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Molecular survey of enteric viruses in commercial chicken farms in Korea with a history of enteritis

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ABSTRACT Several enteric viruses have increasingly received attention as potential causative agents of runting-stunting syndrome (RSS) in chickens. A molecular survey was performed to determine the presence of a broad range of enteric viruses, namely chicken astrovirus (CAstV), avian nephritis virus (ANV), chicken parvovirus (ChPV), infectious bronchitis virus (IBV), avian rotavirus (AvRV), avian reovirus (ARV), and fowl adenovirus (FAdV), in intestinal samples derived from 34 commercial chicken flocks that experienced enteritis outbreaks between 2010 and 2012. Using techniques such as PCR and reverse-transcription PCR, enteric viruses were identified in a total of 85.3% of investigated commercial chicken flocks in Korea. Furthermore, diverse combinations of 2 or more enteric viruses were simultaneously identified in 51.7% of chicken farms positive for enteric viruses. The rank order of positivity for enteric viruses was as follows: ANV (44.1%), CAstV (38.2%), ChPV (26.5%), IBV (20.6%), ARV (8.8%), AvRV (5.9%), and FAdV (2.9%). Additionally, other pathogens such as *Escherichia coli*, *Salmonella* spp., *Eimeria* spp., and FAdV were detected in 79% of chicken flocks positive for enteric viruses using PCR, bacterial isolation, and microscopic examination. The results of our study indicate the presence of several enteric viruses with various combinations in commercial chicken farms that experienced enteritis outbreaks. Experimental studies are required to further understand the roles of enteric viruses in RSS in commercial chickens.

Key words: molecular survey, runting-stunting syndrome, enteric virus, chicken

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INTRODUCTION

Enteritis is one of the most economically important diseases in poultry industry because it directly decreases feed absorption, resulting in growth retardation, impaired feed efficiency, immunosuppression, and sometimes increased mortality due to secondary infection (Hoerr, 1998; Yegani and Korver, 2008). Genetic improvements in broiler chickens to increase feed conversion have made intestinal health particularly important, because fast-growing broilers have a tendency to be hyperphagic and can be severely affected by decreased feed absorption (Dekich, 1998). Various factors can adversely affect intestinal health, including microorganisms, suboptimal environments, and feed, because the intestines have an extensive surface that is exposed to the external environment (Smart et al., 1988; Goodwin et al., 1993). Moreover, the intestines have a delicate and balanced bacterial microenvironment referred to as the microflora that can also be adversely affected by factors such as feed, infectious agents, antibiotics, and the environment (Yegani and Korver, 2008). An imbalanced microflora could result in decreased vitamin production, immunosuppression, and increased growth of harmful bacteria in the intestines of chickens (Yegani and Korver, 2008). Therefore, enteric diseases are a common occurrence in the poultry industry worldwide.

Among various factors affecting intestines, enteric viruses have been a focus of interest because enteritis in broiler chickens can lead to runting-stunting syndrome (**RSS**; Guy, 1998; Ono et al., 2001; Otto et al., 2006; Nili et al., 2007; Pantin-Jackwood et al., 2008; Palade et al., 2011). Several enteric viruses have been identified in a high proportion of chickens suffering from RSS in the fields using molecular surveys, including chicken astrovirus (**CAstV**), avian nephritis virus (**ANV**), chicken parvovirus (**ChPV**), infectious bronchitis virus (**IBV**), avian rotavirus (**AvRV**), avian reovirus (**ARV**), and fowl adenovirus (**FAdV**; Yu et al., 2001; Otto et al., 2006; Pantin-Jackwood et al., 2006; Smyth et al., 2009; Hewson et al., 2010; Palade et al., 2011; Canelli et al., 2012). However, it is usually difficult to isolate enteric viruses in vitro, especially CAstV, AvRV, and ChPV for virus purification; furthermore, the severity of enteritis in experimental infections is less than

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that of enteritis in the field (Yason and Schat, 1986; Guy, 1998; Songserm et al., 2003; Baxendale and Mebatsion, 2004). Enteric viruses such as CAstV, ANV, AvRV, and ARV are also present in a high proportion of healthy broiler flocks (Pantin-Jackwood et al., 2008). Therefore, the exact role of enteric viruses in RSS in chickens has not been clarified.

In experimental infections, significant growth retardation has been observed in broiler chickens inoculated with intestinal contents and litters collected from broiler farms showing signs of RSS (Smart et al., 1988; Songserm et al., 2000; Kang et al., 2012). Additionally, broiler chickens inoculated with bacteria-free intestinal contents filtered with 0.22 μm pore filters experienced a significant reduction in weight compared with nontreated chickens, although the severity of weight depression in these chickens was less than that of broilers inoculated with nontreated intestinal contents (Nili et al., 2007; Sellers et al., 2010). Based on these findings, enteric viruses are suspected to be the etiologic agents of RSS, but other pathogens, such as bacteria and protozoa, may also cause intestinal damage in broilers infected with enteric viruses.

A molecular survey was performed for a broad range of enteric viruses, namely CAstV, ANV, IBV, AvRV, ARV, and FAdV, in commercial chicken flocks suffering from enteritis. Additionally, other viral and bacterial infections were documented, because secondary infections need to be identified in RSS cases due to immunosuppression.

