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Genetic characterization of bovine rotavirus isolates in Korea

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Abstract : Throughout the world, rotavirus infections cause extensive morbidity in human infants and diarrhea in animals such as white scour caused by bovine rotavirus in calves. We isolated three rotavirus strains designated KV0407, KV0418, and KV0426 from 103 fecal samples of diarrheic calves. The genes coding for proteins VP4, VP6, VP7, and NSP4 from strain KV0407 were sequenced and compared with the nucleotide sequences of other known strains of rotavirus. The KV0407 VP4 gene was highly homologous to the OSU (99.4%) and JL94 (99.4%), but not the B223 (62.4%) and K33 (62.4%) VP4 genes. The KV0407 and KV0418 VP7 genes were most similar to the OSU and super-short type VMRI VP7 genes. Based on nucleotide sequence analysis, the KV0407 strain was tentatively assigned to A serogroup (SG I), G5P[7], NSP4 genotype B and the KV0418 and KV0426 strains were assigned to A serogroup (SG I), G6P[5], NSP4 genotype A. The genetic characterization of these bovine rotavirus isolates could be useful for the diagnosis and prevention of diarrhea in calves.

Keywords : bovine rotavirus, NSP4, VP4, VP6, VP7

Introduction

Group A rotaviruses from the family *Reoviridae* are an important cause of gastroenteritis in young children and animals worldwide [3, 4, 12]. Rotaviruses consist of three concentric protein layers surrounding 11 segments of double-stranded RNA that encode six structural proteins, i.e., VP1, VP2, VP3, VP4, VP6, and VP7, and five non-structural proteins, i.e., NSP1, NSP2, NSP3 (NS34), NSP4, and NSP5. Proteins VP3, VP4, VP7, NSP1, NSP2, and NSP4 are associated with rotavirus virulence in some animals, and proteins VP4, VP7, NSP1, and NSP4 exhibit sequence heterogeneity with the animal hosts from which they originate [8, 14, 16]. The three concentric protein layers are composed primarily of VP2 for the inner layer, VP6 for the middle layer, and VP4 and VP7 for the outer layer. Three major antigenicities classified as group, subgroup, and serotype have been demonstrated in the rotaviruses, with the group and subgroup (SG) specificities defined by VP6 and the serotype specificity defined by VP7 (G) and VP4 (P) [9]. In human and animal rotaviruses, 15 G serotypes and 26 P serotypes (22 genotypes) have been identified, respectively [15, 20]. Bovine rotavirus (BoRV) is also recognized as a significant cause of calf diarrhea and causes clinical symptoms that include watery diarrhea, depression, anorexia, and dehydration. Rotavirus infection is transmitted via the fecal-oral route and by contact with contaminated fomites. Infections are most common in the cold season. Ball *et al*. [2] reported a new mechanism of rotavirus-induced disease for which the non-structural protein NSP4 functions as a viral enterotoxin. Rotaviruses have evolved via several mechanisms, including reassortment, single point mutation, and genetic rearrangement [17]. Therefore, the genetic characterization of rotaviruses in cattle is important to develop diagnostic and preventive measures, including development of more effective vaccines. To investigate the molecular characteristics of Korean BoRV strains isolated from the fecal samples of calves with diarrhea, we sequenced some of the important genes from these isolates and established their genetic relationships to existing BoRV strains isolated from different regions around the world.

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Materials and Methods

Virus isolation

Fecal samples (N = 103) were collected from diarrheic calves that had been submitted to the Virology Division, National Veterinary Research and Quarantine Service, Korea, in 2004. Rotaviruses were isolated from fecal samples by inoculating monkey kidney cells (TF-104) with the samples in the presence of 0.5 μg/ml trypsin (Type IX; Sigma, USA), as described previously [19]. The cells were incubated for 7 days in 24-well microplates and then screened for cytopathic effects (CPE). Cells that showed specific CPE were examined using immunofluorescent antibody staining with a BoRV-specific monoclonal antibody.

