Characterization of H5N2 influenza viruses isolated in South Korea and their influence on the emergence of a novel H9N2 influenza virus

Hye-Ryoung Kim,1 Choi-Ku Park,1 Jae-Ku Oem,1 You-Chan Bae,1 Jun-Gu Choi,2 O-Soo Lee1 and Youn-Jeong Lee2

1Animal Disease Diagnosis Center, National Veterinary Research and Quarantine Service, 335 Joongangro, Manangu, Anyangsi, Gyeonggido 430-824, Republic of Korea
2Avian Disease Division, National Veterinary Research and Quarantine Service, 335 Joongangro, Manangu, Anyangsi, Gyeonggido 430-824, Republic of Korea

We characterized low pathogenic avian influenza (LPAI) H5N2 and H9N2 viruses isolated in South Korea from 2008 to 2009. Genetic analysis of the H5N2 viruses isolated from wild birds and domestic ducks demonstrated that they were related to the recently isolated southern Chinese LPAI H5 viruses and various influenza viruses circulating in Eurasia. Three H9N2 viruses obtained at live bird markets and duck farms were reassortant viruses generated from the H5N2 viruses of domestic ducks and the H9N2 virus endemic in Korean chickens. The H5N2 viruses did not replicate well in experimentally infected chickens and mice, but novel H9N2 viruses, without pre-adaptation, were recovered at high titres in chickens. Our results show that reassortment between H5N2 and H9N2 viruses must have occurred in domestic ducks and may have contributed to the diversity expansion of the gene pool, which has potential to alter the pathogenicity and host range of the influenza virus.

Avian influenza viruses (AIV) are classified in the family Orthomyxoviridae, genus Influenzavirus A. To date, 16 haemagglutinin (HA) and nine neuraminidase (NA) subtypes have been identified, and all known subtypes of AIV have been implicated in various clinical signs ranging from asymptomatic infections to respiratory diseases with low mortality to severe pathogenicity with high mortality (Swayne & Halvorson, 2003).

Low pathogenic avian influenza (LPAI) viruses of H5 and H7 subtypes have been recognized to evolve into highly pathogenic avian influenza (HPAI) viruses once transmitted to domestic poultry (Banks et al., 2001; Horimoto et al., 1995; Lee et al., 2005a). Thus, many countries have emphasized the significance of H5 and H7 subtypes in surveillance programmes for HPAI detection and the LPAI H5 and H7 subtypes viruses have been classified as avian influenza together with HPAI viruses, according to World Organization for Animal Health (OIE)’s International Animal Health Code (OIE, 2004).

There have been reports of LPAI H5 viruses detected in several Asian countries. In 2005, the LPAI H5N2 viruses isolated in Japan originated from H5N2 strains prevalent in Central America (Okamatsu et al., 2007). LPAI H5N2/N3 viruses isolated from food products in Singapore clustered with the Eurasian H5 lineage viruses (Yeo et al., 2009). Southern Chinese LPAI H5 viruses isolated from wild ducks were related to the LPAI H5 viruses isolated in Italy in late 1990 (Duan et al., 2007). In Korea, one LPAI H5N2 virus was isolated from domestic ducks in 2004 (OIE, 2005), but there have been no reports on the characterization of the virus. In addition, avian- and avian/swine-like LPAI H5N2 viruses have been identified in swine (Lee et al., 2009).

We are the first to characterize the H5N2 AIV (two isolates) from domestic ducks in Korea, together with the H5N2 AIV isolated from wild birds, as the result of a systematic surveillance programme conducted from September 2008 to June 2009. In addition, some H9N2 subtype AIV obtained from duck farms and live bird markets (LBMs) during the same time were selected and tested to elucidate their relationship with the H5N2 viruses. We performed a phylogenetic analysis to investigate their genetic relationships and conducted animal experiments to determine their pathogenicity.

