



## Continuing evolution of H9 influenza viruses in Korean poultry

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### Abstract

We analyzed the evolution of H9 influenza viruses isolated from Korean chicken farms from 2002 to 2004. Korean H9 viruses formed two antigenically distinct groups: those isolated from 1996 to mid-2003, and those isolated from late 2003 through 2004. Most of the 2004 isolates showed greater cross-reactivity with the second group than with the first group. Phylogenetic analysis of the 12 viruses studied revealed three genotypes of H9N2 viruses and showed that reassortment had occurred. One isolate, Ck/Kor/164/04, belonged to the H9N8 subtype. Its HA and PB1 genes were similar to those of the H9N2 viruses, but its other genes were closely related to H3N8 viruses. This report is the first (to our knowledge) of H9N8 infection in this host. The pathogenicity of the early isolates altered due to antigenic drift and reassortment, leading to H9 avian influenza viruses in Korea that potentially can expand their host range to mammals.

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H9N2 influenza viruses have become panzootic in Eurasia during the past decade and have been isolated from terrestrial poultry worldwide (Cameron et al., 2000; Guo et al., 2000; Li et al., 2003; Choi et al., 2004). Detailed antigenic and molecular analyses have identified three groups of H9N2 influenza virus in avian species in southeastern China: the G1 group, represented by A/quail/Hong Kong/G1/97; the Y280 group, represented by A/chicken/Hong Kong/Y280/97; and the Korea group, represented by A/duck/Hong Kong/Y439/97 (Guan et al., 1999). The two H9N2 viruses isolated from humans in Hong Kong belong to the G1 group, whereas at least one human isolate from China and the two isolates from pigs in Hong Kong belong to the Y280 group (Guo et al., 1999, 2000; Peiris et al., 1999). G1- and Y280 group H9N2 viruses are still widespread in domestic poultry in

China, and their reassortment events and genotypes are relatively well characterized (Li et al., 2003; Choi et al., 2004). However, the genetics and pathogenicity of the Korea group, like H9N2 viruses, are not characterized as well.

The first avian influenza outbreak on Korean chicken farms was caused by A/chicken/Kor/96006/96 (H9N2), a virus closely related genetically to the A/duck/Hong Kong/Y439/97 (Korea group) virus later isolated from aquatic birds (Lee et al., 2000). We recently reported that the HA genes of nine H9N2 viruses isolated from live-bird markets in Korea in 2003 were closely related only to Korea group-like H9N2 viruses and differed from the HAs of H9N2 viruses isolated in southeastern China (Choi et al., 2005). In contrast, the internal genes of these viruses represented at least 4 genotypes, and some were closely related to those of viruses isolated from aquatic birds in southeastern China. However, the viruses described in that report (5) were isolated solely from live-bird markets (not poultry farms) during a brief period (2003 only), and the infected birds showed no clinical signs of illness.

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In 2004, avian influenza viruses seriously affected several chicken farms in Korea, causing severe clinical signs and increasing mortality in the chickens. We therefore investigated whether avian influenza viruses isolated in Korea during 2002–2004 had undergone genetic and pathogenic changes. This report describes the expanded gene pool of Korean H9 influenza viruses circulating in chicken farms and demonstrates their continued evolution. Furthermore, we document the reassortment of the 2004 H9 viruses with wild avian influenza viruses, which altered the pathogenicity of the H9 isolates in experimentally infected chickens and mice.

## Results

### *Virus isolation and selection*

Most of the chicken viruses studied were isolated by NVRQS in embryonated eggs from tracheal and cecal tonsil swab specimens submitted during the period January 2002 through December 2004. The clinical signs of illness in chickens observed at affected farms included depression, coughing, reduced feed intake, diarrhea, decreased egg production, and variable mortality. H9 influenza virus was isolated from 20 (2.1%) of 958 specimens tested (452 in 2002, 327 in 2003, and 179 in 2004). For genetic analysis, we selected three H9 viruses isolated in 2002, three isolated in 2003, and six isolated in 2004, on the basis of the clinical signs and mortality rates apparent at the time of specimen collection (Table 1). We genetically characterized all 12 isolates and tested their pathogenic potential in chickens and mice.