MATERIALS AND METHODS

Chicken Flocks

Between 2010 and 2012, a total of 34 commercial chicken flocks including 32 broiler and 2 layer flocks from various provinces in Korea were selected for molecular examination of the following enteric viruses: CAstV, ANV, ChPV, IBV, AvRV, ARV, and FAdV (Table 1). Flocks ranging in age from hatching to 4 wk old that had a history of growth retardation (5 cases), increased mortality (21 cases), and both of them (5 cases) were submitted to the Avian Disease Laboratory (College of Veterinary Medicine, Chungbuk National University) for clinical diagnosis. During routine necropsy procedures, one or more pathologic lesions were observed in the intestines, including watery and undigested food contents, intestinal dilatation with thin wall and gaseous contents in the ceca, and we aseptically collected these intestines for molecular studies. Anatomical regions from the duodenum to the cecum were collected. In addition to intestinal pathologic lesions, various other gross lesions were observed in submitted chicken flocks (data not shown).

Samples and DNA/RNA Extraction

Intestinal samples collected from each flock were pooled and diluted with 10 volumes of PBS and homogenized at 30 Hz for 5 min using TissueLyser II (Qiagen,

Table 1. Characteristics of chicken flocks investigated by PCR or reverse-transcription PCR targeting enteric viruses[1]

Year of submission	Enteric virus	Concomitant infection with other pathogen	Tissues used for other pathogen isolation
2010	AvRV	—	—
2010	ANV	SAL	Liver
2010	CAstV, ANV, AvRV	—	—
2011	CAstV, IBV	*Eimeria*	Cecum
2011	CAstV, ANV	—	—
2011	CAstV, ANV	—	—
2011	CAstV, ANV, ChPV	*Escherichia coli*	Liver
2011	ARV	FAdV	Liver
2011	CAstV, ANV, ARV	SAL	Liver
2011	ANV, ChPV	*Eimeria*, *E. coli*	Duodenum, liver
2012	FAdV	—	—
2012	ANV, ChPV	*E. coli*	Liver
2012	CAstV, ANV, ChPV	SAL	Liver
2012	ANV, ChPV	SAL	Liver
2012	ChPV	SAL	Liver
2012	CAstV, ANV, ChPV	SAL	Liver
2012	ANV	*E. coli*	Liver
2012	CAstV	*E. coli*	Liver
2012	CAstV, ANV, ChPV	FAdV, *E. coli*	Liver
2012	IBV	SAL, *E. coli*	Liver
2012	ARV	*E. coli*	Liver
2012	ChPV, IBV	FAdV	Liver
2012	CAstV	*E. coli*	Yolk
2012	CAstV	—	—
2012	IBV	*Eimeria*, *E. coli*	Duodenum, liver
2012	IBV	*E. coli*, *Staph.*	Liver, joint
2012	IBV	SAL	Liver
2012	IBV	*E. coli*	Liver
2012	CAstV	*E. coli*	Liver

[1]CAstV = chicken astrovirus; ANV = avian nephritis virus; ChPV = chicken parvovirus; IBV = infectious bronchitis virus; AvRV = avian rotavirus; ARV = avian reovirus; FAdV = fowl adenovirus; SAL = *Salmonella* spp.; *Staph.* = *Staphylococcus* spp.; *Eimeria* = *Eimeria* spp..

Valencia, CA). The homogenized intestines were then centrifuged at 2,095 × *g* at 4°C for 10 min in an Allegra X-15R (Beckman Coulter, Fullerton, CA), and the supernatant was harvested into 1.5-mL Eppendorf tubes and stored at −4 or −80°C for subsequent procedures. Viral DNA/RNA was extracted from 300 μL of intestine sample supernatant using the Viral Genespin DNA/RNA extraction kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions.

PCR for ChPV and FAV

Polymerase chain reaction targeting a partial region of the nonstructural (**NS**) gene of ChPV and the hexon gene of FAdV was performed using a commercial DNA kit (iNtRON Biotechnology, Seongnam, Korea) as per the manufacturer's instructions (Raue and Hess, 1998; Zsak et al., 2009). Briefly, 20-μL reaction mixtures contained 10 pmol/μL of forward and reverse primers specific for the target gene, 2 μL DNA template, *Taq* DNA polymerase, distilled water, and other reaction solutions including deoxyribonucleoside triphosphates. The thermal cycling conditions for amplifying the ChPV gene were as follows: one cycle of denaturation (94°C for 2 min) followed by 35 cycles of amplification (94°C for 20 s, 50°C for 20 s, and 72°C for 35 s), and finally one cycle of extension (72°C for 5 min). The thermal cycling conditions for amplifying the FAdV gene were the same as above except for the annealing temperature (50 to 52°C) and extension time (35 to 80 s). Cycling reactions were conducted using a TaKaRa PCR thermal cycler dice gradient (TaKaRa, Shiga, Japan). All primers used in this report are summarized in Table 2.

Reverse-Transcription PCR for CAstV and ANV

Reverse-transcription (**RT**) PCR targeting a partial region of open reading frame (**ORF**) 1b gene of CAstV and ANV was performed using a commercial one step RT-PCR kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Briefly, reaction mixtures with a total volume of 30 μL contained forward and reverse primers (10 pmol/μL), 2 μL DNA template, 2× reaction buffer, 1.2 μL M-MLV RT-polymerase, and distilled water. To amplify CAstV and ANV target genes, RT-PCR was performed using 2 different primer sets described below because of the relatively high possibility of primer template mismatch caused by the high nucleotide (**nt**) sequence diversity of astroviruses (Tang et al., 2005a; Day et al., 2007). For simultaneous amplification of CAstV and ANV with the primers TAPG-L1 and R1, the thermal cycling conditions were as follows: one cycle of reverse transcription (50°C for 30 min), one cycle of initial denaturation (94°C for 2 min), followed by 40 cycles of amplification (94°C for 30 s, 50°C for 30 s, and 72°C for 45 s). To amplify genes from CAstV and ANV viruses, the primer sets CAS pol 1F and 1R and ANV pol 1F and 1R were used, respectively. The thermal cycling conditions were as follows: one cycle of reverse transcription step (50°C for 30 min), one cycle of initial denaturation (94°C for 2 min), followed by 40 cycles of amplification (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s).