Electron microscopy

Three Korean isolates were propagated in TF-104 cells. After freezing and thawing three times, cell debris was removed by centrifugation. The supernatants were precipitated with polyethylene glycol (PEG; MW 8000), pelleted by centrifugation, and resuspended in 1/100 of the original volume with GTNE buffer (200 mM glycine, 50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5). Concentrated supernatants were overlaid on discontinuous sucrose gradients, centrifuged at 100,000 × g for 3 h, and recovered from the interface between the two sucrose layers. Following dialysis against distilled water, the viral particles were observed using a Hitachi 7100 electron microscope (Hitachi, Japan).

Sequencing and phylogenetic analysis

BoRV RNA was extracted from cell-cultured isolates using a commercial kit (Bioneer, Korea) according to the manufacturer's instructions. Reverse transcriptase polymerase chain reaction (RT-PCR) with primers specific for BoRV was used to amplify the genes for VP4, VP6, VP7, and NSP4 (Table 1). In short, RT-PCR was performed in a reaction mixture containing 20 μl of denaturated RNA, 10 μl of 5 × buffer (12.5 mM $MgCl_2$), 2 μl of enzyme mix (reverse transcriptase and *Taq* polymerase), 1 μl of each primer (50 pM) and 16 μl of distilled water (Qiagen, Germany), for a 50 μl final volume. The cycling profile was as follows: cDNA synthesis at 42°C for 30 min; followed by 35 cycles with denaturation at 95°C for 45 sec, annealing at 50°C for 45 sec, and extension at 72°C for 45 sec; and a final extension at 72°C for 5 min. RT-PCR products were visualized using electrophoresis on 1.5% agarose gels containing ethidium bromide. Purified PCR products were ligated with the pGEM-T easy vector (Promega, USA). Sequencing reactions were performed using recombinant plasmids and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, USA). Phylogenetic analysis was performed on the VP4, VP6, VP7, and NSP4 nucleotide sequences of the three Korean isolates and reference BoRV strains obtained from GenBank.

Table 1. List of the oligonucleotide primers used for reverse transcriptase polymerase chain reaction against rotavirus

Primer designated	Oligonucleotide sequence (5'- 3')	Target gene	Size of amplicon
VP4 -1F	ATG GCT TCG CTC ATA TAC AGA CAG	VP4	1,085 bp
VP4-1R	AAT GCT TGT GAA TCG TCC CA		
VP4-2F	GGG ATG ATT CAC AAG CAT T	VP4	714 bp
VP4-2R	ATC TCC GTC CAA GCG GAT GC		
VP4-3F	CCA TGG CAA CGA ACG TGA TG	VP4	718 bp
VP4-3R	ACC TCT TGA CAC TGC TTA CAG TCT		
VP6 F	GGC TTT TAA ACG AAG TCT TC	VP6	1,320 bp
VP6 R	GGT CAC ATC CTC TCA CTA TG		
VP7 F	GGC TTT AAA AGC GAG AAT TT	VP7	1,062 bp
VP7 R	GGT CAC ATC ATA CAA CTC TA		
NSP4 F	CCA TGG AW*A ARC TTR CCG	NSP4	528 bp
NSP4 R	AWW CKR GCY GTC ACT TC		

*W= A/T, R= A/G, K=G/T, Y=C/T.

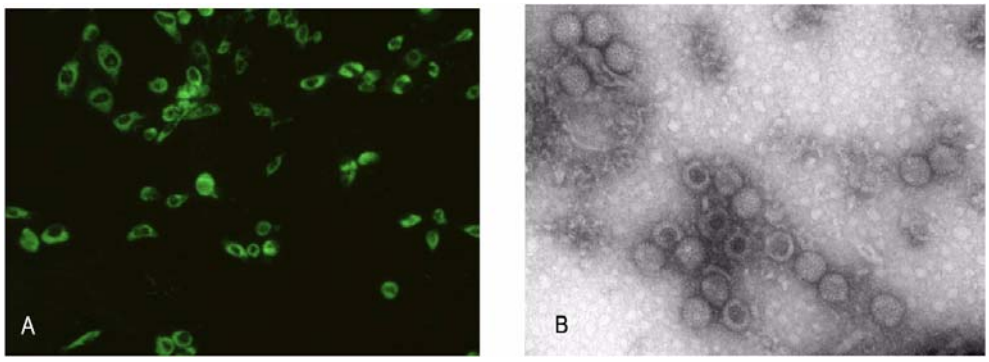

Fig. 1. Immunofluorescence of TF-104 cell infected with the KV0407 isolate using a bovine rotavirus specific monoclonal antibody (A) and electron micrograph of bovine rotavirus of the KV0407 isolate (B).

