequence analyses of the VP7 and VP4 genes suggested that the Dai-10 strain belongs to new G and P genotypes. Hence, we carried out complete ORF analyses.

The entire coding regions of the VP7 and VP4 genes of Dai-10 were found to be 987 and 2325 nt in length and to code 329 and 775 aa, respectively. Based on the online RotaC genotyping tool, the Dai-10 strain was considered to belong to new G and P genotypes. In phylogenetic analyses, the Dai-10 strain was located in a new branch (Fig. 1). The VP7 and VP4 sequences of Dai-10 strain were assigned to new VP7 (G) and VP4 (P) genotypes, G24P[33], by the RCWG.

Genotype determination of AzuK-1 and Dai-10 strains

For the NSP3 gene, the AzuK-1 and Dai-10 strains have only up to 80% nucleotide sequence identities compared to other rotavirus sequences. Phylogenetic analysis showed that neither of the strains belonged to any of the established T1–T8 genotypes (Fig. 1). They were confirmed to belong to a new NSP3 genotype, T9, by the RCWG, while the NSP3 genotype of most bovine rotaviruses is T6. AzuK-1 and Dai-10 strains share the same genotype for eight gene segments, although their nucleotide/amino acid similarities are rather variable (Table 1). Thus, excluding the G and P genotypes, the complete genotype assignment of the AzuK-1 and Dai-10 strains was as follows: I2-R2-C2-M2-A13-N2-T9-E2-H3. These genotypes are common among bovine rotaviruses (Matthijnssens et al., 2008a) with only the NSP3 genotype being different.

Phylogenetic analyses

To investigate the genetic relatedness of AzuK-1, Dai-10 and other rotavirus strains, we performed phylogenetic analyses for each gene. AzuK-1 and Dai-10 strains were closely related to other bovine and bovine-like rotavirus strains when the gene segments VP1, VP2, VP3, NSP1, NSP4 and NSP5 were analysed (Figs 2 and 3). With regard to the NSP1 gene, other bovine rotaviruses were divided into two clusters, A3 and A13. AzuK-1 and Dai-10 strains were clustered closely with bovine rotaviruses, Arg/B383/98 and B223 strains belonging to the A13 genotype.

On the other hand, with regard to the VP6 and NSP2 gene segments, AzuK-1 and Dai-10 strains were clustered away from other bovine rotaviruses within the same corresponding genotypes with only the exception of the bovine RUBV51 strain in the VP6 gene (Fig. 4). For the VP6 gene, AzuK-1 and Dai-10 strains were clustered with simian SA11-H96 strain, which is believed to most closely resemble the original SA11 isolate. Surprisingly, comparative analysis using the deduced amino acid sequences of AzuK-1, Dai-10, RUBV51 and SA11-H96 strains showed high identities (98.5–99.8%) and 6 aa substitutions (D62N, T205I, Q213P, Y248F, M342L and V396I) that were unique to these four strains (Supplementary Fig. S1, available in JV online). As for the NSP2 gene, the Dai-10 strain was closely related to a guanaco strain, Gu/Arg/Rio Negro/98, Gu/Argentina/Rio Negro/98 and Gu/Argentina/Rio Negro/98.
whereas the AzuK-1 strain was located rather far apart from other rotavirus strains. Interestingly, AzuK-1, Dai-10 and Gu/Arg/Rio Negro/98 strains were clustered, albeit distantly, within the same genotype as canine, feline and canine/feline-like human rotavirus strains.

To date, the NSP3 gene has been classified into T1–T8 genotypes (Matthijnssens et al., 2008a; Schumann et al., 2009). Among these genotypes, bovine rotaviruses belong to T6 or T7 genotypes. On the other hand, as mentioned above, the NSP3 gene of the AzuK-1 and Dai-10 strains belongs to a new genotype, T9 (Fig. 1). Interestingly, the T9 genotype was clustered with T2, T3 and T5 genotypes, which were distinct from the bovine genotypes, T6 and T7.