Table 2. Detailed information about the primers used in PCR and reverse-transcription PCR

Virus[1]	Target gene[2]	Polarity	Primer sequence (5′ to 3′)	Size of amplicon (bp)	Reference
Astrovirus	ORF1b	Forward	TGGTGGTGYTTYCTCAARA	601	Tang et al., 2005a
		Reverse	GYCKGTCATCMCCRTARCA		
CAstV	ORF1b	Forward	GAYCARCGAATGCGRAGRTTG	362	Day et al., 2007
		Reverse	TCAGTGGAAGTGGGKARTCTA		
ANV	ORF1b	Forward	GYTGGGCGCYTCYTTTGAYAC	473	Day et al., 2007
		Reverse	CRTTTGCCCKRTARTCTTTRT		
ChPV	NS	Forward	TTCTAATAACGATATCACTCAAGTTTC	561	Zsak et al., 2009
		Reverse	TTTGCGCTTGCGGTGAAGTCTGGCTCG		
IBV	S1	Forward	AGGAATGGTAAGTTRCTRGTWAGA	620 to 640	Mase et al., 2004
		Reverse	GCGCAGTACCRTTRAYAAAATAAGC		
		Forward	TGAAAACTGA ACAAAAGAC		Lee et al., 2000
	3′ UTR	Forward	GAGAGGAACAATGCACAGC	351	Mardani et al., 2006
		Reverse	CATTTCCCTGGCGATAGAC		
AvRV	VP6	Forward	GGCTTTTAAACGAAGTCTTC	1,350	Ito et al., 1995
		Reverse	GGTCACATCCTCTCACT		
	NSP4	Forward	GGG CGT GCG GAA AGA TGG AGA AC	630	Day et al., 2007
		Reverse	GGG GTT GGG GTA CCA GGG ATT AA		
ARV	S4	Forward	GTG CGT GTT GGA GTT TCC CG	1,120	Pantin-Jackwood et al., 2008
		Reverse	TAC GCC ATC CTA GCT GGA		
FAdV	Hexon	Forward	TGGAC ATGGGGGCGACCTA	1,219	Raue and Hess, 1998
		Reverse	AAGGG ATTGACGTTGTCCA		

[1]CAstV = chicken astrovirus; ANV = avian nephritis virus; ChPV = chicken parvovirus; IBV = infectious bronchitis virus; AvRV = avian rotavirus, ARV = avian reovirus; FAdV = fowl adenovirus.

[2]ORF1b = open reading frame 1b; NS = nonstructural gene; S1 = spike 1 gene; 3′ UTR = 3′ untranslated region; VP6 = viral structural protein 6; NSP4 = nonstructural protein 4; S4 = small segment 4.

RT-PCR for IBV

First, we performed RT-PCR targeting a partial region of IBV 3′ untranslated region (**UTR**), which is known to be more conserved in IBV compared with the IBV spike gene (**S**; Mardani et al., 2006). For positive IBV 3′ UTR samples, we conducted RT-PCR targeting part of the IBV S1 gene using 2 primer sets: IBV S1 and S2, and new S1 oligo5′ and IBV S2 (Lee et al., 2000; Mase et al., 2004). To amplify the IBV 3′ UTR gene, we used the following thermal cycling conditions: one cycle of reverse transcription (50°C for 30 min), one cycle of initial denaturation (94°C for 2 min), and 40 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 25 s). To amplify the IBV S1 gene, the thermal cycling conditions were one cycle of reverse transcription (50°C for 30 min), one cycle of initial denaturation (94°C for 2 min), and 40 cycles of amplification (94°C for 30 s, 50°C for 30 s, and 72°C for 45 s).

RT-PCR for AvRV and ARV

Reverse-transcription PCR was conducted targeting a partial region of the AvRV nonstructural protein (**NSP**) 4 and ARV small segment (**S**) 4 (Day et al., 2007; Pantin-Jackwood et al., 2008). For samples positive for the AvRV NSP4 gene, we performed RT-PCR targeting AvRV VP6 (Ito et al., 1995). The same procedure described above was used except for the thermal cycling conditions. Thermal cycling conditions were as follows: one cycle of reverse transcription step (50°C for 30 min), one cycle of initial denaturation (94°C for 2 min), and 40 cycles of amplification (94°C for 30 s, 52°C for 30 s, and 72°C for 80 s).

Sequencing and Molecular Analysis

The PCR products were separated by electrophoresis on 1% agarose gels with 0.5 μg/mL of ethidium bromide and identified via UV transillumination. Target size amplicons for each virus were purified using a commercial gel extraction kit (GeneAll, Seoul, Korea). The nt sequences were obtained by direct nt sequencing using an ABI3730XL DNA sequencer (Perkin-Elmer, Foster City, CA). Sequences were manipulated using Bioedit software, version 7.0.9.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and assembled using CLC sequence viewer 6.7 (CLC bio A/S, Aarthus, Denmark). Multiple alignments of nt sequences and the predicted amino acids (**aa**) were obtained using CLUSTAL W method. The phylogenetic trees were constructed using the neighbor-joining algorithm with 1,000 bootstrapping replicates in the software package Molecular Evolutionary Genetics Analysis (**MEGA**, version 5.01). Sequence pairwise similarities of nt and aa sequences were calculated using default values in the LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html). The nt sequences of the reference strains were obtained from the GenBank database.