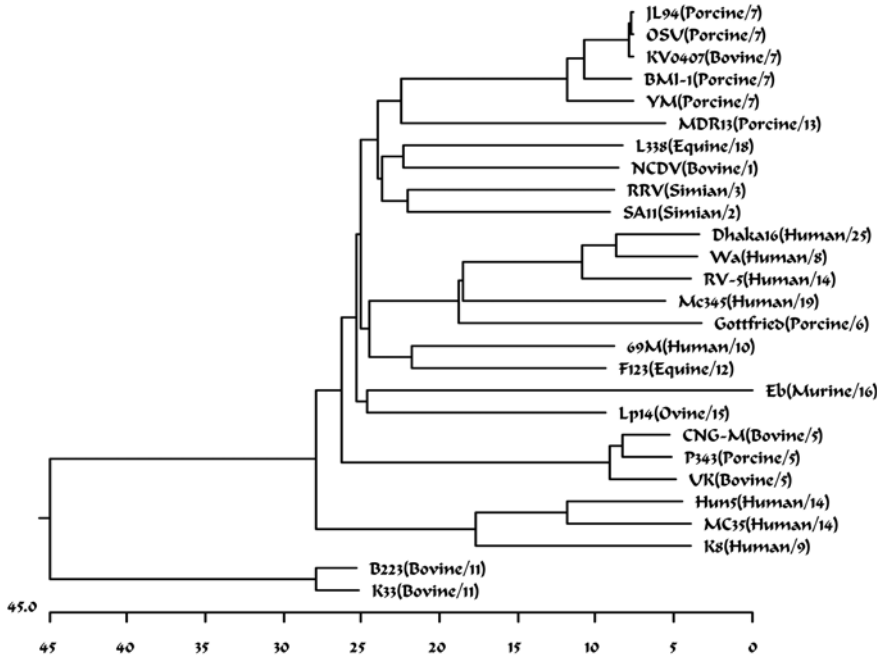

Fig. 2. Phylogenetic tree based on the VP4 genes of KV0407 isolate showing its genetic relationship with other P type rotavirus strains.

Phylogenetic trees and sequence pair distances of the nucleotides were obtained using the DNASTAR software program (Madison, USA). Homology analysis was performed using DNASIS software (Hitachi Software, Japan).

Results

Identification of bovine rotaviruses

Of the 103 fecal diarrhea samples, 3 produced obvious CPE characterized by the formation of granules and detachment of cells from the culture plates 3 or 4 days after inoculation. Following three passages of the isolates in TF-104 cells, viral titers of $10^{5.5}$ $TCID_{50}$/ml were obtained. The three isolates were designated KV0407, KV0418, and KV0426. For positive identification as rotaviruses, TF-104 cells infected with the CPE-producing isolates were fixed with cold acetone and stained with a rotavirus-specific monoclonal antibody. The BoRV-specific fluorescence appeared in the cytoplasm of the infected cells (Fig. 1A). Electron microscopy revealed that the virus particles ranged from 60 to 70 nm in diameter, with typical rotavirus morphology (Fig. 1B).

Sequence analysis of the VP4 gene from the KV0407 strain

A total of 2,362 nucleotide sequences for the VP4 gene from the KV0407 strain were identified and compared with the VP4 genes from 26 reference strains. The KV0407 VP4 gene was most similar to the VP4 genes from the OSU strain (99.4%) and the JL strain P7 genotype (99.4%). The lowest sequence similarity was observed with the VP4 gene from the B223 strain (62.4%). Phylogenetic analysis also indicated that KV0407 clustered most closely with the JL94 strain P7 genotype (Fig. 2).