**DISCUSSION**

In order to elucidate the infection cycles of pathogens in nature, it is necessary to keep track of the infection status in host populations, regardless of whether they are symptomatic or asymptomatic. To understand the ecology of rotaviruses in nature, we have examined cattle and wild animals for rotavirus infection since 2006 in Japan (Abe et al., 2009, 2010). During this survey, the AzuK-1 (G21P[29]) strain was identified as a bovine rotavirus belonging to new G and P genotypes. Furthermore, in this study, we identified the Dai-10 strain (G24P[33]) as a bovine rotavirus belonging to other new G and P genotypes. Recently, a number of new genotypes have been found in humans and animals (Collins et al., 2010; Lamhoujeb et al., 2010; Martella et al., 2006, 2007; Matthijnssens et al., 2009, 2010a; McNeal et al., 2005; Parra et al., 2007; Rahman et al., 2005; Schumann et al., 2009; Solberg et al., 2009; Ursu et al., 2009). However, the genetic background of most strains has remained to be identified. In this study, we determined the complete ORF sequences of AzuK-1 and Dai-10 strains and investigated the evolutionary relationships among the two strains and other rotaviruses.
Table 1. Comparison of complete full-genome ORF sequences between the AzuK-1 and Dai-10 strains

<table>
<thead>
<tr>
<th>Gene encoding</th>
<th>Nucleotide cut-off value (%)*</th>
<th>Identity between AzuK-1 and Dai-10 strains (%)</th>
<th>Genotype</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nucleotide</td>
<td>Amino acid</td>
</tr>
<tr>
<td>VP1</td>
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<tr>
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<td>85</td>
<td>97.7</td>
<td>99.7</td>
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<tr>
<td>NSP5</td>
<td>91</td>
<td>96.1</td>
<td>95.5</td>
</tr>
</tbody>
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*Established by Matthijnssens et al. (2008b).

Fig. 2. Phylogenetic trees based on the full-length nucleotide sequences of VP1, VP2, VP3 and NSP1 genes of AzuK-1, Dai-10 and established reference R, C, M and A genotype strains, respectively. The tree was generated by the neighbour-joining method using MEGA version 4. Bootstrap values (1000 replicates) above 50 are shown. Dark circles, strain data and codes for species of origin are as for Fig. 1. Bars indicate nucleotide substitutions per site.
We previously reported that G21P[29] rotaviruses have been widely prevalent among cattle in Japan for over 10 years (Abe et al., 2009). On the other hand, G24P[33] rotaviruses were detected from only two cows (a calf and the mother, data not shown), suggesting that G24P[33] genotypes are rare in cattle populations. Thus, G24P[33] rotaviruses may not efficiently transmit from cattle to cattle compared with G21P[29] rotaviruses. Complete ORF analyses revealed that AzuK-1 (G21P[29]) and Dai-10 (G24P[33]) strains shared a similar genetic background except for VP4 and VP7 genes (Figs 2, 3 and 4). VP4 has been shown to participate in several important functions, such as cell attachment, entry into cells, neutralization and protease-enhanced infectivity of rotavirus (Estes & Kapikian, 2007; López & Arias, 2004). VP7 is the major neutralization antigen and is involved in the cell entry
process (Estes & Kapikian, 2007; Graham et al., 2003). Therefore, compared with G24P[33] strains, G21P[29] strains might have an advantage in cell attachment and/or cell entry in the gut of cattle. However, considering the fact that nucleotide/amino acid similarities of other gene segments between the AzuK-1 and Dai-10 strains are rather variable (Table 1), the possibility of involvement of some gene segments other than VP4 and VP7 in efficiency of transmission cannot be excluded.

Excluding the VP4 and VP7 genotypes, I2-R2-C2-A3/A13-N2-T6/T7-E2-H3, corresponding to VP6, VP1–VP3 and NSP1–NSP5 genotypes, are commonly found in bovine rotaviruses (Matthijnssens et al., 2006, 2009). Since most of the gene segments of bovine rotavirus strains are clustered together within the same corresponding genotypes regardless of the geographical origin or time of isolation, it is thought that these are typical gene segments of bovine rotaviruses. In addition, it was recently shown that other members of the family Bovidae or the order Artiodactyla share a largely preserved consensus genotype constellation with bovine rotaviruses (Matthijnssens et al., 2009).

