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Genetic Diversity of Avian Paramyxovirus Type 6 Isolated from Wild Ducks in the Republic of Korea

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ABSTRACT: Eleven avian paramyxovirus type 6 (APMV-6) isolates from Eurasian Wigeon (n=5; *Anas penelope*), Mallards (n=2; *Anas platyrhynchos*), and unknown species of wild ducks (n=4) from Korea were analyzed based on the nucleotide (nt) and deduced amino acid sequences of the fusion (F) gene. Fecal samples were collected in 2010–14. Genotypes were assigned based on phylogenetic analyses. Our results revealed that APMV-6 could be classified into at least two distinct genotypes, G1 and G2. The open reading frame (ORF) of the G1 genotype was 1,668 nt in length, and the putative F0 cleavage site sequence was ^{113}PAPEPRL^{119}. The G2 genotype viruses included five isolates from Eurasian wigeons and four isolates from unknown waterfowl species, together with two reference APMV-6 strains from the Red-necked Stint (*Calidris ruficollis*) from Japan and an unknown duck from Italy. There was an N-truncated ORF (1,638 nt), due to an N-terminal truncation of 30 nt in the signal peptide region of the F gene, and the putative F0 cleavage site sequence was ^{103}SIREPRL^{109}. The genetic diversity and ecology of APMV-6 are discussed.

Key words: Avian paramyxovirus type 6, F gene, genetic diversity, phylogenetic analysis, wild duck.

Avian paramyxoviruses (APMVs) belong to the genus *Avulavirus* in the family *Paramyxoviridae* and their natural hosts are wild or domestic bird species (Mundt 2013). There are 13 recognized avian avulavirus species (AAvV-1 to AAvV-13) in the genus *Avulavirus*, and each has a single member, called APMV 1–13 (Amarasinghe et al. 2017). In recent years, at least five putative novel avulavirus species from wild waterfowl and penguins were reported worldwide, including Antarctica (Yamamoto et al. 2015; Lee et al. 2017; Neira et al. 2017; Thampaisam et al. 2017; Thomazelli et al. 2017). The virulent form of AAvV-1 (Newcastle disease virus) causes fatal infection in poultry. In addition to AAvV-1, AAvV-2, AAvV-3, AAvV-6, and AAvV-7 are reportedly associated with diseases of poultry (Miller and Koch 2013).

In 1977, avian paramyxovirus type 6 (APMV-6), a member of AAvV-6, was first isolated from a domestic duck in Hong Kong (Shortridge et al. 1980). Since then, APMV-6 has been isolated from various wild bird species, including ducks, geese, Common Egrets (*Egretta alba*), and Red-necked Stint (*Calidris ruficollis*) worldwide. Despite some APMV-6 isolates being antigenically and genetically distinct from the prototype strain (APMV-6/duck/HK/18/199/77), the genetic diversity and ecology of APMV-6 are unclear (Xiao et al. 2010; Bui et al. 2014; Sobolev et al. 2016).

In this study, APMVs were isolated accidentally from free-living wild ducks during the winter season (November to February) through an avian influenza surveillance program at wintering sites in Korea between 2010 and 2014. Approximately 18,500 fresh fecal droppings from wild ducks were collected during this period, and were inoculated into the allantoic cavity of 9–11-day-old specific-pathogen-free chicken embryonated eggs (World Organisation for Animal Health 2012). Avian influenza-negative viral hemagglutinating agents were subjected to a cross-hemagglutination-inhibition test using a reference of APMV-1 to APMV-9 (except for APMV-5) antigen/antiserum panel for the serotyping of APMV (World Organisation for Animal Health 2012). Seventy-nine APMVs were isolated from wild ducks during the study period, including eleven isolates (14%, 11/79) confirmed by a cross-hemagglutina-