Sequence analysis of the VP6 and VP7 genes

A total of 1,320 nucleotide sequences for the VP6 gene from the KV0407, KV0418, and KV0426 strains were identified. There are reports that SG specificity shows a correlation with comparative analysis of partial amino acids of the VP6 (aa 241 to 367) [10, 17]. The partial sequences (378 bp) of the three Korean strains were compared with those of VP6 gene sequences of 21 rotavirus reference strains. Based on the comparative analysis of partial VP6 gene, all KV0407, KV0418, and KV0426 strains were associated with SG I specificity (Fig. 3). Complete nucleotide sequences for the VP7 genes of the three strains were also determined and compared with the VP7 sequences of 36 rotavirus strains obtained from GenBank. KV0407 exhibited high nucleotide identity with the G5 rotaviruses, including porcine OSU, JL94, and the bovine KJ75 and KJ44 strains (99.4% homology). In contrast, KV0418 and KV0426 were most homologous to the G6 rotaviruses, including the bovine VMRI and IND strains (97.3% homology). Phylogenetic trees also revealed that KV0407 was closely related to the G5 genotype strains, and KV0418 and KV0426 clustered most closely with the G6 genotype strains (Fig. 4).

Sequence analysis of the NSP4 genes

The NSP4 gene of rotaviruses can be classified into at least five genetic groups using nucleotide sequence analysis [5], so the full-length NSP4 genes from KV0407, KV0418, and KV0426 were sequenced and compared with the published NSP4 sequences to determine their genetic groups. KV0407 NSP4 displayed nucleotide identity ranging between 87.9 and 99.4% with NSP4 genotype B sequences. The KV0418 and KV0426 NSP4 sequences were 100% identical to each other and ranged between 86.3 and 96.8% identical to NSP4 genotype A sequences. Phylogenetically, KV0407 belonged to genotype B and KV0418 and KV0426 were classified as genotype A (Fig. 5).

Discussion

Recent studies [1, 5] have indicated that new bovine rotavirus strains are emerging as a result of natural genetic reassortment with the animal host. Three strains

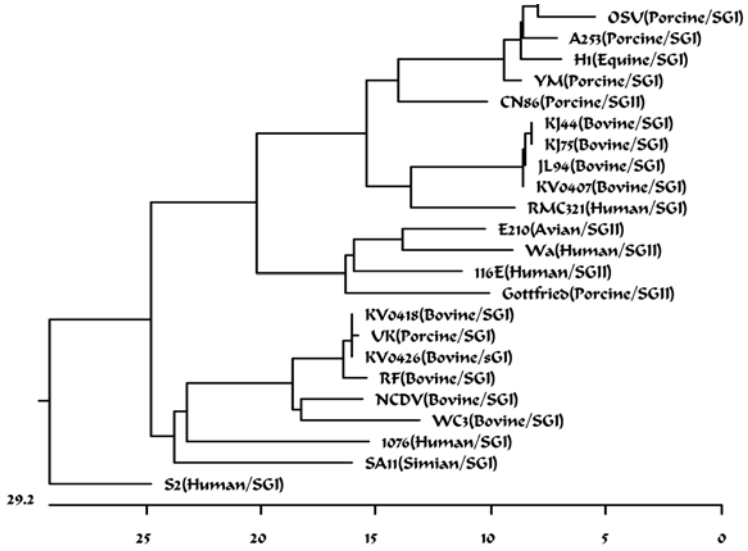

Fig. 3. Phylogenetic tree based on the VP6 genes of the KV0407, KV0418, and KV0426 isolates showing its genetic relationship with other rotavirus strains.