### *Antigenic analysis*

Three genetically distinct lineages of H9 virus have recently been reported in Asia (Guan et al., 2000; Choi et al., 2004; Li et al., 2003). To investigate the antigenic properties of the H9 viruses of Korea, we tested them by HI assay for cross-reactivity with polyclonal antisera to H9N2 viruses isolated in Asia. All of the H9 viruses isolated during 2002–2004 in Korea reacted

weakly with antisera to Qa/HK/G1/97 (G1 group) and Ck/HK/Y280/97 (Y280 group) but reacted relatively strongly with antisera to Ck/Kor/MS/96 (Korea group) and its homologs. Therefore, most of the H9N2 viruses circulating in chicken farms in Korea during 2002–2004 were antigenically distinct from those circulating in southeastern China. Interestingly, the H9 viruses of Korea formed two antigenically distinct groups: the H9N2 viruses isolated from 1996 to mid-2003 were distinct from those isolated in late 2003 and 2004. Most of the 2004 isolates reacted less strongly (HI titers, 80 to 320) with vaccinated chicken antisera of the first group than with vaccinated chicken antisera of the second group (HI titers, 320 to 1280) (Table 2). Therefore, the H9 viruses circulating in Korea appear to have undergone significant antigenic drift during late 2003 and 2004.

To determine the NA subtype of H9 isolates, we performed neuraminidase inhibition (NI) tests. Nineteen of the 20 isolates were of the H9N2 subtype, but the A/Ck/Kor/164/04 isolate was subtyped as an H9N8 virus. Therefore, at least two subtypes of H9 viruses are circulating in poultry farms in South Korea, although the H9N2 subtype markedly predominates (19 of 20 isolates).

### *Prevalence of H9N8 viruses (virologic and serologic evidence)*

To confirm that the H9N8 and H9N2 viruses are co-circulating, we performed NI tests of 30 additional H9 viruses, which were isolated at Chungbuk National University (Korea). The tested viruses were obtained from Korean chicken farms and slaughter houses during 2003–2004. Only 1 of the 30 viruses was identified as being of the N8 subtype. These data suggest that H9N8 virus is co-circulating with H9N2 viruses but has a very low prevalence.

### *Genotypes*

#### *H9N2 viruses*

The results of antigenic analysis suggested that most of the H9N2 viruses isolated from Korean chicken farms were similar to Ck/Kor/96006/96-like viruses of the Korea group rather than those of the H9N2 viruses isolated in southeastern China (Choi

Table 1  
Avian H9 influenza A viruses tested

Year	Isolate	Subtype	Source	Chicken type (breed)	Age (weeks)	Clinical signs	Mortality (%)	Genotype
2002	Ck/Kor/249/02	H9N2	Trachea	LL (Brown)	30	Reduced egg production (86%→39%)	2	C
2002	Ck/Kor/252/02	H9N2	Trachea	NB (Brown)	10	None	4	C
2002	Ck/Kor/311/02	H9N2	Trachea	NB (Brown)	32	None	0	C
2003	Ck/Kor/150/03	H9N2	Cecal tonsil	LL (Brown)	56	Reduced egg production	1	C
2003	Ck/Kor/188/03	H9N2	Feces	LL (Brown)	40	Reduced egg production	0	A
2003	Ck/Kor/236/03	H9N2	Cecal tonsil	LL (Brown)	40	Reduced egg production	0	A
2004	Ck/Kor/112/04	H9N2	Cecal tonsil	NB (Brown)	44	Reduced egg production, diarrhea, decreased feed intake	15	A
2004	Ck/Kor/116/04	H9N2	Trachea	NB (Brown)	12	Diarrhea, weight loss	30	A
2004	Ck/Kor/136/04	H9N2	Trachea	LB (Brown)	40	Reduced egg production, depression diarrhea, reduced feed intake	18	C
2004	Ck/Kor/164/04	H9N8	Cecal Tonsil	NB (Brown)	10	Reduced feed intake	20	H9N8
2004	Ck/Kor/Q19/04	H9N2	Cecal Tonsil	LL (Brown)	30	Reduced egg production, respiratory signs, reduced feed intake	16	A
2004	Ck/Kor/Q30/04	H9N2	Cecal tonsil	LL (White)	30	Acute respiratory signs	65	B

LB, Leghorn broiler; LL, Leghorn layer; NB, Korea Native, Korea native broiler (*Gallus gallus domesticus*).