A total of 159 345 faeces from domestic ducks in duck farms, 1816 oropharyngeal and cloacal swabs of captured migratory birds, 12 327 faeces of wild birds at migratory bird habitats and 3900 oropharyngeal swabs and faeces of...
poultry from LBMs were sampled according to the active surveillance of HPAI conducted in South Korea from September 2008 to June 2009. The swabs and faecal samples were examined by virus isolation in 9–11-day-old embryonated chicken eggs (ECE). The presence and subtype of AIV in the ECE were determined by a haemagglutination assay and RT-PCR methods, as described previously (Fereidouni et al., 2009; Fouchier et al., 2000; Lee et al., 2001; Munch et al., 2001). A total of 201 LPAI viruses of various subtypes were identified by the National Veterinary Research and Quarantine Service (NVRQS), but no HPAI viruses had been isolated. Only four H5N2 subtype AIV were isolated from two duck farms and two migratory bird aggregation sites and all were selected for this study (Supplementary Table S1, available in JGV Online). In addition, we selected three H9N2 viruses that were grouped distinctly from the phylogenetic analysis of the M genes of 77 H9N2 viruses obtained during the same duration (data not shown).

We further sequenced the genomes of the selected AIV isolates by using a method described previously (Kim et al., 2010b). The PQRET R amino acid motif at the HA cleavage site was identified from three H5N2 viruses tested, although the wb/Kr/L60-2/08 virus had a PQKETK motif. The HA peptide motif of the selected H9 isolates was PATSGR, similar to that of H9 viruses isolated in Korea during 2002 and early 2003 (Lee et al., 2007).

A phylogenetic analysis of the HA gene showed that three H5N2 viruses (Dk/Kr/A14/08, Dk/Kr/A93/08 and wb/Kr/A81/09) clustered in the Eurasian lineage and were similar to viruses recently isolated in southern China. However, the wb/Kr/L60-2/08 virus did not cluster in the American and Eurasian lineages but had similarity (86 %) to the Tk/Ramon/73 virus of the H5 subtype viruses known so far (Fig. 1). These data suggested that three of the viruses tested clustered with group A and that the other virus clustered with group B. In the NA gene tree, three NA genes of the tested H5N2 viruses were related to those of H5N2 viruses recently isolated in southern China, but the wb/Kr/L60-2/08 virus clustered in the American lineage (Supplementary Fig. S1, available in JGV Online). These N2 genes were different from those of H3, H6 and H9 subtype viruses, which were endemic and had circulated previously in Korea. Therefore, the genes for the surface proteins of the three Korean H5N2 viruses were closely related to the southern Chinese H5N2 virus, whereas the wb/Kr/L60-2/08 virus appeared to originate from unidentified avian sources.

A phylogenetic analysis of polymerase basic protein 2 (PB2) demonstrated that two H5N2 viruses from domestic ducks were distinct from the two H5N2 viruses from wild birds (Supplementary Fig. S2, available in JGV Online). The PB2 genes of H5N2 viruses isolated from wild birds were similar to those of H5N2 viruses isolated in southern China from 2005 to 2006, whereas the viruses of domestic ducks clustered with the H5N2 viruses isolated in Hong Kong from 1976 to 1980 and to the AIV circulating in wild birds and poultry in Eurasia. The nucleoprotein (NP) genes were separated into three different lineages (Supplementary Fig. S3, available in JGV Online). In particular, the Korean H5N2 viruses of domestic ducks were divided into two groups that were related to viruses from migratory birds and poultry in Eurasia, including the recently isolated H5N2 viruses of southern China. The PB1 gene and polymerase acidic protein (PA) gene of the tested H5N2 viruses clustered with the recently isolated H5N2 viruses from southern China, except for the PA gene of the wb/Kr/L60-2/08 virus (Supplementary Figs S4 and S5, available in JGV Online). A phylogenetic analysis of the matrix (M) and non-structural (NS) genes showed that only the wb/Kr/A81/09 virus of wild birds clustered with the Gs/GY/3799/05 virus, one of the LPAI H5 viruses isolated in southern China, and that the H5N2 viruses of domestic ducks belonged to the wild bird lineage circulating in the Eurasia region (Supplementary Figs S6 and S7, available in JGV Online). The NS genes of the wb/Kr/A81/09 virus and most of the H5N2 viruses isolated from southern China clustered to a different NS allele (allele B) that was distinct from the alleles of other subtype viruses isolated in Eurasia.

