Genetic analysis of duck circovirus in Pekin ducks from South Korea

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ABSTRACT The genetic organization of the 24 duck circovirus (DuCV) strains detected in commercial Pekin ducks from South Korea between 2011 and 2012 is described in this study. Multiple sequence alignment and phylogenetic analyses were performed on the 24 viral genome sequences as well as on 45 genome sequences available from the GenBank database. Phylogenetic analyses based on the genomic and open reading frame 2/cap sequences demonstrated that all DuCV strains belonged to genotype 1 and were designated in a sub-cluster under genotype 1. Analysis of the capsid protein amino acid sequences of the 24 Korean DuCV strains showed 10 substitutions compared with that of other genotype 1 strains. Our analysis showed that genotype 1 is predominant and circulating in South Korea. These present results serve as incentive to add more data to the DuCV database and provide insight to conduct further intensive study on the geographic relationships among these virus strains.

Key words: duck circovirus, Pekin duck, genotype, phylogenetic tree

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INTRODUCTION

Ducks infected with duck circovirus (DuCV) exhibit feathering disorders, growth retardation, and low BW. A histopathological examination of the bursa of Fabricius demonstrates lymphocyte depletion, necrosis, and histiocytosis (Soike et al., 2004). Duck circovirus, a tentative species in the genus Circovirus of the family Circoviridae, is a small, round, nonenveloped, single-stranded DNA virus with a circular genome of less than 2 kb (Hattermann et al., 2003; Wang et al., 2011). Three major open reading frames (ORF) have been identified such as ORF1, ORF2, and ORF3 (Johne et al., 2006). The ORF1 (rep gene) is located in the viral strand and encodes a 33.6-kDa protein involved in virus replication, and ORF2 (cap gene) is located in the opposite orientation and encodes a 29.7-kDa major immunogenic capsid protein (Hattermann et al., 2003; Liu et al., 2010). Another major conserved ORF3 was recently located in the complementary strand of ORF1, encodes the ORF3 protein, and has apoptotic activity (Xiang et al., 2012). The 5′-end intergenic regions (IR) located between the initiation codons of the 2 major ORF contain rolling circle replication motifs such as stem-loop structures and nonamer sequences. The 3′-end IR of the 2 major ORF contain 4 repeats of a 44-bp sequence; this sequence feature is a distinctive characteristic of DuCV when compared with other circoviruses (Fu et al., 2011).

Duck circovirus was first described in mallards from a farm in Germany in 2003 (Hattermann et al., 2003). A similar DuCV was detected in Muscovy, Pekin, and mule ducks with stunting and feather abnormalities in Taiwan (Chen et al., 2006). Since then, DuCV infections have been reported in Hungary, the United States, and China (Fringuelli et al., 2005; Banda et al., 2007; Zhang et al., 2009).

The South Korean duck industry has been growing dramatically in the past several years according to the Food and Agriculture Organization, as duck meat is presumed to be a healthy food (Cha et al., 2013). However, management and sanitation on duck farms are poor compared with those on chicken farms. A DuCV infection has been detected in South Korea by our laboratory, but information regarding the genetic variation in the DuCV was unclear until now. In this study, we investigated the genomic sequence diversity and phylogenetic analysis of 24 DuCV from South Korea and compared them with available reference DuCV strain sequences obtained from databases. The aim of this study was to provide more information on the molecular characteristics and evolutionary diversity of the DuCV genomes.

MATERIALS AND METHODS

Sample Collection

Of the 147 samples collected from 92 farms in South Korea from 2011 to 2012, 32 of 147 samples (21.9%)...
were positive for DuCV. Among the 32 PCR-detected DuCV-positive samples, co-infection by Riemerella anatipestifer and Salmonella Enteritidis was detected. All Pekin ducks were euthanized by cervical dislocation, and samples were collected at necropsy and had no gross lesions. A portion of the bursa of Fabricius was prepared as a 10% suspension by placing approximately 1 g of tissue in a screw-topped container containing sea sand and 10 mL of PBS containing 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA). The samples were centrifuged at low speed after freezing and thawing, and the supernatant was kept at −80°C until use.

**PCR and Sequencing**

Total viral DNA was extracted from tissue homogenates and allantoic fluid stocks using the Viral GeneSpin Viral DNA/RNA Extraction kit (iNtRON Biotechnology, Daejeon, Korea). The DNA of the tissue homogenates was used in PCR assays to detect DuCV pathogens. The common gene was detected using common DuCV detection primers (408 bp; DuCVaF 5′-MGA GCT GCC GCC CTT GAG-3′ and DuCVaR 5′-TCC CGA GTA ACC GTC CCA CCA C-3′; Banda et al., 2007). The remaining portion of the DuCV genome was amplified to obtain the full-length genomic sequence according to a method described previously (Banda et al., 2007). The amplified products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI), and nucleotide sequences were determined using an ABI PRISM 310 Genetic Analyzer autosequencer (Applied Biosystems, Foster City, CA).