Table 2  
Antigenic analysis of avian influenza viruses isolated from Korean chicken farms during 2002–2004

Virus	Qa/HK/G1/97	Dk/HK/Y280/97	Ck/Kor/MS/96	Ck/Kor/311/02	Ck/Kor/150/03	Ck/Kor/164/04	Ck/Kor/Q19/04
Ck/Kor/MS/96	40 <sup>a</sup>	40	<b>640</b>	<b>640</b>	<b>640</b>	160	320
Ck/Kor/311/02	40	20	320	<b>640</b>	<b>640</b>	320	160
Ck/Kor/236/03	40	40	160	<b>640</b>	320	320	320
Ck/Kor/250/02	40	40	320	320	<b>640</b>	160	160
Ck/Kor/150/03	40	40	320	<b>640</b>	<b>640</b>	80	80
Ck/Kor/188/03	40	40	160	<b>640</b>	320	160	80
Ck/Kor/112/04	20	20	80	80	80	320	<b>640</b>
Ck/Kor/116/04	40	20	80	80	160	320	<b>640</b>
Ck/Kor/136/04	20	20	40	80	80	320	<b>640</b>
Ck/Kor/Q19/04	20	20	80	160	80	<b>640</b>	<b>1280</b>
Ck/Kor/Q30/04	20	20	80	160	80	<b>640</b>	<b>640</b>
Ck/Kor/164/04	40	20	80	160	80	<b>640</b>	320
Qa/HK/G1/97	<b>1280</b>	160	40	40	40	40	20
Dk/HK/Y280/97	160	<b>640</b>	20	20	40	20	20

<sup>a</sup> The HI titer of each serum that reacted with the virus listed at the top of the column. Titers in bold are the highest HI titers.

et al., 2005). To examine the genetic diversity of these viruses, we compared full sequences of the eight gene segments of each virus, including two H9N2 viruses isolated from wild birds in 2004 (aquatic bird/Kor/W02/04 and aquatic bird/Kor/W03/04).

Phylogenetic analysis based on the HA1 region of the HA gene and on the NA gene showed that, as predicted by antigenic comparison, all 12 of the H9 viruses studied belonged to the Ck/Kor/96006/96-like lineage (Fig. 1a and b). Although H9N2 viruses from aquatic birds were detected in the same areas, they were genetically, completely different from H9N2 isolates from chicken farms. We found no evidence of Qa/HK/G1/97-like or Dk/Hk/Y280/97-like genes circulating in southeastern China in Korea.

Phylogenetic analysis of the six internal genes revealed three genotypes of virus and showed that reassortment had occurred. The M and NS genes of the H9N2 viruses isolated in this study formed at least two groups: the Ck/Kor/96006/96-like lineage and that of an unknown aquatic avian influenza virus (Fig. 2). However, the other internal genes were clustered with the Ck/Kor/96006/96-like or Ck/Kor/99029/99-like lineage. No viruses tested in this study contained genes of the Korean H5N1 virus lineages (Ck/Kor/ES/03 and Dk/Kor/ESD1/03) that caused outbreaks in 2003. Our phylogenetic analysis identified at least three different genotypes (designated A, B, and C) among the H9N2 viruses isolated from chicken farms (Fig. 2 and Table 1). These findings suggest that reassortment had occurred between H9N2 viruses circulating in poultry farms and unknown avian influenza viruses but not between H9N2 viruses and the highly pathogenic avian H5N1 viruses that caused outbreaks in Korea in 2003.

#### H9N8 virus

Although only one H9N8 isolate in this study was associated with clinical signs of disease, H9N8 is a completely new subtype in domestic chickens. Antigenic analysis and phylogenetic analysis of the HA1 region showed the Ck/Kor/164/04 isolate to belong to the Ck/Korea/96006/96-like lineage (Fig. 1a). To investigate the genetic origin of the Ck/Korea/164/04 virus, we compared the sequences of its internal genes with

those of H3N8 avian influenza viruses isolated from wild aquatic birds in Korea in 2004 and with GenBank data. Phylogenetic analysis revealed the NA gene of Ck/Korea/164/04 to be closely related to those of H3N8 influenza viruses (aquatic bird/Kor/W01/04 and aquatic bird/Kor/W04/04) isolated from aquatic birds in 2004 in Korea. Furthermore, all of the internal genes (except PB1) of Ck/Kor/164/04 were clustered with those of aquatic bird/Kor/W04/04. The PB1 gene of Ck/Kor/164/04 was in the same lineage as that of the Ck/Kor/99029/99 virus (Fig. 2). These results suggest that the Ck/Kor/164/04 virus may be the product of reassortment of an H9N2 subtype virus of the Korean lineage with an H3N8 subtype virus circulating in wild aquatic birds, such as aquatic bird/Kor/W04/04.