Genotype determination of the AzuK-1 and Dai-10 strains revealed that most of the 11 gene segments of both strains possessed genotypes typically observed in bovine rotaviruses, except for VP4, VP7 and NSP3 genotypes (Figs 2, 3 and 4). Unexpectedly, VP6 and NSP2 gene segments of both strains, which have the same genotypes as those of bovine rotaviruses, are clustered separately from those of other bovine rotaviruses. In regard to the NSP2 gene segment, the Dai-10 strain is closely related to the Gu/Arg/Rio Negro/98 strain. This finding corresponds with those of a previous study, suggesting a relationship between rotaviruses from guanacos and cows, perhaps transmitted through a third host species in contact with both animals (Matthijnssens et al., 2009). On the other hand, the NSP2 of AzuK-1 strain exhibited low nucleotide sequence identity (<90%) compared with those of other rotavirus strains. Of note, the AzuK-1 and Dai-10 strains were clustered with canine, feline and canine/feline-like human rotavirus strains, although the phylogenetic relationships among them are rather distant. These findings suggest that the AzuK-1 and Dai-10 strains might have a shared ancestry with canine/feline rotaviruses. If that is the case, a long time must have passed since the interspecies transmission between a feline/canine host and a bovine/guanaco host. For the VP6 gene segment, phylogenetic analysis and comparative analysis of deduced amino acid sequences suggested a close evolutionary relationship among AsuK-1, Dai-10 and SA11-H96 strains (differing only in 2–3 aa residues, Supplementary Fig. S1). These findings indicate that the AzuK-1 and Dai-10 strains might have originated by interspecies transmission and multiple reassortment events involving bovine, simian, canine/feline and unknown rotavirus strains.

As previously reported, the VP6 gene segment of the bovine RUBV51 strain, which belongs to rare G/P genotypes G15P[21], was also closely related to the SA11-H96 strain (Ghosh et al., 2008) (Fig. 4). AzuK-1 and Dai-10 were detected in Japan in 2006 and 2007, respectively, whereas the RUBV strain was detected in India in 2005. From the fact that these strains were detected in different places, the SA11-H96-like VP6 gene segments may circulate in cattle in South and South-east Asia. Considering that these three bovine strains were detected recently (2005–2007), it would be interesting to investigate whether the SA11-H96-like VP6 gene is currently prevalent among bovine rotaviruses.

The origins of the VP4 (P[29] or P[33]), VP7 (G21 or G24) and NSP3 (T9) gene segments of the AzuK-1 and Dai-10 strains remain obscure. For G21 and P[29] genotypes, considering the fact that G21P[29] genotypes have been found to be endemic in cattle, G21 and P[29] genotypes could be typical bovine genotypes in asymptomatic cattle. Interestingly, the novel T9 genotype formed a clade with T2, T3 and T5 genotypes, which are commonly found in human, canine, feline and simian hosts, not in cattle (Fig. 1). The T9 genotype might be phylogenetically related to the simian SA11-H96 strain, although this is supported only by a low bootstrap value. Taken together with the fact that the VP6 gene segments of the AzuK-1 and Dai-10 strains showed a close evolutionary relationship with those of the SA11-H96 strain (Fig. 4 and Supplementary Fig. S1), the origins of the NSP3 gene segments of the AzuK-1 and Dai-10 strains might also be related to simian rotaviruses.

It should be noted that both of the new rotavirus strains, AzuK-1 (G21P[29]) and Dai-10 (G24P[33]), were isolated from asymptomatic cows. In spite of the fact that rotaviruses cause asymptomatic infection (Collins et al., 2010; Estes & Kapikian, 2007; Lamhoujeb et al., 2010; Martella et al., 2010; McNulty & Logan, 1983; Parra et al., 2008; Reynolds et al., 1985; Steyer et al., 2008; Swiatek et al., 2010), there have been few studies on rotaviruses in asymptomatic animals. Investigation and characterization of rotaviruses in asymptomatic animals would be helpful to understand the ecology and evolution of rotaviruses.

In this study, we characterized the whole genome of two new bovine rotavirus strains, AzuK-1 (G21P[29]) and Dai-10 (G24P[33]). Our data indicate the possibility that both strains emerged by interspecies transmission and multiple reassortment events involving bovine, simian, canine/feline and unknown rotavirus strains. Considering the fact that the available genetic database of animal rotaviruses is limited, global surveillance and epidemiological study on rotaviruses in animals are crucial to fully understand the true origin of the AzuK-1 and Dai-10 strains.