Clinical Diagnosis of Other Pathogens

For field cases of suspected bacterial septicemia during necropsy, liver samples were selected and cultured onto blood and MacConkey agar plates, and then incubated at 37°C for 24 h. Coccidial infection in the intestines was examined by both the direct smear method and microscopic examinations of the intestines under a light microscope. Inclusion body hepatitis (**IBH**) was diagnosed using RT-PCR and microscopic examination (Raue and Hess, 1998).

GenBank Accession Numbers

All nt sequences of enteric viruses identified in this report were submitted to GenBank. The GenBank accession numbers are as follows: CAstV (KC593403-KC593414, JN635502), ANV (KC593387-KC593401), ChPV (KC593415-KC593423), IBV (KC593425-KC593431), AvRV (KC593402, JN635503), ARV (KC593432-KC593434), and FAdV (KC593424).

RESULTS

Molecular Epidemiology of Enteric Viruses

Enteric viruses were identified in 85.3% of Korean commercial chicken flocks. The breakdown of enteric viruses was as follows: ANV (44.1%), CAstV (38.2%), ChPV (26.5%), IBV (20.6%), ARV (8.8%), AvRV (5.9%), and FAdV (2.9%). Two or more enteric viruses were identified in 51.7% of chicken flocks positive for enteric viruses. In particular, CAstV, ANV, and ChPV were simultaneously identified in investigated flocks at a high rate (Table 3). Additionally, bacterial septicemia, IBH, and coccidial infection were identified in 79% of chicken flocks positive for enteric viruses (data not shown). Other bacterial and protozoa infections were identified in all cases of ChPV infection. The CAstV, ANV, and ARV were identified in a high proportion of chickens under 2 wk old, whereas ChPV was detected in chickens between 1 and 3 wk of age (Table 4). No geographic or seasonal patterns were identified.

Sequence Analysis of Astroviruses

Polymerase chain reaction products and corresponding nt sequences (330 bp) of a partial region of the ORF1b gene of CAstV and ANV were successfully obtained using RT-PCR. The nt and predicted aa sequences of the CAstV ORF1b gene had low sequence identities ranging from 76.7 to 98.5% and 89.1 to 100%, respectively, among the CAstV identified in this report. Phylogenetic analysis based on a 330-bp segment of the ORF1b gene revealed 4 subgroups of Korean CAstV

Table 3. Polymerase chain reaction or reverse-transcription PCR results for avian enteric viruses in Korean chicken flocks[1]

Infection type	CAstV	ANV	ChPV	IBV	AvRV	ARV	FAdV	Number of flocks positive (%)
Single infection (48.3%)[2]	○							4 (13.8)
		○						2 (6.9)
			○					1 (3.4)
				○				5 (17.2)
					○			1 (3.4)
						○		1 (3.4)
							○	0 (0)
Dual infection (31.0%)	○	○						2 (6.9)
	○			○				1 (3.4)
		○	○					3 (10.3)
		○				○		1 (3.4)
		○					○	1 (3.4)
			○	○				1 (3.4)
Triple infection (20.7%)	○	○	○					4 (13.8)
	○	○			○			1 (3.4)
	○	○				○		1 (3.4)

[1]CAstV = chicken astrovirus; ANV = avian nephritis virus; ChPV = chicken parvovirus; IBV = infectious bronchitis virus; AvRV = avian rotavirus, ARV = avian reovirus; FAdV = fowl adenovirus.

[2]Percentage rates according to infection types among total chicken flocks positive for enteric viruses.

strains that showed a 12% difference in nt sequences (Figure 1). The ANV identified in this study were more similar to each other based on ORF1b region than the CAstVs, with nt and aa sequence identities ranging from 91.2 to 99.2% and 94.7 to 100%, respectively.

Sequence Analysis of ChPV

A fragment of the ChPV NS gene (430 bp) was successfully amplified using PCR. The nt and aa sequence similarities of the ChPV NS fragments identified in this study ranged from 96.7 to 100% and 96.5 to 100%, respectively (data not shown), consistent with previous studies (Biđin et al., 2011; Palade et al., 2011).

Sequence Analysis of IBV

The nt sequences were successfully amplified from all IBV strains that showed positive RT-PCR results for the IBV 3′ UTR gene using 2 primer sets. In the phylogenetic tree based on analysis of the nt sequence (549–589 bp) of the partial IBV S1 gene, most IBV were classified into the QX-like IBV group except for 2 IBV that did not group with any of the IBV subtypes previously identified in commercial chickens (Figure 2). Strain ADL121459 had 85 to 88% nt sequence identities to IBV reference strains belonging to the K-I group, which is a Korean indigenous IBV type (Lee et al., 2008). Furthermore, the ADL120942 strain clustered with turkey corona virus in the phylogenetic tree and showed nt sequence identities of 90 to 91% to turkey corona virus reference strains (Maurel et al., 2011).