#### Characterization of the HA gene

Matrosovich et al. (2001) recently described avian H9N2 viruses whose receptor specificity was like that of human influenza A viruses. To determine what proportion of the viruses isolated during 2002–2004 in Korea had this trait, we compared their HA amino acid sequences (deduced from the nucleotide sequences of the genes) with those of representative strains. All 12 of the H9 viruses had glutamine at position 226 of the HA molecule, as did the earlier Korean group isolates (e.g., A/Ck/Kor/96006/96 and A/Ck/Kor/99029/99), and there was 93.2% to 99.7% nucleotide identity among the HA genes of these 12 viruses. Gln226 is in the receptor-binding site of the HA1 region and confers high binding affinity to 2,3-linked sialic acid (SA) moieties (typical of avians and horses) but low binding affinity to the 2,6-linked SA moieties found in most mammals (Matrosovich et al., 2001). Receptor affinity was confirmed by HA tests with horse red blood cells (which exclusively have 2,3-linked SA) and guinea pig red blood cells (which exclusively have 2,6-linked SA). The HA titers of the analyzed viruses were lower with guinea pig red blood cells than with horse red blood cells by a factor of 4 or more (data not shown).

The first H9N2 virus isolated in Korea (Ck/Kor/9006/96) has the HA-cleavage site peptide sequence Ala-Ser-Tyr-Arg