The HA and NP genes of selected H9N2 viruses isolated from LBMs and duck farms were closely related to those of the Ck/Kr/96006/96-like viruses that were endemic in Korea (data not shown and Supplementary Fig. S3). However, six gene segments, excluding HA and NP genes, clustered with those from the H5N2 viruses isolated from domestic ducks (Dk/Kr/A14/08 and Dk/Kr/A93/08) with more than 99.4 % similarity (Supplementary Figs S4–S7). The genetic diversity of H5N2 viruses was recognized based on the phylogenetic analysis of eight genes from each virus, including wild bird isolates. The wb/Kr/A81/08 (H5N2) virus isolated from wild birds belonged to the goose/Guixiang/3799/05-like lineage (>99 % similarity), which was isolated recently at a retail market in southern China (Duan et al., 2007). This may be evidence of the direct introduction of LPAI H5 viruses from southern China through migratory birds. The wb/Kr/L60-2/08 virus, the other H5N2 virus isolated from wild birds, clustered with the wb/Kr/A81/09 virus in the PB2 and PB1 phylogeny, whereas the six gene segments were different from those of the other three H5N2 isolates tested and these gene segments were originated from unknown sources. Genes from the Dk/Kr/A14/08 and Dk/Kr/A93/08 viruses of domestic ducks were closely related to each other, excluding the NP gene. Some genes of both these viruses were similar to those of the wb/Kr/A81/08 virus, whereas PB2, M and NS genes had similarity with those genes of viruses circulating in Eurasia. These results suggested that the H5N2 subtype AIV in southern China could be introduced to Korea through wild migratory birds and that H5N2 viruses isolated from domestic ducks in Korea might be generated by exchanging genes of internal viral proteins between AIV of various subtypes and the H5N2 viruses of wild birds. Moreover, it was suggested that the
H9N2 viruses tested in this study have been generated by the reassortment between pre-existing H9N2 viruses from chickens and the H5N2 viruses of domestic ducks at LBMs (Fig. 2).

To investigate the cross-reactivity of the isolated H5 viruses, we performed a haemagglutinin inhibition (HI) assay (Palmer et al., 1975) using chicken polyclonal antibodies to three H5 subtype AIV: Dk/Kr/A14/08, A/chicken/Korea/Gimje/08 (Ck/Kr/Gimje/08) and A/duck/Hong Kong/820/80 (Dk/HK/820/80). The HI assay was performed to investigate the antigenic diversity of H5N2 viruses isolated in Korea using antisera against three H5 subtype AIV (Supplementary Table S2, available in JGV Online). As expected from the phylogenetic analysis, the H5N2 AIV studied separated into two antigenically distinct groups: group A and group B. H5N2 viruses of both groups A and B did not cross-react with the Ck/Kr/Gimje/08 (H5N1) HPAI virus. All H5 subtype AIV tested with the Dk/HK/820/80 virus showed antigenic reaction of various titres. This result demonstrated that the H5 LPAI viruses isolated from southern China in the 1970s have contributed to the evolution of different AIV among poultry and migratory birds for a long period of time (Duan et al., 2007).

We compared the replication capacity of four H5N2 AIV and selected two H9 AIV (Ck/Kr/A146/08 and Dk/Kr/A174/09) in chickens, using methods described previously (Li et al., 2003). Of the four H5N2 viruses, none were detected in the oropharyngeal and cloacal swabs through 9 days post-infection (p.i.), whereas two H9N2 viruses were replicated in the intestines to relatively high titres (Table 1). None of the H5N2 and H9N2 subtype viruses tested induced signs of disease in inoculated chickens. In addition, we compared the viral re-isolation of the two H5N2 AIV (Dk/Kr/A14/08 and wb/Kr/L60-2/08) in mice. The Dk/Kr/A14/08 virus was not detectable in murine

![Fig. 1. Phylogenetic diagram of the H5 HA.](image-url)
lungs through 9 days p.i. (data not shown), and the wb/Kr/L60-2/08 virus displayed poor replication (1.1 logEID₅₀/0.1 ml titre only at 1 day p.i.).

In the present study, we isolated just four H5N2 viruses during the collection period of 9 months, but the genotypes of these viruses did not coincide with one another. Phylogenetic analysis demonstrated that one H5N2 virus isolated from wild birds was introduced directly from recently isolated LPAI H5 viruses from southern China, and two H5N2 viruses of domestic ducks had high homology to most LPAI H5 viruses recently circulating in southern China, with the exception of their PB2, M and NS genes. Another H5N2 virus isolated from wild birds (wb/Kr/L60-2/08) clustered with the duck/Korea/GJ54/2004 (H5N2) virus (additional virus

**Table 1. Replication of selected H5N2 and H9N2 isolates in 4-week-old chickens**

Values shown are the number of infected animals per number inoculated. The values shown in parentheses are virus titre (logEID₅₀/0.1 ml). Virus titre is the average of positive samples taken on days 1, 3, 5, 7 and 9 after inoculation. The dose of inoculation for chickens was 6.5 logEID₅₀/0.1 ml. OP, Oropharyngeal; CL, cloacal.