**METHODS**

**Viruses.** The Dai-10 strain was isolated in African green monkey kidney (MA-104) cells from an asymptomatic cow in Hyogo Prefecture, Japan in 2008. G and P genotyping based on partial nucleotide sequences of VP7 and VP4 genes revealed that the Dai-10 strain did not belong to any of the established G and P genotypes.
The AzuK-1 (G2P[29]) strain was isolated in 2006 from an asymptomatic calf in Gifu Prefecture, Japan (Abe et al., 2009).

**Complete ORF analyses of VP4 and VP7 genes of Dai-10 strain.**

Primers were designed and PCR or semi-nested PCR was performed to determine the complete ORF sequences of the VP4 and VP7 genes. Outer PCR was performed with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. The cycle conditions for inner PCR were 25 cycles of 95 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min. All the primers used to determine the complete ORF sequences of the VP4 and VP7 genes of the Dai-10 strain are listed in Supplementary Table S1.

**RNA extraction and RT-PCR for complete ORF analysis.**

Viral RNA was extracted from the AzuK-1 and Dai-10 strains by using a QIAamp Viral RNA Mini kit (QIagen). Synthesis of the cDNA was performed using a PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa BIO) with random hexanucleotides (for VP4, VP6–7, NSP2–5 genes) and specific primers (for VP1–3 and NSP1 genes) as described previously (Matthijssens et al., 2008a). Genomic RNAs were heated at 95 °C for 5 min and immediately chilled on ice, and then the reverse transcription reaction was carried out. The cDNAs were amplified by PCR with TaKaRa Ex Taq (TaKaRa BIO). The full lengths of the ORFs of VP1, VP2, VP3, VP6, NSP1, NSP2, NSP3, NSP4 and NSP5 genes were amplified with primer pairs MAX-1aF and MAX-1bR, MAX-2aF and MAX-2bR, GEN-VP3Fe and GEN-VP3Rc, GEN-VP6F and GEN-VP6R, BOV-5F and BOV-5R1, GEN-NSP2F and GEN-NSP2R, GEN-NSP3F and NSP3-endR (Supplementary Table S1, modified slightly from MAX-NSP3R), GEN-NSP4F and GEN-NSP4R and GEN-NSP5F and GEN-NSP5R, respectively, as described previously (Matthijssens et al., 2008a). PCR was carried out with an initial step at 95 °C for 5 min, followed by 40 cycles of amplification and a final extension at 72 °C for 5 min on a PC-320 Program Temp Control System (ASTEC) and TP600 TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa BIO). The cycle conditions for the amplification of VP1, VP2, VP3 and NSP1 genes were 45 s at 95 °C, 1 min at 45 °C and 2 min at 68 °C; for the other gene segments, the conditions were 45 s at 95 °C, 1 min at 45 °C and 1 min at 68 °C.

**Direct sequencing and phylogenetic analysis.**

The PCR products were purified with NucleoSpin Extract (Macherey-Nagel) and sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism 3100 DNA analyser (Applied Biosystems). The sequencing was performed with the forward and reverse primers used for the PCR. Primer walking sequencing was performed to cover the complete sequences of the respective fragments. The entire coding regions were assembled from the sequences and analysed using A plasmid Editor v1.10.4. Sequence alignments and construction of phylogenetic trees were performed using CLUSTAL W and MEGA version 4.0 software (Tamura et al., 2007) by the neighbour-joining method (with 1000 bootstrap replicates).

**Assignment of newly identified genotypes.**

The genotypes of each of the 11 genome segments for the AzuK-1 and Dai-10 strains were determined according to the genotyping recommendations of the RCWG (Matthijssens et al., 2008b) using the online RotaC genotyping tool (http://rotac.regatools.be/) (Maes et al., 2009). The assignment of novel genotypes was approved by the RCWG.

**Nucleotide sequence accession numbers.**

The nucleotide sequence data presented in this paper have been deposited in the GenBank database. The accession numbers of the genes encoding VP4, VP7, VP1, VP2, VP3, VP6, NSP1, NSP2, NSP3, NSP4 and NSP5 of the Dai-10 strain are AB513836, AB513837 and AB573070–AB573078, respectively. The accession numbers of the genes encoding NSP3, VP1, VP2, VP3, VP6, NSP1, NSP2, NSP4 and NSP5 of AzuK-1 strain are AB513838 and AB573079–AB573086, respectively.

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