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Molecular characterization of avian rotavirus isolated in Korea

Jun-Hui Wang, Bon-Sang Koo, In-Pil Mo, Shien-Young Kang*

College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea

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Abstract

An avian rotavirus (AvRV-2) was isolated from feces of broilers suffering from acute gastroenteritis in 2011. It was the first avian rotavirus isolated in Korea. To investigate the molecular characteristics of AvRV-2, the VP4, VP6, VP7 and NSP4 gene nucleotide sequences were determined and compared with those of rotavirus strains available in the GenBank database. The phylogenetic tree of VP7 gene showed that AvRV-2 had a high degree of nucleotide sequence homology (93.4% to 94.7%) with those of rotaviruses belonging to genotype G19 cluster. The phylogenetic tree of the VP4 gene revealed a high degree of nucleotide sequence homology (95.8% to 95.9%) with genotype P[30] rotaviruses isolated from chickens. The VP6 and NSP4 gene nucleotide sequences showed the highest identities with those of avian strains with 95.3% to 96.4% and 90.3% to 92.2%, respectively. Genetic characterization of the VP4, VP6, VP7 and NSP4 showed that AvRV-2 strain was most closely related to chicken rotavirus strains from Germany and Japan. Comparative nucleotide sequences and phylogenetic analysis indicated that avian rotavirus isolated from broilers belonged to genotype G19P[30] and it was the first report on avian rotavirus infection in Korea.

Key words : Avian rotaviruses, VP4, VP6, VP7, NSP4 gene

INTRODUCTION

Rotaviruses are classified in the genus *Rotavirus* within the family *Reoviridae*. They have 11 segments of double-stranded RNA genome and consist of six structural proteins (VP1-4, VP6 and VP7) and six non-structural proteins (NSP1-6). A mature infectious virion has a diameter of approximately 100 nm and includes three layers with no lipid-containing envelope (Raming et al, 2005). The inner core is mainly formed by VP2 which surrounds genomic RNA. VP1 and VP3, minor components of the core, are presumed to have a link with RNA polymerase and the guanylyl transferase, respectively (Ito et al, 2001). The intermediate layer is formed by VP6, which was the first rotavirus protein used for classification. The outer layer consists of VP4 and VP7. They also independently elicit neutralizing an-

tibodies which contain the serotype-specific epitopes that were defined P (protease sensitive) serotype and G (glycoprotein) serotype, respectively (Gorziglia et al, 1990). However, antigenic characterization by virus neutralization test is time-consuming and requires virus collections and proper immunological reagents that are not available in all the laboratories. And due to the increasing cases of nucleotide sequences, the antigenic serotypes were replaced by VP4 and VP7 genotypes. So far, at least 27G (G1-G27) and 35P (P[1]-P[35]) genotypes of rotaviruses have been identified globally. Recently, new classification strategy was proposed by determining the percentage identity cut-off values of each nucleotide sequence of eleven rotavirus segmented genomes (Matthijnsens et al, 2011). Nonstructural proteins may play a role in viral replication and morphogenesis, but the effects remain to be studied in more detail. The nonstructural proteins interact with nucleic acid except for NSP4, which is the only nonstructural protein that does

*Corresponding author: Shien-Young Kang, Tel. +82-43-261-2598,
Fax. +82-43-267-2595, E-mail. sykang@cnu.ac.kr

not bind to RNA. It has been reported that NSP4 plays an important role in viral morphogenesis, pathogenesis and enterotoxin activity (Ball et al, 1996; Estes and Kapikian, 2007).

The rotaviruses include at least seven group (A-G). Group A rotaviruses are important enteric pathogens in human, mammalian and avian species. Group A, B and C rotaviruses are currently found in both human and animal species, whereas group D, E, F and G have been isolated only in animal to date (Estes and Kapikian, 2007). Group A, D, F and G have been detected in several avian species including chicken, turkey and pigeon (Hines et al, 1995). Rotavirus infection in avian species was first reported in 1977 (Bergeland et al, 1977). Avian rotaviruses have been detected in Argentina, Belgium, Brazil, Germany, Japan, the United Kingdom, and the United States (Bellinzoni et al, 1987; Meulemans et al, 1985; Gusmal et al, 1994; Elschner et al, 2005; Takase et al, 1986; McNulty et al, 1978; Yason and Schat, 1985). Avian rotaviruses cause enteritis and diarrheal disease in avian species. Clinical signs are associated with depression, dehydration, impaired appetite, poor weight gains and increased mortality. However, clinical symptoms of avian rotavirus infection are not obvious and subclinical infection is also common. So, diagnosis of avian rotavirus infection is necessary to the laboratory confirmation.