**Sequence Analysis**

The full gene sequences of DuCV from South Korea (Table 1) were compared with the sequences of select DuCV strains from various geographic origins. The accession numbers for these complete gene nucleotide sequences were obtained from GenBank. The deduced amino acid (AA) sequences were aligned using the MEGA software package version 4.0 (DNAStar, Madison, WI). The antigenic index was predicted using the Jameson and Wolf algorithm (Jameson and Wolf, 1988) in the Protein program (DNAStar). The phylogenetic analysis was conducted using the neighbor-joining method, and the P distance was set to the algorithm of correction. The robustness of the phylogenetic analysis was assessed by bootstrap analysis with 1,000 replicates using the MEGA software package version 4.0 (DNAStar).

**RESULTS**

**Viral Full Genome Detection from Pekin Ducks**

Twenty-four of 32 full genome positive samples were successfully sequenced. The characteristics of the DuCV are listed in Table 1. Twelve DuCV (D11-JW-001, D11-JW-004, D11-JW-006, D11-JW-007, D11-JW-008, D11-JW-024, D11-JW-037, D12-JW-047, D11-KD-001, D12-KD-002, D12-KD-027, and D12-MR-020) obtained from the bursa of Fabricius of Pekin ducks were used as the representative DuCV in this study.

**Sequence Analysis**

The complete gene of DuCV from Pekin ducks was analyzed and compared with sequence data from Germany, United States, and Chinese strains available under the accession numbers in GenBank (Hattermann et al., 2003; Banda et al., 2007; Wang et al., 2011).
parative full genomic sequence analysis of the 24 DuCV strains indicated that they were closely related to each other (96.7–100% identity), and with genotype 1 (92.4–98.7%) and genotype 2 (82.4–86.0%). Sequence analysis of the ORF1, ORF2, and ORF3 genes of the 60 DuCV strains, including genotype 1 and genotype 2 strains, showed 92.6 to 99.5%, 88.2 to 98.7%, and 91.9 to 99.6% identity compared with genotype 1 and 87.0 to 92.3%, 77.9 to 80.4%, 91.2 to 98.3% identity compared with genotype 2, respectively. The deduced AA sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed. There was 95.2 to 100%, 93.4 to 100%, and 81.4 to 97.9% similarity to the published AA sequences of genotype 1 and 92.1 to 97.6%, 85.2 to 100%, and 81.4 to 97.9% similarity to the published sequence was analyzed.

**Phylogenetic Analysis**

A phylogenetic tree was constructed to investigate the genetic relationships among the Pekin duck strains with other DuCV strains. Phylogenetic analysis of the ORF2/cap deduced AA (Figure 1) sequence revealed that all present strains grouped together in one branch in genotype 1. Twenty-two sequences formed a subcluster in genotype 1 and 3 strains (D11-JW-008, D12-KD-001, and D12-MR-021) were a very closely related group with sequences from China and the United States.

**Analysis of AA Sites and the ORF2 Antigenic Index**

Open reading frame 2, which is believed to cause antigenic differences, displayed differences from reference strains. The AA present at sites 134, 194, 205, 213, 223, 226, 227, 232, 235, and 235 were determined. Detailed information about AA variations within the ORF2 is summarized in Table 2. The antigenic traits of the ORF2 of the DuCV strains were compared, and the antigenic peaks were identified in representative US, German, Chinese, and South Korean strains using the same algorithm (data not shown).