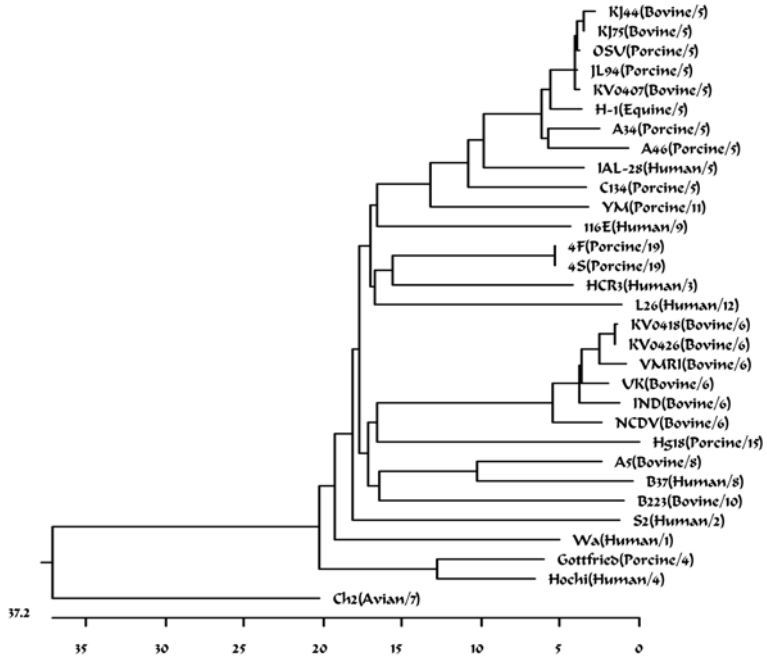

Fig. 4. Phylogenetic tree based on the VP7 genes of the KV0407, KV0418, and KV0426 isolates showing its genetic relationship with other G genotype rotavirus strains.

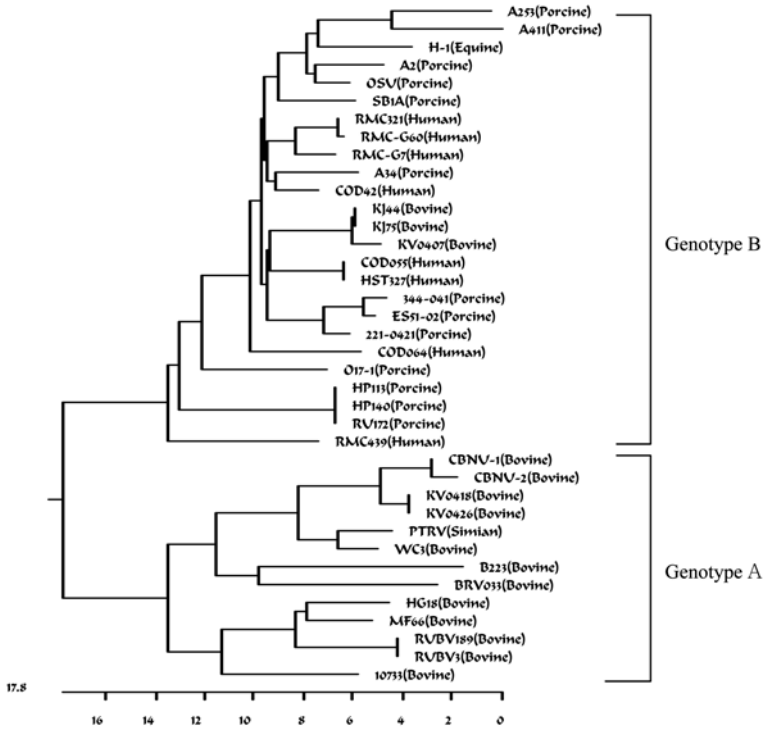

Fig. 5. Phylogenetic tree based on the NSP4 genes of the KV0407, KV0418, and KV0426 isolates showing their genetic relationship with other rotavirus strains.