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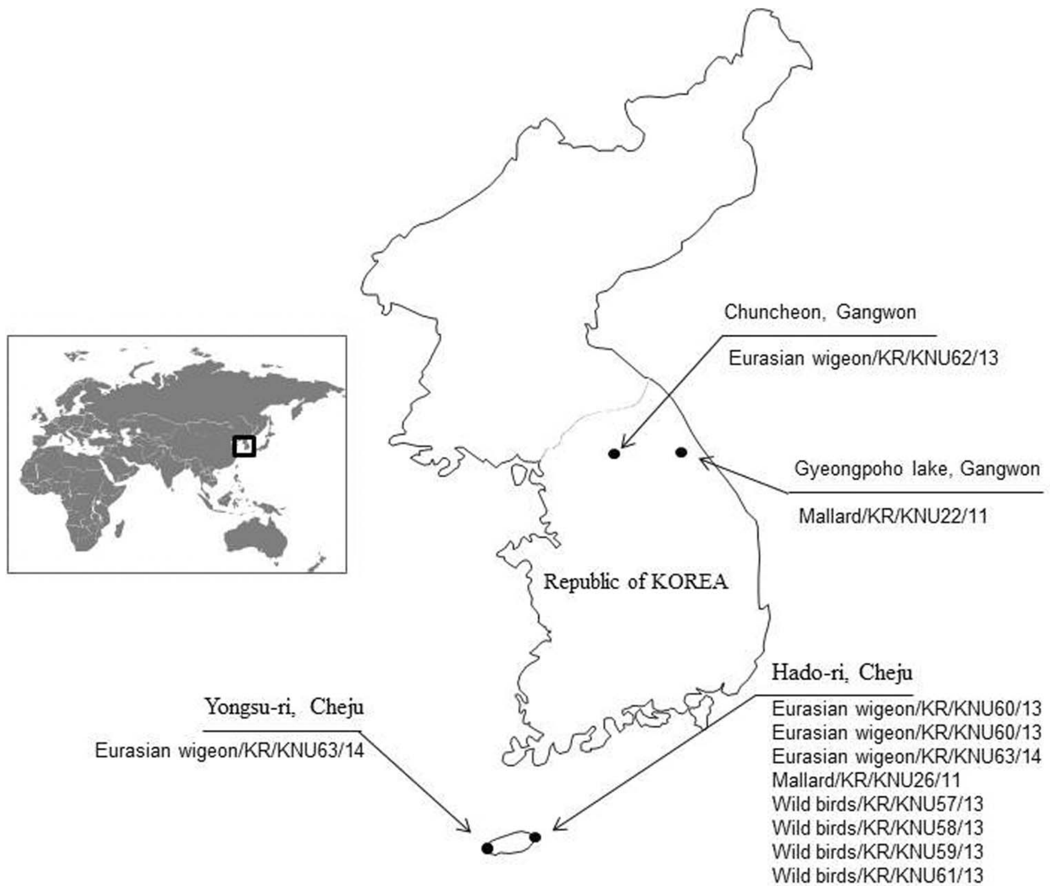

FIGURE 1. Map of the Republic of Korea showing the location from which fecal samples containing avian paramyxovirus type 6 were collected from wild birds in 2010–14.

tion-inhibition test to be APMV-6. The APMV-6 positive fecal samples were subjected to DNA barcoding to identify the bird species (Lee et al. 2010). Five isolates were isolated from Eurasian Wigeons (*Anas penelope*), two from Mallards (*Anas platyrhychos*), and the other four from wild ducks (unknown species). Information regarding the APMV-6 isolates is provided in Figure 1 and Supplementary Table 1.

We sequenced the complete open reading frame (ORF) of the fusion protein (F) gene of the APMV-6 isolates. Viral RNAs were extracted from allantoic fluid using Gene-spin™ Viral DNA/RNA Extraction Kit (Intron Biotechnology, Seongnam, Korea), according to the manufacturer's instructions. We performed the reverse transcriptase-PCR using a One-step RT-PCR Kit (Qiagen, Hilden, Germany) and primers designed for the study (Supplementary Table 2). The PCR products were separated by 1% agarose-gel electrophoresis, purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions, and subjected to direct nucleotide (nt) sequencing using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) followed by analysis with an ABI Prism (ver. 377) DNA autosequencer (Applied Biosystems). Following amplification by reverse transcription PCR, the F genes of the APMV-6 isolates were successfully sequenced. The resultant nucleotide data were submitted to GenBank (accession nos. MF072421 to MF072431; Supplementary Table 1). The

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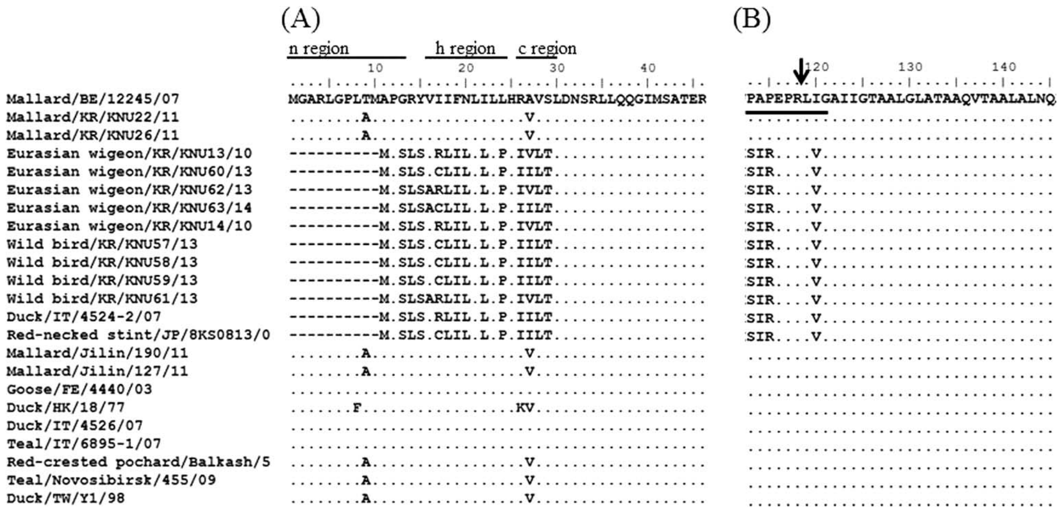