In this study, avian rotavirus was first isolated from feces of broilers suffering from acute gastroenteritis. The nucleotide sequences of major structural proteins (VP4, VP7 and VP6) and nonstructural protein (NSP4) were determined and compared with those of reference rotavirus strains from GenBank database to determine the genetic relatedness of the virus.

MATERIALS AND METHODS

Virus isolation

The fecal samples were collected from 8-day-old broiler chickens with severe diarrhea, which were submitted to the Avian Disease Laboratory, College of Veterinary Medicine, Chungbuk National University, for

diagnosis in 2011. The fecal samples were diluted at 1:10 in phosphate buffered saline (PBS, pH 7.2) and centrifuged at 3,000 rpm for 20 min. The supernatant was filtrated with 0.2 μ m syringe filter. Avian rotavirus was isolated from filtered supernatants using MA104 cells as described previously (Kang et al, 1986a). The AEQ strain, which was isolated from turkey in USA and provided by Dr. LJ saif (Ohio State University), was used as a reference avian rotavirus.

Virus identification

Isolated virus was identified as avian rotavirus by electron microscope (EM), electropherotyping and indirect immunofluorescence antibody (IFA) test.

EM

Tissue culture-infected viral fluid was centrifuged at 2,500 rpm for 20 min after freeze-thawed three times. The supernatant was centrifuged at 25,000 rpm for 2 h in a SW 41 Ti Rotor (Beckman, USA). The pellet was diluted with 0.5 ml of distilled water and negatively stained with phosphotungstate (pH 7.2). The viral particles were observed using an electron microscopy (Libra/20, Carl Zeiss, Germany).

Electropherotyping

The viral RNA was extracted with QIAamp Viral RNA Mini Kit (Qiagen, Germany) and electropherotyping was performed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Otto et al, 1999). After finishing SDS-PAGE, the gel was stained with 1% ethidium bromide and observed under the UV illuminator.

IFA test

IFA test was performed as described previously (Kang et al, 1989). In brief, MA104 cells were inoculated with isolated virus. When CPE appeared after 2~3 days, infected MA104 cells were fixed with 80% acetone for 10 min and washed three times with PBS. Rotavirus VP6-specific

monoclonal antibody (25A11) was applied and incubated at 37°C for 1 h. After washing three times with PBS, the FITC-conjugated affinipure goat anti-mouse IgG + IgM (Jackson, USA) was applied and incubated at 37°C for 1 h. Finally, after washing three times with PBS, 80% glycerin was applied and the fluorescence was observed under a fluorescence microscope (Olympus, Japan).

Reverse transcription polymerase chain reaction (RT-PCR)

Viral RNA was extracted from avian rotavirus infected cell culture supernatant using the QIAamp viral RNA mini Kit (QIAGEN, USA). To determine the complete nucleotide sequence of VP4, VP7, VP6 and NSP4 gene, primers used for RT-PCR of each gene were synthesized based on previously reported data of NCBI GenBank by commercial company (Cosmo Genetech Company, Korea) (Table 1). cDNA was synthesized using the Prime Script 1st strand cDNA Kit (TakaRa, Japan). PCR was performed using PCR Thermal Cycler Dice (TakaRa, Japan). The 50 µl reaction mixtures in-

cluded 10 µl of cDNA template, 6 µl of dNTP (2.5 mM each), 5 µl of 10× ExTaq buffer, 2 µl of forward and reverse primer (10 pmole/µl), 0.5 µl of Taq polymerase (5 unit/µl) and 24.5 µl of RNase free distilled water. The PCR condition for amplification of VP4, VP7, VP6 and NSP4 gene was 95°C for 5min, 35 cycles of 95°C for 35s (denaturation), 52~56°C for 35s (annealing) and 75°C for 1 min (extension), and a final incubation at 75°C for 10 min.