Sequence Analysis of AvRV, ARV, and FAdV

Target genes of AvRV, ARV, and FAdV were all successfully amplified. Two AvRV grouped into group A in a phylogenetic tree and shared a high nt identity (94 to 96%, 1,162 bp) with group A AvRVs according to the nt sequence query in the National Center for Biotechnology Information Basic Local Alignment Search Tool program (Figure 3). Three ARV identified in this report showed high sequence similarity (95.4 to 98.7%) to one another based on alignment of a 963-bp fragment of the ARV S4 gene, and formed a cluster separated from the other avian reoviruses based on a 6% difference in nt sequences (Figure 3). One FAdV identified in this report was classified as a serotype 11, genotype D FAdV based on sequence analysis of 630-bp of the hexon gene.

Table 4. Positive rates for enteric virus infection according to chicken flock age[1]

Item	CAstV	ANV	ChPV	IBV	AvRV	ARV	FAdV
Age (d)							
0 to 7	30.8[2] (4/13)[3]	26.7 (4/15)	0 (0/9)	0 (0/7)	50.0 (1/2)	33.3 (1/3)	0 (0/1)
8 to 14	46.2 (6/13)	46.7 (7/15)	55.6 (5/9)	0 (0/7)	0 (0/2)	66.6 (2/3)	0 (0/1)
15 to 21	7.7 (1/13)	20.0 (3/15)	33.3 (3/9)	28.6 (2/7)	50.0 (1/2)	0 (0/3)	100 (1/1)
22 to 28	15.4 (2/13)	6.7 (1/15)	11.1 (1/9)	71.4 (5/7)	0 (0/2)	0 (0/3)	0 (0/1)
Total positive rate	38.2 (13/34)	44.1 (15/34)	26.5 (9/34)	20.6 (7/34)	5.9 (2/34)	8.8 (3/34)	2.9 (1/34)

[1]CAstV = chicken astrovirus; ANV = avian nephritis virus; ChPV = chicken parvovirus; IBV = infectious bronchitis virus; AvRV = avian rotavirus, ARV = avian reovirus; FAdV = fowl adenovirus.

[2]Percentage of flock positive for enteric virus.

[3]Positive/total number of each enteric virus detected.