FIGURE 2. Predicted amino acid sequence alignment of the N-terminal region (A) and the cleavage site (B) of the fusion (F) protein of avian paramyxovirus type 6 isolates obtained from fecal samples of wild birds in the Republic of Korea in 2010–14. Amino acid identity and amino acid deletion compared to strain Mallard/BE/12245/07 (top sequence) are indicated by dots and dashes, respectively.

complete ORF sequences of F gene were aligned using the ClustalW multiple alignment algorithm in the BioEdit sequence alignment editor (Hall 1999) and their predicted amino acid (aa) sequences were compared with those of known APMV-6 strains. The F gene of APMV-6 isolates in the study had ORFs of 1,668 nt (555 aa) or 1,638 nt (545 aa) in length. In particular, aa sequence variation in the N-terminal region and the F cleavage site were observed among APMV-6s (Fig. 2). An N-terminal truncation of 30 nt (10 aa) accounted for the difference in the ORF length (Fig. 2A), as previously reported (Xiao et al. 2010). Two of our isolates (KNU22 and KNU26) from Mallards had an ORF 1,668 nt in length, as also observed in 10 known APMV-6 strains from Mallards (n=3), Common Teal (*Anas crecca*; n=2), Red-crested Pochard (*Netta rufina*; n=1), Domestic Goose (*Anser anser*; n=1), and unknown species of waterfowl (n=3) found worldwide. The APMV-6 strains having the N-truncated ORF gene included five isolates (KNU10, KNU14, KNU60, KNU62, and KNU63) from Eurasian Wigeons, and four isolates (KNU57, KNU58, KNU59, and KNU61) from unknown species of waterfowl. The N-truncated ORF was also present in two strains of APMV-6 (duck/IT/4524-2/2007 and Red-necked Stint/JP/8KS0813/2008). For all APMV-6s, F0 cleavage site sequences consisted of monobasic aa and leucine, which are typically found in APMV-1s with low virulence (Miller and Koch 2013). The APMV-6 strains having an ORF 1,668 nt in length had amino acid sequences ^{113}PAPEPRL^{119} at the F cleavage site, whereas those having the N-truncated ORF had the sequence ^{103}SIREPRL^{109} (aa positions 103–109) in the corresponding position (Fig. 2B).

The homology of the F gene sequences of APMV-6 isolates and reference strains was analyzed. This resulted in the division of APMV-6 isolates into two genetic groups (Supplementary Table 3). The APMV-6 strains (n=12) having an ORF 1,668 nt in length had high within-group nt (aa) sequence identities of 93.2–97.7% (98.1–100%). The APMV-6 strains (n=11) having the N-truncated ORF also had high within-group nt (aa) sequence identities of 95.1–100% (97.9–100%). There was a relatively low nt and aa similarity between the two groups of 71.0% and 84.1%, respectively.

We assessed the genetic diversity of APMV-6 using phylogenetic analysis including esti-