Nucleotide sequences and phylogenetic analysis

The PCR products were acquired with electrophoresis on 1.5% agarose gel and purified using Expin Gel SV Kit (GeneAll Biotechnology, Korea). The nucleotide sequences of VP4, VP7, VP6 and NSP4 gene were compared with those of reference rotavirus strains available in the NCBI GenBank database using MegAlign program (DNASTAR, USA) and the phylogenetic trees were constructed based on Clustal W method (Thompson et al, 1994).

Table 1. Oligonucleotide primers used in this study

Gene	Primer	Sequence (5'-3')	Expected size (bp)
VP4	FP1 (1~23)	GGC TAT AAA ATG GCT TCA CTC GT	1,080
	RP2 (1,080~1,061)	TGC TTG TGA ATC ATC CCA GT	
	FP3 (848~872)	CAG GTG GAT TAG GTT ATA AAT GGT C	865
	RP4 (1,712~1,690)	GCT GCG TCT GAT AAT TCT TCC GT	
	FP5 (1,500~1,518)	GCG TGA AGA GTT TAA TCAA G	855
	RP6 (2,354~2,333)	GGT CAA ATC CTC ATA GAC AGC T	
VP6	FP5' (1,607~1,628)	CAC TGG ATG CTG TTA AAT CGG T	748
	RP6 (2,354~2,333)	GGT CAA ATC CTC ATA GAC AGC T	
	FP1 (1~27)	GGC TTT TAA ACG AAG TCT TCA TCA TGG	340
	RP2 (359~340)	TCC ATT ACG CTG AGA TTC AC	
	FP3 (939~919)	TAG ACC ACC GAA TAT GAC ACC	429
	RP4 (1,347~1,322)	GGT CAC ATC CTC TCA CTA TAC TGC TG	
VP7	FP1 (1~27)	GGC TTT TAA ACG AAG TCT TCA TCA TGG	1,347
	RP4 (1,347~1,322)	GGT CAC ATC CTC TCA CTA TAC TGC TG	
	FP1 (1~28)	GGC ATT TAA ATT AGT AAT TTC CGT TTC T	640
	RP2 (640~617)	CCC ATT GCT ATC CAT TTA TTA GCC	
	FP3 (690~711)	GGA ATT GGG TGT CAG ACA ACT	377
	RP4 (1,066~1,042)	GGT CAC ATC AAT CCT TTC AAT ATG A	
NSP4	FP1 (1~28)	GGC ATT TAA ATT AGT AAT TTC CGT TTC T	1,066
	RP4 (1,066~1,042)	GGT CAC ATC AAT CCT TTC AAT ATG A	
	FP (1~24)	AAC TCA ATA TCG AGA GAG CCC GTG	629
	RP (629~605)	GGA TTA AGT CTT CTT GAC ACA CCC G	

Nucleotide sequence accession numbers

The nucleotide sequences of VP4, VP6, VP7 and NSP4 gene of avian rotavirus (AvRV-2) were submitted to GenBank under the accession numbers: JQ085405, JQ085406, JQ085407, and JQ085408, respectively.

RESULTS

Virus isolation and identification

The MA-104 cells inoculated with the supernatant of processed fecal samples showed CPE from the second passage. Purified virus was confirmed by EM, SDS-PAGE electropherotyping and IFA. Characteristic rotavirus morphology was shown by EM (Fig. 1). SDS-PAGE electropherotyping result exhibited a RNA migration pattern of 5:1:3:2, which was typical for avian group A rotaviruses (Fig. 2). To add weight to isolated virus that belonged to avian group A rotaviruses, IFA was performed using a group A rotavirus VP6-specific monoclonal antibody. The IFA results confirmed that isolated rotavirus belonged to group A rotaviruses (Fig. 3).