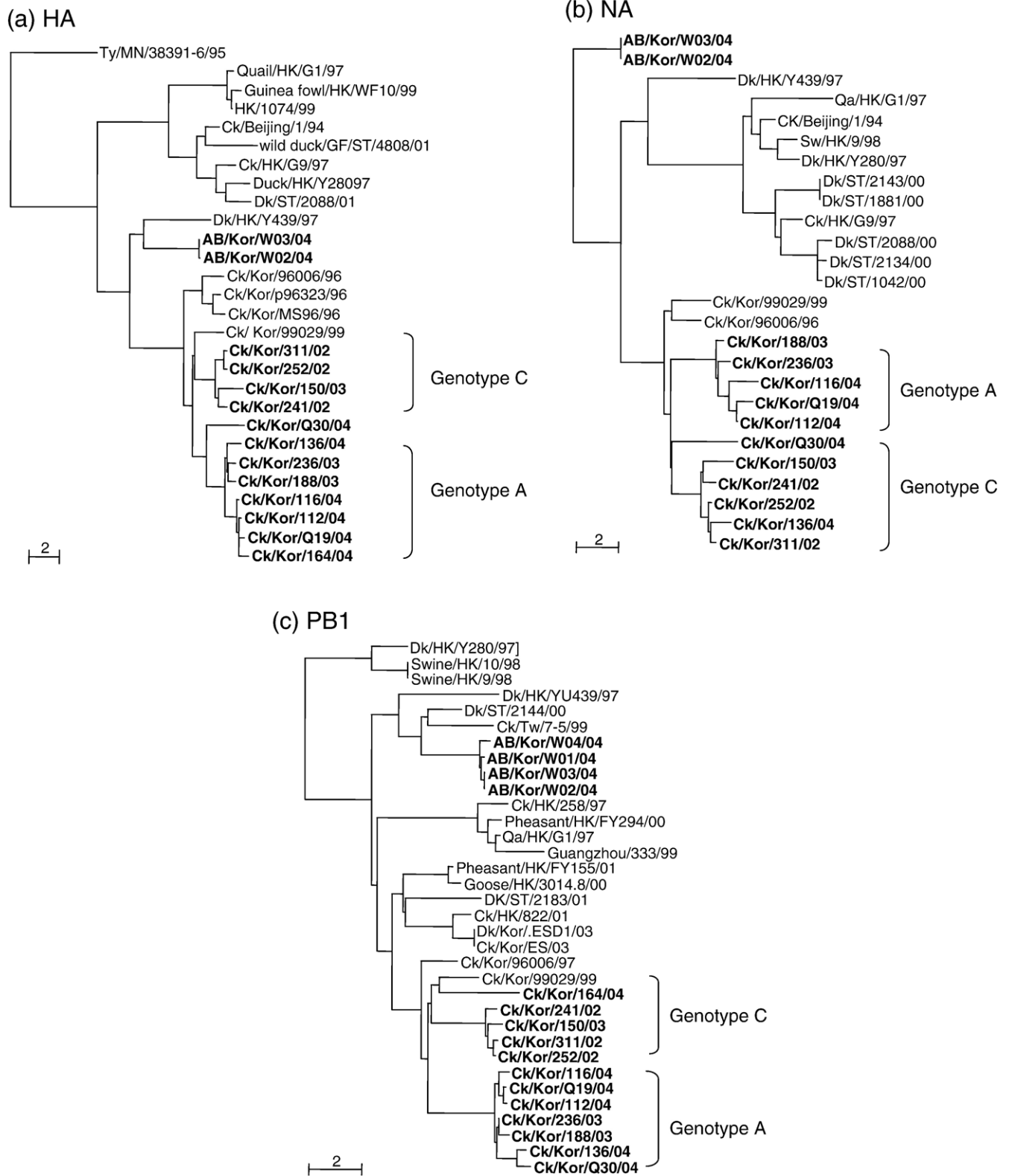


Fig. 1. Phylogenetic analysis of the H9 influenza viruses isolated on Korean chicken farms during 2002–2004. (a) HA1 region of the *H9* gene; (b) *NA* gene. As predicted by antigenic comparison, all isolates belong to the Ck/Kor/96006/96-like lineage. However, the H9 viruses recently isolated from chickens in Korea form two antigenic groups. (c) the *PB1* genes of all H9 isolates, including Ck/Kor/164/04 (H9N8), were clustered in the Ck/Kor/96006/96-like lineage and formed two distinguishable subgroups. Ck, chicken; Dk, duck; Qa, quail; AB, aquatic bird; Ty, turkey; HK, Hong Kong; Kor, Korea. Bold type indicates the viruses tested in the current study.