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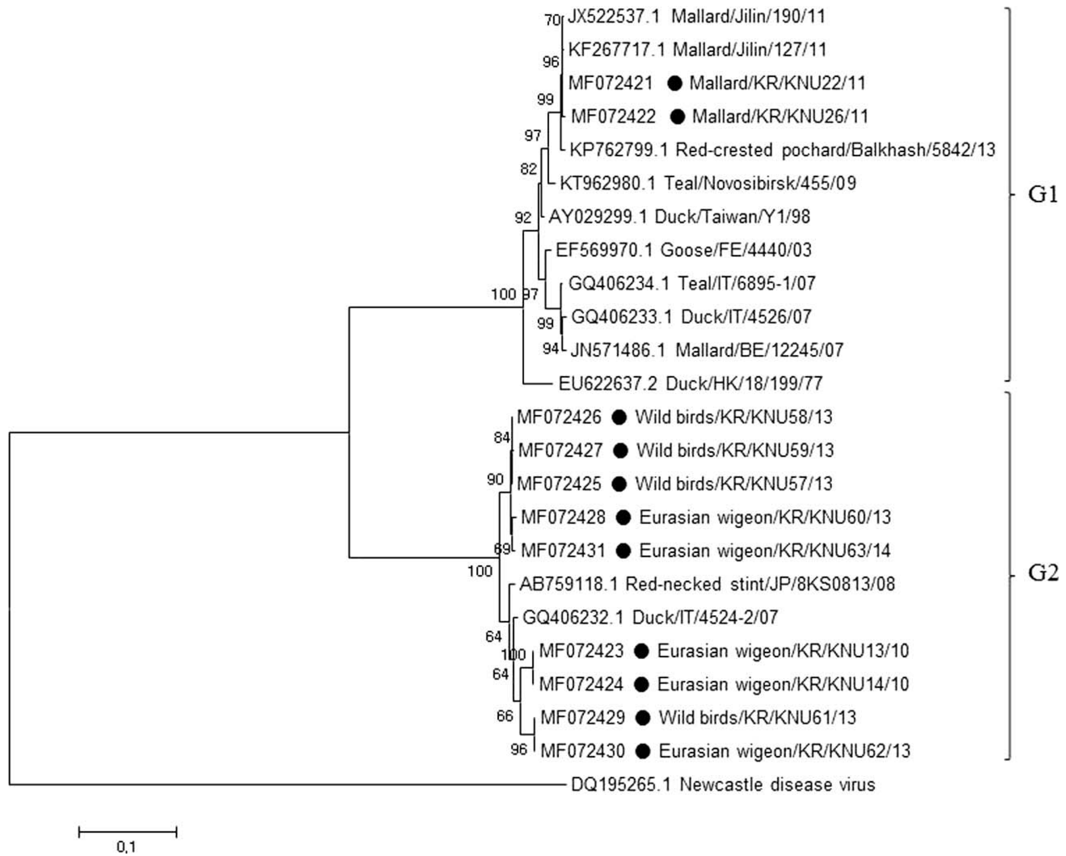

FIGURE 3. Phylogenetic analysis based on the complete nucleotide sequence of the fusion (F) gene of avian paramyxovirus type 6 (APMV-6) isolated from fecal samples collected in 2010–14 in the Republic of Korea. The phylogenetic analysis was performed in MEGA 7.0 using the maximum likelihood method, based on the Tamura-Nei model with 1,000 bootstrap replicates. APMV-1 (Newcastle Disease Virus) was used as an outgroup for rooting the tree. Filled circle=APMV-6 sequences isolated in this study.

mates of evolutionary distance. A phylogenetic tree based on the complete ORF nt sequence of the F gene was constructed in MEGA 7.0 (Kumar et al. 2016) using the maximum-likelihood method based on the Tamura-Nei model, with 1,000 bootstrap replicates. The APMV-1 (Newcastle Disease Virus) strain was used as an outgroup to construct the phylogenetic tree. We also included the F sequences of 12 known APMV-6 strains retrieved from the GenBank database. The results revealed that APMV-6 strains were divided into two distinct genetic groups: G1 and G2 (Fig. 3). Two isolates from Mallards formed the G1 with 10 APMV-6 strains having an ORF 1,668 nt in length. Nine isolates from Eurasian Wigeons and unknown species of waterfowl were assigned to group G2 with two APMV-6 strains, all of which had the N-truncated ORF.

We calculated the evolutionary distance between and within groups, based on the nt sequences of the complete F gene using MEGA 7.0 (over 1,000 bootstrap replicates; Kumar et al. 2016; Supplementary Table 4). Currently, no criteria are proposed for the genotype classification of APMV-6. Thus, the criteria for genotype classification of APMV-1 (Diel et al. 2012), which requires at least four independent isolates within each group with complete F gene sequences, was also applied directly for APMV-6. Estimates of the evolutionary intragroup distances for groups G1 (n=12) and G2 (n=11) were 1–6.8% and 0–5.4%, respectively. The mean estimate of the evolutionary distance

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between G1 and G2 (44.7%) was greater than 10%, a cutoff used to validate genotypes of APMV-1 identified by phylogenetic analysis (Diel et al. 2012). Our results revealed that there are at least two distinct genotypes (G1 and G2) within APMV-6, based on the criteria of Diel's classification system.

In summary, G1 viruses included two isolates derived from Mallards in the study. Strains of APMV-6 from Mallards in China and Belgium also belonged to the G1 genotype. In addition, G1 viruses were isolated from other waterfowl species, such as geese, teal, and Red-crested Pochards. On the contrary, G2 viruses included five isolates from Eurasian Wigeons and four isolates from unknown waterfowl species, together with an APMV-6 strain from a Red-necked Stint in Japan, and an unknown duck in Italy. From our findings, it seemed that G2 viruses had a different ecology from G1 viruses, but this might have been influenced by sampling bias due to the distributions of the bird species, the migration patterns of the birds, and the sampling seasons. Thus, there is simply not enough genetic data at this time to infer host specificity or the evolution of APMV-6 in a phylogeographical context. Further research will improve our understanding of APMV-6, and with additional genetic data, there will be a time in the future when these questions can be addressed.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2017-07-158.

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