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Molecular epidemiology of rotaviruses among healthy calves in Japan: Isolation of a novel bovine rotavirus bearing new P and G genotypes

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ABSTRACT

A total of 171 fecal specimens collected from healthy calves on a beef farm in Gifu Prefecture, Japan in 2006–2007 were examined for group A rotaviruses by RT-semi-nested PCR targeting the coding region for VP8*. Nine specimens were positive for rotavirus. G and P genotyping indicated that one strain was G10P[11]-like and six strains were considered to be the same unknown G and P genotypes. Among these six untypeable strains, one strain, AzuK-1, was adapted to cell culture and analyzed. Sequence and phylogenetic analyses of the full lengths of VP4 and VP7 genes revealed that AzuK-1 strain is a novel bovine rotavirus bearing new G21 and P[29] genotypes as confirmed by the RCWG. Furthermore, we detected G21P[29] rotaviruses in fecal specimens collected from healthy calves in Hokkaido, Japan during the period from 1997 to 1998. These findings suggest that novel G21P[29] rotaviruses have been widely prevalent among cattle for over 10 years in Japan.

1. Introduction

Group A rotaviruses are known to be major agents of severe acute gastroenteritis in infants and young animals worldwide. Approximately 611,000 children worldwide die from rotavirus diarrhea every year, the numbers of deaths being particularly high in developing countries (Estes, 2001; Glass et al., 2006; Parashar et al., 2006). Even in developed countries, rotavirus remains an important cause of morbidity. For example, in the United States, it has been estimated that rotavirus accounted for 47,000–60,000 hospitalizations each year of children less than 5 years of age, suggesting that 1 in 67 to 1 in 85 children will be hospitalized with rotavirus by the age of 5 years (Malek et al., 2006). Rotavirus-induced diarrhea is therefore a serious public health problem throughout the world.

Rotaviruses are members of the *Reoviridae* family. The virus particles have a triple-layered protein capsid that contains a genome of 11 segments of double-stranded RNA, which encode six viral structural proteins (VP1-4, VP6 and VP7) and six nonstructural proteins (NSP1-NSP6). Each RNA segment is monocistronic, with the exception of segment 11, which has an additional overlapping open reading frame, encoding NSP6. Because of the segmented nature of the genome, infection of one cell with two different rotaviruses could result in a reassortment event, which is known to generate new viruses, so-called "reassortants" with combinations of genome segments from two parental viruses (Palombo, 2001; Ramig, 1997). Reassortment is one of the important mechanisms for generating genetic diversity of rotaviruses and eventually for viral evolution.

VP4 and VP7, which are encoded by segments 4 and 9, respectively, are components of the viral outer capsid. VP4 forms viral spikes that project from the surface of viral particle. VP4 has been shown to participate in several important functions, such as cell attachment, entry into cells, haemagglutination, neutralization, virulence, and protease-enhanced infectivity of rotavirus (Dunn et al., 1995; Estes, 2001; Fuentes-Panana et al., 1995; Kaljot et al., 1988; Ludert et al., 1996; Offit et al., 1986). By protease treatment, VP4 is cleaved into two polypeptides, VP8* and VP5*. VP7, the most abundant external glycoprotein, forms a smooth surface of the virion. VP7 is the major neutralization antigen and is involved in the cell entry process (Fukuhara et al., 1988; Sabara et al., 1985).

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According to diversities of VP4 and VP7 genes, group A rotaviruses are classified into P and G genotypes. Rotavirus strains with VP4 and VP7 sharing 89% or greater amino acid identities belong to the same P and G genotypes, respectively (Estes, 2001; Gorziglia et al., 1990; Nishikawa et al., 1989). Recently, a novel classification system for rotaviruses has been proposed based on the nucleotide sequences of a complete open reading frame (ORF) (Matthijnssens et al., 2008a). In this system, 80% VP4 and VP7 nucleotide sequence identities are the cut-off values for the classification of P and G genotypes. To date, 30 P-genotypes (P[1]-P[28] and P[30]-P[31]) and 21 G-genotypes (G1-G20 and G22) have been described in literature (Matthijnssens et al., 2008a; Schumann et al., 2009; Solberg et al., 2009). The G and P genotypes are peculiarly distributed across various animal species, suggesting host species barriers and restriction. For example, G1P[8], G2P[4], G3P[8] and G4P[8] strains are globally predominant in humans (Estes, 2001). However, human rotaviruses with unusual G genotypes (e.g., G5, G6, G8, G9, G10 and G12) and P genotypes (e.g., P[3], P[6], P[9], P[11] and P[14]) have been emerging, suggesting that natural reaassortments have occurred between human and animal rotaviruses, especially in cattle and pigs (Dhama et al., 2009; Ghosh et al., 2007; Li et al., 2008; Nguyen et al., 2007; Parra et al., 2008; Rahman et al., 2007; Santos and Hoshino, 2005; Sharma et al., 2008). G9 rotavirus, of which the VP7 gene is presumably derived from a porcine rotavirus, is recognized as the emerging pathogen for humans worldwide (Hoshino et al., 2005; Phan et al., 2007b; Yang et al., 2007). Therefore, animal rotaviruses are regarded as a potential reservoir for genetic diversity of human rotaviruses.