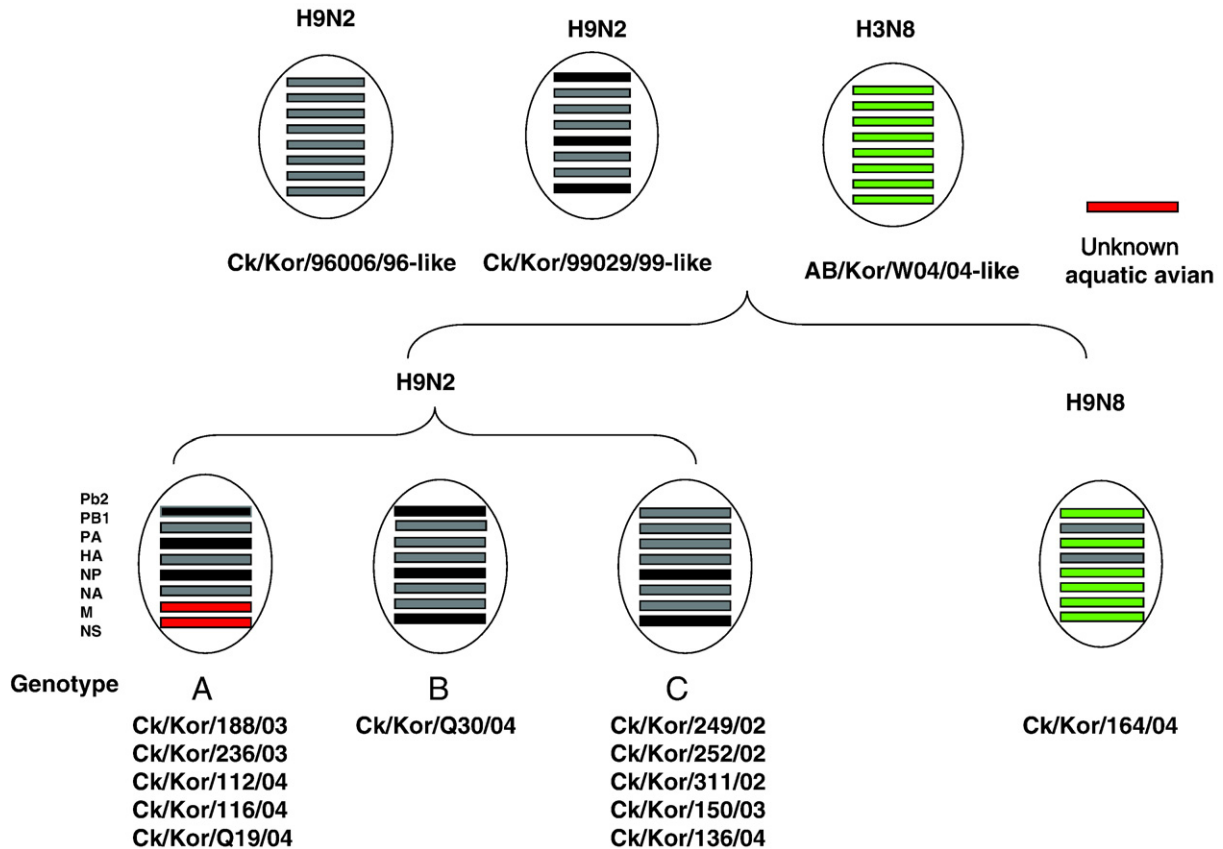


Fig. 2. To determine the genetic diversity of the 12 studied viruses, we compared partial sequences of their 8 gene segments. We identified 3 genotypes (designated A, B, and C) of H9N2 viruses and 1 genotype of H9N8 virus.

(ASYR). In all 12 of our H9 isolates, this HA peptide motif differed from ASYR by at least one amino acid (data not shown). The H9 viruses isolated during 2002 and early 2003 (Ck/Kor/249/02, Ck/Kor/252/02, Ck/Kor/311/02, and Ck/Kor/150/03) had threonine in the place of alanine and had glycine in the tyrosine position (TSGR), and one 2003 isolate (Ck/Kor/236/03) had valine in the alanine position (VSGR). However, the H9 viruses isolated in late 2003 and 2004 (Ck/Kor/188/03, Ck/Kor/136/04, Ck/Kor/116/04, Ck/Kor/112/04, Ck/Kor/Q19/04, and Ck/Kor/Q30/04), including the H9N8 virus (Ck/Kor/164/04), had glycine in the tyrosine position (ASGR). None of the 2002–2004 isolates had the series of basic amino acids in the HA-cleavage site peptides that are characteristic of highly pathogenic avian influenza viruses (Xu et al., 1999), and there were no changes in potential glycosylation sites (data not shown).

#### Replication and transmission of the viruses in animals

To determine whether the genetic heterogeneity we observed is accompanied by biologic heterogeneity, we compared the replication of representative avian influenza viruses isolated in 2004 in chickens and mice. The H9N2 viruses we tested were Ck/Kor/116/04 (genotype A), Ck/Kor/Q30/04 (genotype B), and Ck/Kor/136/04 (genotype C). We included the viruses Ck/Kor/MS/96 (a previous Korean-lineage H9N2 virus), Ck/Kor/164/04 (H9N8), aquatic bird/Kor/W02/04 (H9N2), and aquatic bird/Kor/W04/04 (H3N8) for comparison.

#### Replication in mice

Most of the Korean genotype A viruses (4 out of 5) isolated in 2004 replicated in mice without pre-adaptation, except the Ck/Kor/Q19/04 (H9N2) virus (data not shown). The Ck/Kor/116/04 (H9N2) and Ck/Kor/164/04 (H9N8) isolates replicated well in mice without prior adaptation. Virus titers in the lungs ranged from 2.3 to 3.7 log<sub>10</sub> EID<sub>50</sub>/0.1 ml on day 3 p.i. and disappeared on day 7 p.i. Although the inoculated mice showed slight loss of body weight, neither virus caused death during the 14-day experiment. Mice inoculated with the other H9 isolates (all isolates of genotype B and C) showed no clinical signs of disease, and virus was not detected in any organs, including lungs, during the experiment (Table 3 and data not shown). A wild H3N8 virus, AB/Kor/W04/04, replicated in mice without prior adaptation but disease signs were not observed.