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>Isolates</th>
<th>Swab</th>
<th>Virus replication (days p.i.)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>H5N2</td>
<td>Dk/Kr/A14/08</td>
<td>OP</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CL</td>
<td>0/8</td>
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<tr>
<td></td>
<td>Dk/Kr/A93/08</td>
<td>OP</td>
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<td>CL</td>
<td>0/8</td>
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<td></td>
<td>wb/Kr/L60-2/08</td>
<td>OP</td>
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<td>wb/Kr/A81/09</td>
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<td>CL</td>
<td>0/8</td>
</tr>
<tr>
<td>H9N2</td>
<td>Ck/Kr/A146/09</td>
<td>OP</td>
<td>5/8(1.1)</td>
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<tr>
<td></td>
<td></td>
<td>CL</td>
<td>0/8</td>
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<td>Dk/Kr/A174/09</td>
<td>OP</td>
<td>7/8(1.1)</td>
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<td>CL</td>
<td>0/8</td>
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*Undetectable virus titre (<0.5 logEID₅₀/0.1 ml).
characterized in this study, GenBank accession nos GU351859–GU351863). These results suggested that LPAI H5 viruses, predominant in wild birds in Korea, were directly transmitted to domestic ducks or had generated the reassortant viruses with different subtypes of AIV in poultry or wild birds.

The H9N2 virus had been enzootic, evolving to the various genotypes in chicken farms and LBMs in Korea, since the first outbreak of H9N2 LPAI occurred in 1996 (Kim et al., 2006; Lee et al., 2000, 2007). Interestingly, we identified novel H9N2 viruses from LBMs and duck farms. Phylogenetic analysis confirmed that novel H9N2 viruses were generated by combining the HA and NP segments from the Korean H9N2 lineage and the other six genes from the H5N2 viruses (Dk/Kr/A14/08 and Dk/Kr/A93/08 viruses) isolated from domestic ducks. The reassortment between the H5N2 virus of domestic ducks and the endemic H9N2 virus of chickens might be occurring in domestic ducks at the LBMs, where there is an ideal environment for reassortment and interspecies transfer of the viruses (Liu et al., 2003).

The A/chicken/Ibaraki/1/05 (H5N2) virus isolated from chickens in Japan replicated efficiently in the respiratory tract without clinical signs of infection (Okamatsu et al., 2007). The Korean H5N2 LPAI viruses were isolated from ducks and wild birds, which showed no significant clinical signs of disease, and did not replicate in experimentally inoculated chickens. These results suggested that LPAI H5 viruses isolated in Korea did not prevail in poultry since they had not adapted to surviving in animals such as chickens. However, the novel H9N2 viruses rapidly replicated in chickens without pre-adaptation. Phylogenetic analysis of these viruses showed that the novel H9N2 viruses might be distinguished by two genes, the HA and NP genes, which were related to their pathogenicity and viral replication ability (Murphy et al., 1989; Wagner et al., 2002; Wasilenko et al., 2008). Therefore, further study is necessary to elucidate protein function using reverse genetics.

Although the Korean H5N2 viruses had not directly evolved into highly pathogenic viruses and they were not genetically related to the H5N1 HPAI strains isolated from previous outbreaks in Korea (Kim et al., 2010a; Lee et al., 2005b, 2008), the continuous introduction of LPAI H5 viruses and the subsequent generation of novel reassortant viruses have contributed to the increased viral genetic diversity in Korea, which has the potential to change host range and to evolve into viruses with increased pathogenicity.

Therefore, continuous surveillance and genetic analysis to characterize LPAI viruses, including the H5 and H9 subtype AIV should be strongly encouraged. To ensure successful HPAI outbreak control, it is important to rapidly detect LPAI viruses as well as HPAI viruses that may be introduced through wild birds, resulting in an increase in virulence of endemic LPAI virus or the rise of novel reassortant viruses among poultry.

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References


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