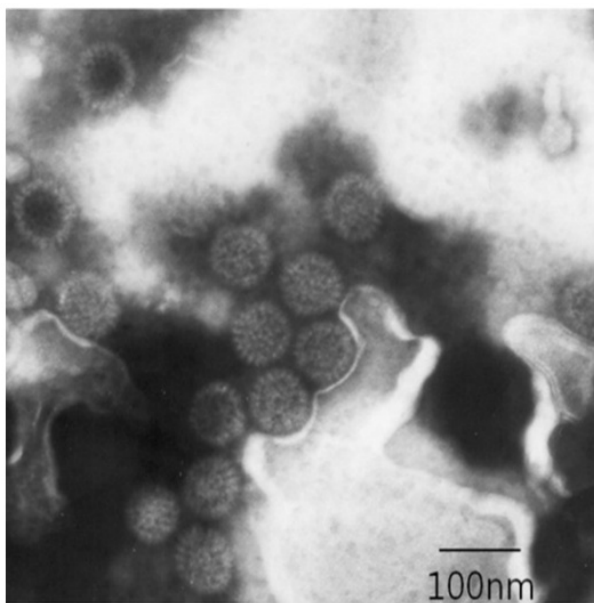


Fig. 1. Electron microscopic detection of avian rotavirus AvRV-2.

Analysis of VP4 gene nucleotide sequences

AvRV-2 VP4 gene was 2,359 bp nucleotides in length and coded for 771 amino acids. Amino acids of AvRV-2 strain were shorter (2 to 6 amino acids) than those of human and mammalian rotaviruses. Comparing the nucleotide sequence of VP4 gene with those of reference rotavirus strains available in GeneBank database, nucleotide sequence homology of the AvRV-2 was 50.2% to 95.9%. The VP4 gene nucleotide sequences of AvRV-2 strain showed a low homology (50.2% to 63.9%) with those of human and mammalian rotaviruses, while it had nucleotide sequence identities of 76.5% to 96.9% with those of avian rotavirus strains. Especially, AvRV-2 strain had a high degree of nucleotide sequence identities (95.8% to 95.9%) with avian rotaviruses isolated from chickens, which belonged to genotype P[30] (Fig. 4).

Analysis of VP7 gene nucleotide sequences

AvRV-2 VP7 gene was 1,067 bp nucleotides in length and coded for 330 amino acids. Amino acids of AvRV-2

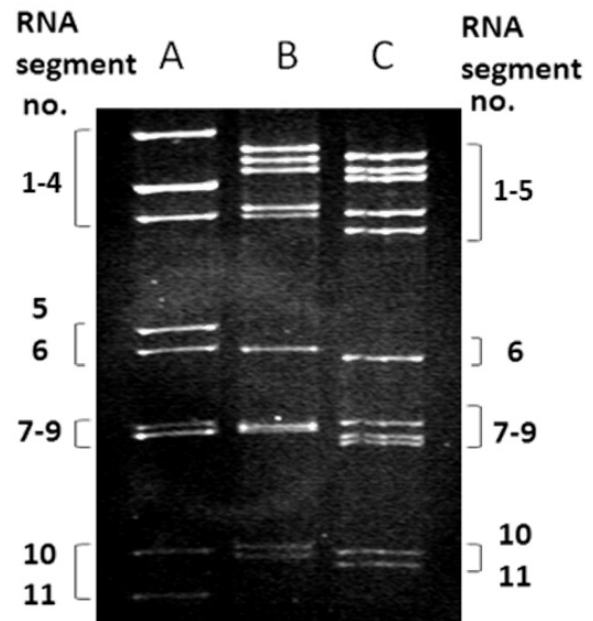


Fig. 2. Electropherotypic RNA migration patterns of genomic RNA. Lane A: Porcine rotavirus OSU strain, Lane B: Avian rotavirus AvRV-2 strain, Lane C: Avian rotavirus AEQ strain.

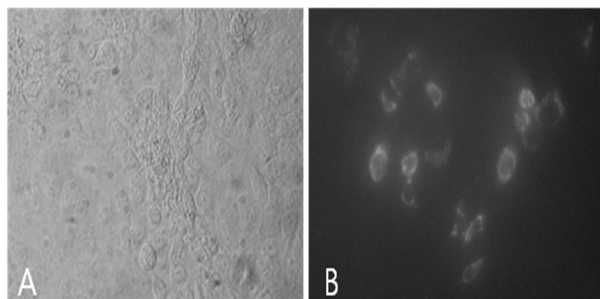


Fig. 3. Cytopathic effects (A) and Immunofluorescences (B) of avian rotavirus AvRV-2 in MA104 cells ($\times 400$).