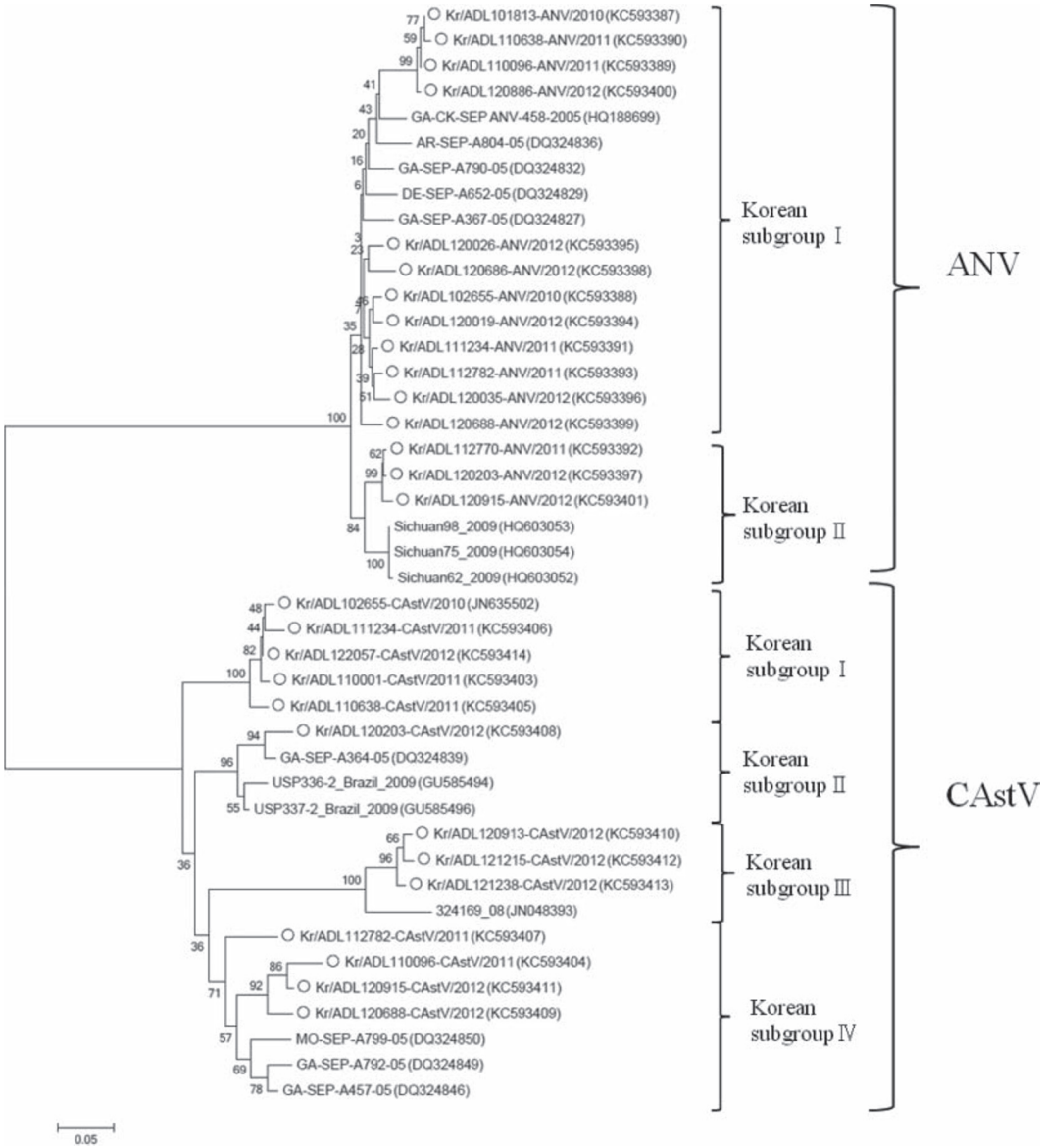

Figure 1. Phylogenetic tree based on analysis of partial nucleotides sequences of the chicken astrovirus (CAstV) and avian nephritis virus (ANV) nonstructural protein (NSP) 4 genes. Molecular Evolutionary Genetics Analysis version 5.01 was used for phylogenetic tree reconstruction using the neighbor-joining algorithm with 1,000 bootstrap replicates. Accession numbers are shown in parentheses. Circles indicate ANV and CAstV identified in this report, respectively.

DISCUSSION

Various enteric viruses have come under the spotlight as potentially causative etiologic agents of RSS in commercial chickens. Using molecular methods, several enteric viruses have been identified in a high proportion of chickens suffering from RSS in the field (Yu et al., 2001; Otto et al., 2006; Pantin-Jackwood et al., 2006; Smyth et al., 2009; Hewson et al., 2010; Palade et al., 2011; Canelli et al., 2012). Moreover, poliovirus and reovirus have been shown to use microflora in the intestines of mice for replication and transmission, indicating that the infectivity of enteric viruses may be closely related to the enteric bacteria present (Kuss et al., 2011). A comprehensive approach is therefore required to further our understanding of RSS. In this study, a molecular survey was performed for a broad range of enteric viruses including CAstV, ANV, ChPV, IBV, AvRV, ARV, and FAdV in intestine samples from commercial chicken flocks suffering from enteritis. Additionally, the presence of bacterial and protozoan infections was documented in each flock.

At least one of the following enteric viruses was identified in 85% of the commercial chicken flocks in Korea that we investigated: CAstV, ANV, ChPV, IBV, AvRV, ARV, and FAdV. Diverse combinations of 2 or more enteric viruses were identified in 51.7% of chicken flocks positive for enteric viruses (Table 3). Concomitant infection with several enteric viruses has been shown to result in more severe enteritis than single infection with one enteric virus type in turkeys (Pantin-Jackwood et al., 2006; Spackman et al., 2010). Therefore, the par-

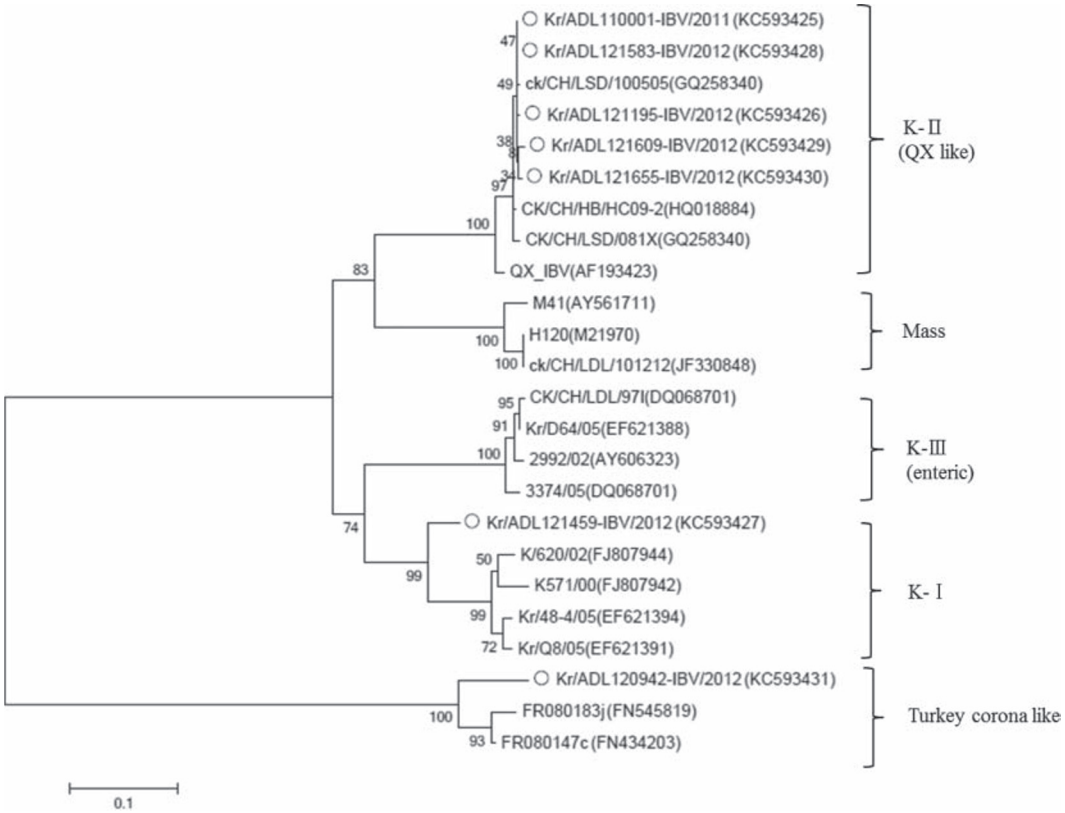

Figure 2. Phylogenetic tree based on analysis of partial nucleotide (nt) sequences of infectious bronchitis virus (IBV) S1. Molecular Evolutionary Genetics Analysis version 5.01 was used for phylogenetic tree reconstruction using the neighbor-joining algorithm with 1,000 bootstrap replicates. Accession numbers are shown in parentheses. Circles indicate IBV identified in this report.