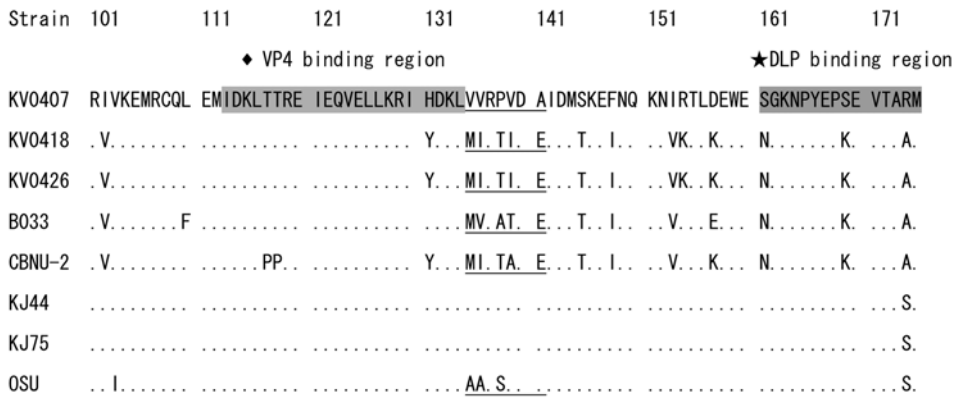

Fig. 6. Multiple sequence alignment of the deduced amino acid sequences of the isolates (KV0407, KV0418 and KV0426) NSP4 protein with those of rotaviruses. The putative VP4 (◆) and double layered particle binding regions (★) and hypervariable region (underline) are indicated.

of BoRV were isolated from 103 fecal samples of diarrheic calves in Korea and confirmed as BoRVs using a specific monoclonal antibody. The five genes encoding the VP4, VP6, VP7, and NSP4 proteins of the three isolates were sequenced, and the nucleotide sequences were compared with published sequences of other rotavirus strains. The rotavirus spike protein VP4 is responsible for a number of important biological functions such as the enhancement of infectivity by proteolytic cleavage into VP8 and VP5, hemagglutination, virulence, and host range restriction [11]. The VP4 gene of the KV0407 strain had a high degree of homology with the VP4 genes of the OSU (99.4%) and JL94 (99.4%) strains, but differed significantly from those of the B223 (62.4%) and K33 (62.4%) strains. The VP6 protein consists of 397 amino acids and is the most antigenic protein of the rotaviruses. Based on reactivity with SG-specific monoclonal antibodies, rotaviruses have been classified into four groups designated SG I, SG II, SG I and SG II, and neither SG I nor SG II [6]. Our phylogenetic analysis indicated that each of the three new strains is assigned to the SGI group. Serotype G of rotaviruses has been differentiated using ELISA with specific monoclonal antibodies, nucleic acid hybridization, restriction fragment length polymorphism, and sequence analysis [10, 11, 13, 18]. The VP7 protein forms the smooth outermost surface of the rotavirus particle and is the determinant of serotype G [6, 7]. BoRVs belonging to serotypes G1, G6, G8, and G10 have been reported in cattle, with most BoRVs assigned to the G6 and G10 genotypes [6, 11], but the G5 genotype has been recently reported in Korean cattle [17]. We identified both the G5 and G6 genotypes of BoRV from Korean cattle. Our comparison of the VP7 sequences from the new strains with the VP7 sequences of existing strains indicated that KV0407 is most homologous with the G5 genotype from the OSU strain, whereas KV0418 and KV0426 are most homologous with the G6 genotype of the super-short type VMRI strain [16]. Therefore, both the VP7 sequence and phylogenetic analysis suggest that KV0407 originated in pigs, whereas KV0408 and KV0426 originated in cattle. Residues 114-135 of the enterotoxic peptide of simian rotaviral NSP4 are considered to be a key domain for enterotoxigenic activity [6]. The NSP4 genes from humans and animals have been classified into five genotypes designated A through E, and the NSP4 proteins exhibit the characteristic activity of viral enterotoxins [2, 4]. KV0407 NSP4 was most homologous (74.3-94.1%) with NSP4 sequences of the other rotavirus strains and the hypervariable region (aa 135-141) associated with altered virulence (Fig. 6).

In conclusion, by analyzing the VP4, VP6, VP7, and NSP4 genes of three new BoRV strains isolated in Korea in 2004, the KV0407 strain was tentatively assigned to the A serogroup (SG I), G5P[7], NSP4 genotype B, and the KV0418 and KV0426 strains were assigned to the A serogroup (SG I), G6P[5], NSP4 genotype A. The genetic information characterizing these new Korean BoRV isolates could be useful in understanding the epidemiology of BoRVs and for choosing candidate target proteins for vaccines or for diagnosis of BoRV infection.

Acknowledgments

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