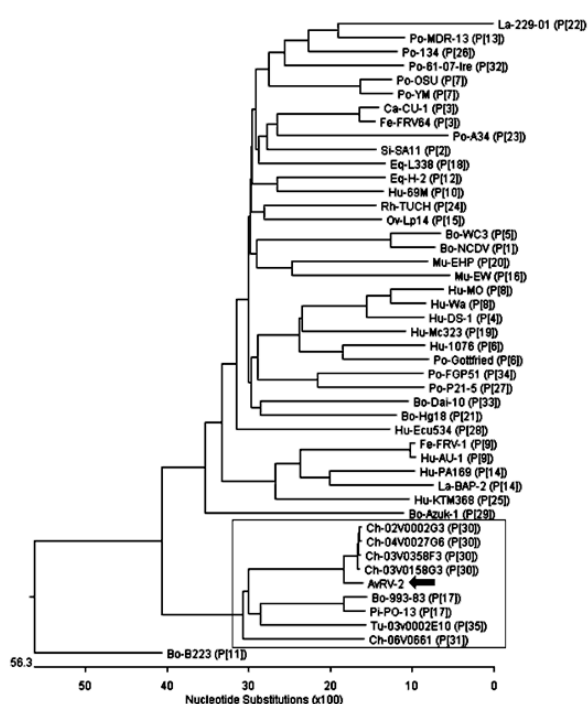


Fig. 4. Phylogenetic analysis of VP4 gene nucleotide sequence of AvRV-2. Bo: bovine, Ch: chicken, Eq: equine, Fe: feline, Gu: guanaco, Hu: human, La: lapine, Mu: murine, Ov: ovine, Pi: pigeon, Po: porcine, Rh: rhesus, Si: simian, Tu: turkey. The number in parentheses indicates the P genotype and rotavirus strains in box were isolated from avian species. The AvRV-2 strain was marked in the black arrow.

strain were longer (3 amino acids) than those of human and mammalian rotaviruses. Phylogenetic tree showed that VP7 genotypes of avian rotaviruses included G7, G17, G18, G19, G22, and G23 (Fig. 5). Comparing the nucleotide sequence of VP7 gene with those of reference avian rotavirus strains available in GeneBank database, nucleotide sequence homology of the AvRV-2 was 69.5% to 94.7%. Especially, AvRV-2 strain has high nucleotide sequence identities of 93.4% to 94.7% with

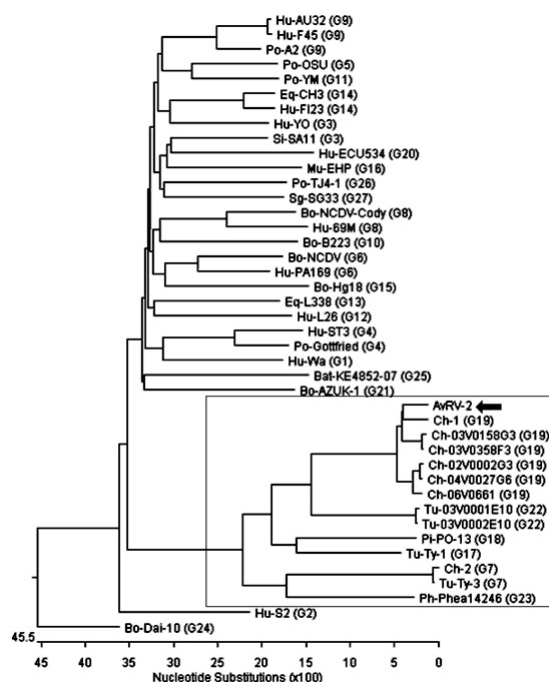


Fig. 5. Phylogenetic analysis of VP7 gene nucleotide sequence of AvRV-2. Bo: bovine, Ch: chicken, Eq: equine, Fe: feline, Gu: guanaco, Hu: human, La: lapine, Mu: murine, Pi: pigeon, Po: porcine, Rh: rhesus, Sg: sugar glider, Si: simian, Tu: turkey. The number in parentheses indicates the G genotype and rotavirus strains in box were isolated from avian species. The AvRV-2 strain was marked in the black arrow.

genotype G19 strains that had been isolated from chickens in Germany and Japan.