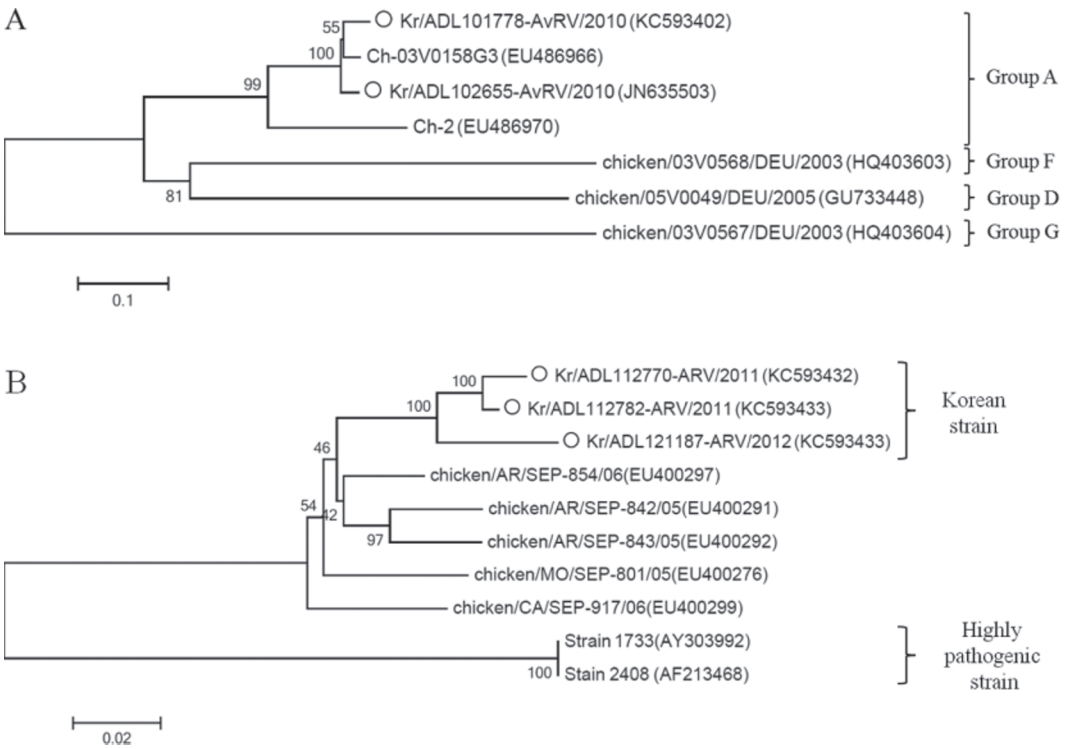

Figure 3. Phylogenetic tree based on analysis of partial nucleotide (nt) sequences of avian rotavirus (AvRV) VP6 (A) and avian reovirus (ARV) S4 (B). Molecular Evolutionary Genetics Analysis version 5.01 was used for phylogenetic tree reconstruction using the neighbor-joining algorithm with 1,000 bootstrap replicates. Accession numbers are shown in parentheses. Circles indicate AvRV and ARV strains identified in this report.

ticular combination of enteric viruses that a flock is infected with likely plays a major role in determining the severity of the enteritis.

Additionally, pathogens such as *Escherichia coli*, *Salmonella* spp., *Eimeria* spp., and FAdV were detected in 79% of the chicken flocks positive for enteric viruses (Table 1). Impaired development of primary immune organs, including the bursa of Fabricius and thymus, is a common finding in chickens suffering from RSS. This impaired development is thought to be caused by nutritional deficiencies due to decreased feed absorption or direct infection of immune organs (Guy, 1998; Tang et al., 2006; Nili et al., 2007). Enteric viruses may also impair mucosal immunity in the intestines (Guy, 1998). Due to impaired immunity, other bacterial infections, mainly *E. coli* and *Salmonella* spp. infection, are frequently identified in RSS cases (Guy, 1998). The presence of enteric viruses needs to be determined in young chickens that show increased mortality, even though infection with other pathogens such as *E. coli*, *Salmonella* spp., and IBH may also contribute to increased mortality.

Avian astroviruses, including CAstV and ANV, were identified in a high proportion of chicken flocks in this report, although positive rates for these viruses were lower than those reported in a previous study (Pantin-Jackwood et al., 2008). Chicken astrovirus and ANV commonly coinfect chickens (Table 3). In an earlier report, CAstV and ANV were simultaneously identified in intestines that showed significant pathological lesions collected from chickens inoculated with contaminated litter derived from flocks suffering from RSS (Kang et al., 2012). Furthermore, high quantities of CAstV and ANV were found to be present in intestine samples of RSS flocks using quantitative RT-PCR (Smyth et al., 2010). These data suggest that concomitant infection of chickens with these viruses may play an important role in RSS outbreaks in Korea.