**DISCUSSION**

Since DuCV, a tentative species in the genus Circovirus of the family Circoviridae was first described in Germany in 2003 (Hattermann et al., 2003), several studies have been reported from Taiwan, Hungary, the United States, and China (Fringuelli et al., 2005; Chen et al., 2006; Banda et al., 2007; Zhang et al., 2009). It has been suggested that the ORF2/cap region of pigeon circovirus displays high genetic variation (Todd et al., 2008). Additionally, the ORF2/cap AA is the major structural protein and the major protective antigen in porcine circovirus 2 (PCV2). It is relatively conserved, but a few mutations have been identified in this gene in recent years (Guo et al., 2010). Two of these regions (57–91 and 121–151) correspond with 2 major variable regions encompassing residues 3 to 15, 31 to 238, which corresponded with our data. Moreover, we observed that the 134, 223, 226, and 227 AA sites were located in conserved regions, but different residues were observed in the DuCV Korean strains. Substitutions in 10 positions were observed (Table 2). Moreover, an analysis of the Korean DuCV ORF2/cap protein using the algorithm developed by Jameson and Wolf to compare the antigenic traits identified 3 antigenic peaks, suggesting that 3 variable AA residue (104–109, 151–158, 237–242) regions might be distinguished by epitope differences compared with other genotype 1 strains (data not shown). The relationships among mu-
Figure 1. Evolutionary relationship of the Korean duck circovirus (DuCV) strains inferred using the neighbor-joining method based on the nucleotide sequences of the complete genome (a), the amino acid sequences of the open reading frame 2/cap gene (b), the open reading frame 1/cap gene (c), and the open reading frame3 gene (d) using the MEGA 4.0 program (DNAStar, Madison, WI). Triangles (▲) indicate the 24 Korean DuCV strains analyzed in this study. The country of origin of each DuCV strain is also indicated: KR, Korea; CN, China; GE, Germany; US, United States; TW, Taiwan.
Figure 1 (Continued). Evolutionary relationship of the Korean duck circovirus (DuCV) strains inferred using the neighbor-joining method based on the nucleotide sequences of the complete genome (a), the amino acid sequences of the open reading frame 2/cap gene (b), the open reading frame 1/cap gene (c), and the open reading frame 3 gene (d) using the MEGA 4.0 program (DNAStar, Madison, WI). Triangles (▲) indicate the 24 Korean DuCV strains analyzed in this study. The country of origin of each DuCV strain is also indicated: KR, Korea; CN, China; GE, Germany; US, United States; TW, Taiwan.
Table 2. Amino acid differences in open reading frame 2 of different genotype duck circoviruses in South Korea, Germany, the United States, and China

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>134</th>
<th>194</th>
<th>205</th>
<th>213</th>
<th>223</th>
<th>226</th>
<th>227</th>
<th>232</th>
<th>235</th>
<th>236</th>
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<td>Q</td>
<td>G</td>
<td>K</td>
<td>S</td>
<td>G</td>
<td>L</td>
<td>R</td>
<td>A</td>
<td>D</td>
<td>E</td>
</tr>
<tr>
<td>Genotype 12</td>
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<td>Q</td>
<td>G</td>
<td>R</td>
<td>T</td>
<td>G</td>
<td>L</td>
<td>R</td>
<td>T</td>
<td>V</td>
<td>N</td>
</tr>
<tr>
<td>D11-JW-001</td>
<td>1</td>
<td>K*</td>
<td>S*</td>
<td>R</td>
<td>T</td>
<td>G</td>
<td>L</td>
<td>R</td>
<td>T</td>
<td>V</td>
<td>N*</td>
</tr>
<tr>
<td>D11-JW-004</td>
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<td>S*</td>
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<td>R</td>
<td>T</td>
<td>G</td>
<td>L</td>
<td>R</td>
<td>T</td>
<td>V</td>
<td>N*</td>
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<td>R</td>
<td>A*</td>
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<td>L</td>
<td>R</td>
<td>T</td>
<td>V</td>
<td>N*</td>
</tr>
<tr>
<td>D12-KD-001</td>
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<td>Q</td>
<td>G</td>
<td>K*</td>
<td>T</td>
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<td>L</td>
<td>R</td>
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<tr>
<td>D12-KD-002</td>
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<td>T</td>
<td>G</td>
<td>F*</td>
<td>W*</td>
<td>P*</td>
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<td>L</td>
<td>R</td>
<td>T</td>
<td>V</td>
<td>N</td>
</tr>
</tbody>
</table>

1Strains from China.
2Strains from other countries including Germany, the United States, and China.
*Amino acids with different positions compared with genotype 1 are shown with an asterisk.

tations and changes in the antigenicity, pathogenicity, and virulence of viruses warrants further investigation. Furthermore, phylogenetic and evolutionary studies are of increasing importance in molecular epidemiological studies on viral pathogens.

Infections of DuCV are highly prevalent in Hungary and are likely to be widespread in farmed ducks (Fringuelli et al., 2005). A comparatively lower level of DuCV has been reported in Germany, Taiwan, the United States, and China (Soike et al., 2004; Chen et al., 2006; Banda et al., 2007; Zhang et al., 2009). An in-depth study could be conducted if more sequence information was available. To date, only 5 countries had 45 genetic reports. The present study might serve as an incentive to add more data to the DuCV database and may provide insight to conduct further intensive study on geographic relationships. Further study is needed to intensively analyze the pathogenesis and transmission of DuCV.

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