Analysis of VP6 gene nucleotide sequences

AvRV-2 VP6 gene was 1,352 bp nucleotides in length and coded for 398 amino acids. The numbers of amino acids of AvRV-2 was the same as other avian, human and mammalian rotaviruses. Phylogenetic tree revealed that avian rotaviruses were divided into two I genotypes, I4 and I11 and AvRV-2 was closely related (95.3% to 96.4%) to I11 genotype (Fig. 6). Avian rotaviruses isolated from turkey and chicken belonged to genotype I4 and I11 respectively, except Ch-2 strain. Ch-2 strain was isolated from chickens and was closely related (92.6~96.6%) to genotype I4, while it had low nucleotide sequence identities (78.0~79.3%) with genotype I11. The rotaviruses isolated from pigeon also belonged to genotype I4.

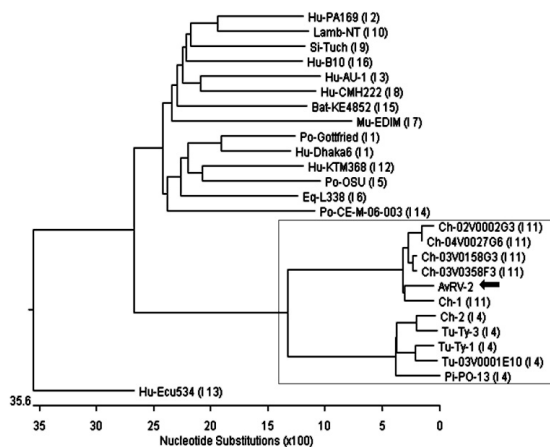


Fig. 6. Phylogenetic analysis of VP6 gene nucleotide sequence of AvRV-2. Bat: bats, Bo: bovine, Ch: chicken, Eq: equine, Fe: feline, Gu: guanaco, Hu: human, Pi: pigeon, Po: porcine, Si: simian, Tu: turkey. The number in parentheses indicates the I genotype and rotavirus strains in box were isolated from avian species. The AvRV-2 strain was marked in the black arrow.

Analysis of NSP4 gene nucleotide sequences

AvRV-2 NSP4 gene was 662 bp nucleotides in length and coded for 169 amino acids. The length of amino acids of AvRV-2 was shorter than those of turkey (1 amino acid), human (7 amino acids) and mammalian (7 amino acids) rotavirus strains. Phylogenetic tree exhibited that avian rotaviruses were divided into three E genotypes, E4, E10 and E11 and NSP4 gene nucleotide sequence of AvRV-2 strain had high degree of nucleotide identities (90.3% to 92.2%) with those of avian rotaviruses from chickens, E10 cluster (Fig. 7). AvRV-2 strain was 76.6~78.0% identical to those of rotaviruses isolated from turkey which belonged to genotype E11.

DISCUSSION

Since rotavirus infection in avian species was first reported in 1977, it became prevalent among several species of domesticated birds (Hines et al, 1995; McNulty and Reynolds, 2008; Minamoto et al, 1988). Rotaviruses isolation from bovine, porcine and human had been reported in Korea (Park et al, 2006; Jeong et al, 2009; Oh et al, 1983). However, rotavirus infection from avian species was not yet reported. Runting-stunting syndrome in broiler chickens was continuous problems for chicken

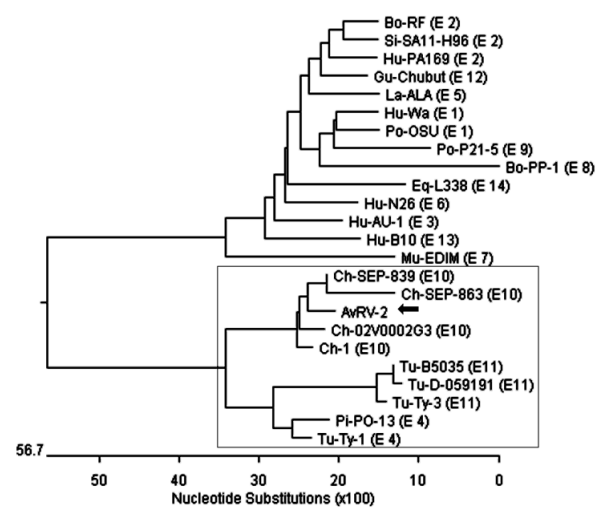


Fig. 7. Phylogenetic analysis of NSP4 gene nucleotide sequence of AvRV-2. Bo: bovine, Ch: chicken, Eq: equine, Gu: guanaco, Hu: human, La: lapine, Mu: murine, Pi: pigeon, Po: porcine, Si: simian, Tu: turkey. The number in parentheses indicates the E genotype and rotavirus strains in box were isolated from avian species. The AvRV-2 strain was marked in the black arrow.