It is notable that a number of new genotypes have recently been found in animals (Liprandi et al., 2003; Martella et al., 2006, 2007; McNeal et al., 2005; Parra et al., 2007; Rao et al., 2000; Schumann et al., 2009). Monitoring of newly emerging rotaviruses in animals is important for the control of rotavirus infection in both humans and animals. Therefore, there has been great interest in rotaviruses circulating in our environment. However, despite the fact that rotaviruses cause asymptomatic infection in calves, pigs, cats and humans (Dea et al., 1985; Debouck and Pensaert, 1983; Hoshino et al., 2003; McNulty and Logan, 1983; Mochizuki et al., 1997; Ray et al., 2007; Reynolds et al., 1985; Roger et al., 2005; Steyer et al., 2008, 2007), there is little information on rotavirus infection in healthy animals. Hence, it remains to be elucidated how asymptomatic infection with rotaviruses affects the ecology and evolution of rotaviruses. Our previous seroepidemiological study showed high prevalences of infection with rotaviruses of several genotypes in cattle (unpublished data). Thus, it is expected that surveillance of rotavirus infection in cattle will provide further information on the natural history of rotaviruses.

To understand infection cycles of rotaviruses in nature, it is necessary to characterize the viruses in subclinically infected animals. For this purpose, we examined healthy calves in Gifu Prefecture, Japan for rotavirus infection during the period from 2006 to 2007. We were able to isolate an unknown bovine group A rotavirus from the feces of healthy cattle. Genetic analysis of the strain demonstrated that VP4 and VP7 have less than 80% nucleotide identities with other established P- and G-genotypes, and therefore the Rotavirus Classification Working Group (RCWG) assigned novel P[29] and G21 genotypes (Matthijnssens et al., 2008b).

Furthermore, to clarify when G21P[29] rotavirus emerged in cattle populations, we retrospectively investigated the prevalence of G21P[29] rotaviruses in healthy calves using the fecal specimens collected in Hokkaido, Japan from 1997 to 1998. The results suggested that G21P[29] rotaviruses have been endemic in calves for at least 10 years.

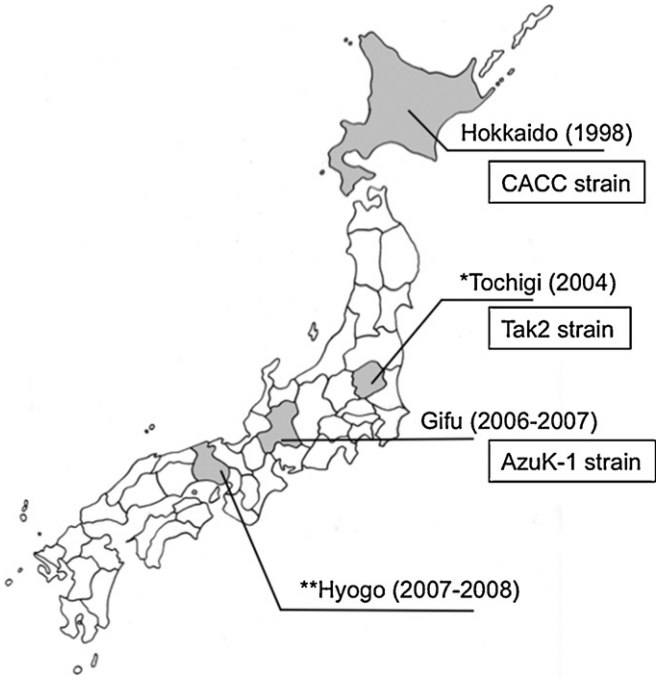

Fig. 1. Geographical distribution of G21P[29] rotaviruses in Japan. *Fukai et al. (2007); **unpublished data.

2. Materials and methods

2.1. Fecal samples

A total of 171 fecal specimens were collected from healthy calves (aged <1year) on a beef cattle farm located in Gifu Prefecture, Japan from July 2006 to June 2007 (Fig. 1). The fecal samples were diluted with phosphate-buffered saline to 20% suspensions and clarified by centrifugation at 750 × *g* for 10 min. The supernatants were collected and stored at −80 °C until use.

In addition, we investigated 298 fecal specimens collected from eight healthy calves in Hokkaido Animal Research Center in Japan during the period from 1997 to 1998 (Fig. 1). These fecal specimens were diluted with phosphate-buffered saline to 10% suspensions and clarified by centrifugation at 750 × *g* for 10 min. The supernatants were collected and stored at −20 °C until use.

2.2. RNA extraction, reverse transcription, and nested and semi-nested PCR (RT-PCR)

Viral RNA was extracted from fecal suspensions by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Synthesis of the cDNA was performed using a PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa BIO, Shiga, Japan) with random hexanucleotides as primers. Genomic RNAs were heated at 95 °C for 5 min and immediately chilled on ice, and then reverse transcription reaction was carried out. The cDNAs were amplified by semi-nested PCR. To detect and analyze the rotavirus genes, we designed primers for amplifying and sequencing VP4 and VP7 genes (Supplementary Table 1). The cDNA was amplified by an outer PCR with the primers VP4-HeadF and VP4-1092R for VP4, resulting in a 1050-bp product, and by an inner PCR with the primers VP4-HeadF and VP4-887R, resulting in an 840-bp product. PCR was carried out with TaKaRa Ex Taq (TaKaRa BIO, Shiga, Japan) on a PC-320 PROGRAM TEMP CONTROL SYSTEM (ASTEC, Fukuoka, Japan) and TP600 TaKaRa PCR Thermal Cycler Dice Gradient (TaKaRa BIO, Shiga, Japan). Inner and outer PCRs were performed with an initial denaturation step at 95 °C for 5 min, followed by 50 cycles of 95 °C for 30 s, 50 °C for