The CAstV identified in this report were classified into 4 subgroups based on phylogenetic analysis of a partial sequence of ORF1b (Figure 1). Of the 4 subgroups, CAstV Korean subgroup I showed large differences (87 to 89% nt similarity) to CAstV reported earlier (Pantin-Jackwood et al., 2006; Canelli et al., 2012). The ANV were divided into 2 subgroups based on phylogenetic analysis of a partial fragment of the ORF1b gene; the sequence divergence between these 2 subgroups was 4%. The capsid gene sequence of astroviruses is quite variable and is closely related to antigenicity (Koci and Schultz-Cherry, 2002; Tang et al., 2005b). Human and turkey astroviruses were classified into different serotypes based on amino acid sequence divergences of 95 and 82.8% of the astrovirus capsid gene, respectively (Tang et al., 2005b). The CAstV and ANV identified in this report were therefore expected to show high nt sequence divergence for the capsid gene and distinct serotypes. Additionally, the presence of CAstV was identified in hatched chicks, strongly indicating vertical transmission of CAstV in broiler chickens. This positive result is unlikely to be due to contamination given the distinctness of the nt sequence of this CAstV strain compared with other CAstV strains identified.

Chicken parvoviruses have also been identified in a high proportion of chicken flocks suffering from enteritis; this virus can cause growth retardation, bad feathering, and bone disorders in broiler chickens when experimentally infected (Kisary, 1985; Zsak et al., 2009; Biđin et al., 2011). In this report, fewer Korean flocks were positive for ChPV (26.5%) than reported from flocks from other countries (Zsak et al., 2009; Biđin et al., 2011). Chicken parvoviruses, except for one case, were identified simultaneously with other enteric viruses, especially CAstV and ANV. Moreover, *E. coli*, *Salmonella* spp., *Eimeria* spp., and FAdV infections were present in all ChPV-positive flocks. In an earlier report, ChPV was detected in the epithelial and local inflammatory cells of the duodenum and jejunum in chickens suffering from enteritis, but pathological lesions in the small intestines did not always correspond to regions of ChPV detection (Palade et al., 2011). This discrepancy between pathological lesions and ChPV IHC positive regions in the intestines indicates that other viral infections and factors such as bacteria and protozoa are responsible for these lesions.

Considering the extent of coinfection with other pathogens in the ChPV-infected flocks, ChPV infection may only occur in immunosuppressed hosts. However, we cannot exclude the possibility that ChPV itself may cause immunosuppression because turkey parvovirus was detected in the bursa of Fabricius that showed atrophy (Palade et al., 2011). In this report, concomitant infection with ChPV and ANV was identified in submitted layer chickens that had generalized failure of ossification in the growth plate and pale intestines with watery contents on necropsy. Although enteric viruses are not significantly problematic in layer flocks (Kisary, 1985; Dekich, 1998), coinfection with these 2 viruses may have contributed to enteritis in this case. The pathogenicity and immunosuppressive abilities of ChPV merit further study.

Most IBV were classified as QX-like type IBV; this is a prevalent and nephropathogenic IBV type in Korea (Lee et al., 2008). Although we did not identify enteric-type IBV that cause proventriculitis and mild hemorrhage of the intestines in this report (Yu et al., 2001), 2 novel IBV were identified by sequence analysis. First, the ADL121459 strain represented sequence identity (89%) to K-I IBV isolated in Korea (Lee et al., 2008). Considering that several *Eimeria* spp. were also observed in intestinal contents in this case, the contribution of this variant IBV to enteritis was unclear. The ADL120942 strain was very different (sequence identity 44.4 to 48.2%) to IBV reported earlier but was relatively similar (90 to 91% sequence identity) to turkey coronavirus (Maurel et al., 2011). In phylogenetic analysis, this virus clustered with the turkey coronavirus-like group (Figure 3). Gross lesions of chickens observed

in this case included enlargement of the proventriculus, failure of muscular development of the ventriculus, and gaseous contents in the cecum. Therefore, the pathogenicity of this turkey coronavirus-like IBV in the intestines should be evaluated in future studies.

Avian rotavirus, ARV, and FAdV were less prevalent in the Korean chicken flocks that we examined than other enteric viruses. Two AvRV were identified as group A AvRV by phylogenetic analysis (Figure 3). In contrast to group A AvRV that have been identified primarily from normal intestines, group D AvRV have been identified in intestines showing severe villous atrophy microscopically (Otto et al., 2006). Therefore, the pathogenicity of the AvRV identified in this report is not clear. Three ARV detected in this report formed a group distinct from other strains, including strains 1733 and 2408 that are known as pathological ARV strains (Rosenberger et al., 1989; Figure 3). One FAdV was assigned to serotype 11 by sequence analysis of the hexon gene. Serotype 1 of FAdV is associated with gizzard erosion and enteritis in commercial chicken flocks; furthermore, IBH and gizzard erosions have simultaneously been identified in chickens infected with FAdV serotype 8 (Ono et al., 2001; Okuda et al., 2004). Severe enteritis and hepatitis without gizzard erosions was observed in the flock infected with FAdV serotype 11. The role of this virus in enteritis is therefore not clear.

To summarize, a molecular survey of various enteric viruses was performed in intestinal samples derived from commercial chicken flocks suffering from enteritis in Korea. Concomitant infections were identified by a variety of combinations of enteric viruses as well as secondary bacterial and protozoa infections. Given primer template mismatches, the presence of PCR inhibitors in feces, and the lower sensitivity of conventional RT-PCR than quantitative RT-PCR (Das et al., 2009; Otto et al., 2012), the actual prevalence of enteric viruses in Korean chicken flocks is likely to be higher than what we reported. Although the presence of various enteric viruses was identified in commercial chickens, their exact role was not clear. The roles of these enteric viruses in the pathogenesis of RSS should be evaluated by experimental infection of chickens with individual viruses and various combinations of these viruses.

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