producers throughout the world. The specific enteric agents for this syndrome were not known. But several viruses such as avian rotaviruses, astroviruses and reoviruses were detected in chickens showing enteric symptoms. In this study, virus isolated from the feces of 8-days old broiler chickens suffering from acute gastroenteritis was confirmed as avian rotavirus by EM, electropherotyping and IFA. Isolated rotavirus AvRV-2, was seen wheel-like appearances and about 70~75 nm in diameter under the electron microscopy. Wheel-like appearances of virus particles in EM was the characteristics of rotaviruses. Based on the migration of 11 RNA segments, genomic RNA migration patterns of AvRV-2 were 5:1:3:2 and were similar with those of AEQ strain used as a reference avian group A rotavirus. From the electropherotyping results, we could assumed that AvRV-2 was a avian group A rotavirus. The genome segments of avian group A rotaviruses had a migration pattern of 5:1:3:2, while avian group D rotaviruses had a migration pattern of 5:2:2:2 (Kang et al, 1986b). On the other hand, mammalian group A rotaviruses had a migration pattern of 4:2:3:2 (McNulty et al, 1981). However, the migration patterns of the genomic segments might be influenced by antigenic shift, drift and rearrangements (Estes and Kapikian, 2007). The AvRV-2 was further

confirmed as a group A rotavirus using a group A rotavirus specific monoclonal antibody by IFA. Monoclonal antibody, 25A11 used in this study was known to be group A rotavirus VP6-specific (Kang et al, 1989).

After the avian rotavirus was confirmed, molecular characterization of VP4, VP7, VP6 and NSP4 genes were analyzed by comparing the nucleotide sequences with those of other rotavirus strains. The avian rotavirus genotypes of VP4 included P[17], P[30], P[31] and P[35]. AvRV-2 strain located in the same cluster (P[30]) with those of chicken strains and had no direct relation with those of mammalian and human rotavirus.

In this study, avian rotaviruses belonged to one of the 6 G genotypes (G7, G17, G18, G19, G22, G23). Interestingly, all avian rotaviruses including AvRV-2 isolated from chicken belonged to the same G19 genotype except Ch-2, which belonged to the G7 genotype. Other avian rotaviruses isolated from different avian species belonged to the different G genotypes except for turkey. Rotaviruses isolated from turkey belonged to the G7, G17 and G22 genotypes. It suggested that cross-infection with avian rotaviruses between chicken and turkey had been reported and the reassortment between these avian rotaviruses might be occurred (Yason and Schat, 1986; Schumann et al, 2009). At the same time, AvRV-2 and other chicken rotavirus strains belonged to the genotype I11 except for Ch-2 strain, which was closely related with turkey strains belonging to the genotype I4. On the other hand, chicken avian rotavirus strains including AvRV-2 located in the same E cluster (E10), while turkey strains located in the cluster E11. Based on above results, the genotypes of AvRV-2 strain were related with those of chicken strains and it suggested that the reassortment between AvRV-2 strain and other species did not yet occurred. Reassortment between mammalian and avian rotaviruses were reported (Wani et al, 2003). Reassortment among bovine, porcine and human rotavirus strains was also reported in Korea (Park et al, 2011). However, in Korea, it was not known whether the potential reassortments between avian and mammalian rotavirus strains occurred or not. Continued surveillance studies for avian rotaviruses will be necessary to verify it.

The phylogenetic trees based on VP4, VP7, VP6 and

NSP4 showed that AvRV-2 was clustered into chicken rotaviruses isolated previously in Germany and Japan, which belonged to the genotype G19P[30]. Further studies were needed to confirm whether AvRV-2 came from foreign strains or not. Prevalences of avian rotavirus infection in chicken farms in Korea should be elucidated. Worldwide collaboration including sharing informations of avian rotaviruses is crucial for effective monitoring of any significant genetic changes that could lead to production of high pathogenic avian rotaviruses.

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