#### Replication and transmission in chickens

We inoculated 5-week-old SPF chickens orally and intranasally with H9N2 viruses of the A, B, and C genotypes and with the 2 viruses of other subtypes that were isolated (Table 3). Most of the viruses replicated to high titers by day 3 p.i., and titers were consistently highest in the tracheal swabs, with the exception of those for AB/Kor/W02/04 (H9N2) and AB/Kor/W04/04 (H3N8). AB/Kor/W04/04 (H3N8) replicated to low titers in the trachea ( $\leq 10^1$  EID<sub>50</sub>/ml), but the AB/Kor/W02/04

Table 3  
Replication of the Korean influenza viruses in 4-week-old chickens and 5-week-old mice<sup>a</sup>

Virus	Chickens						Mice	
	Trachea	Lung	Cecal tonsil	Kidney	Spleen	Disease signs	Lung	Disease signs
<i>H9N2</i>								
Ck/Kor/MS/96	1/8 <sup>b</sup> (4.3/0.5) <sup>c</sup>	1/8 (2.7/0.5)	2/8 (4.7/2.5)	1/8 (0/0.3)	0/8	None	0/8	None
Ck/Kor/150/03	4/8 (3.7/1.7)	3/8 (3.3/1.0)	4/8 (4.3/2.7)	0/8	0/8	None	0/8	None
Ck/Kor/136/04	5/8 (4.7/4.2)	5/8 (3.7/1.0)	8/8 (6.0/6.0)	3/8 (1.3/0.7)	7/8 (1.7/0.7)	None	0/8	None
Ck/Kor/116/04	6/8 (5.0/4.5)	5/8 (4.5/2.3)	5/8 (5.5/5.7)	3/8 (2.3/1.7)	4/8 (2.0/0.5)	Depression	8/8	Weight loss
Ck/Kor/Q30/04	8/8 (4.7/3.5)	7/8 (3.3/2.0)	8/8 (5.7/5.7)	3/8 (1.7/0.5)	7/8 (1.3/0.7)	Depression	0/8	None
AB/Kor/W02/04	0/8	0/8	1/8 (1.3/0.7)	0/8	0/8	None	0/8	None
Control chickens	0/8	0/8	0/8	0/8	0/8	None	0/8	None
<i>H9N8</i>								
Ck/Kor/164/04	4/4 (4.3/3.0)	3/4 (4.7/2.2)	3/4 (5.3/1.8)	2/4 (1.5/1.0)	2/4 (1.3/0.7)	Depression	7/8	Weight loss
<i>H3N8</i>								
AB/Kor/W04/04	2/4 (1.7/0.7)	0/4	2/4 (1.7/0.7)	0/4	0/4	None	6/8	None

Ck, chicken; AB, aquatic bird; Kor, Korea.

<sup>a</sup> The dose of inoculum for chickens and mice was  $10^{5.5}$  EID<sub>50</sub>/0.1 ml.

<sup>b</sup> No. of animals infected/no. inoculated by intranasal and tracheal route.

<sup>c</sup> (in parentheses) The mean virus titer ( $\log_{10}$  EID/0.1 ml) in samples taken on day 3/5 post-inoculation.

(H9N2) virus was not detected in any organ (data not shown). Chickens inoculated with Ck/Kor/MS/96 virus had very low tracheal virus titers on day 5 p.i. ( $10^{0.5}$  EID<sub>50</sub>/ml). However, titers of Ck/Kor/116/04 (genotype A), Ck/Kor/Q30/04 (genotype B), and Ck/Kor/136/04 (genotype C) in the trachea were high on day 5 p.i. ( $\leq 10^{4.5}$ ,  $\leq 10^{3.5}$ , and  $\leq 10^{4.2}$  EID<sub>50</sub>/ml, respectively) (Table 3). On day 7 p.i., tracheal and cloacal viral titers were low or undetectable ( $\leq 10^1$  EID<sub>50</sub>/ml), with the exception of those for Ck/Kor/Q30/04 (H9N2) ( $\leq 10^{2.5}$  EID<sub>50</sub>/ml; data not shown).

To investigate the distribution of virus in infected chickens, we collected samples of trachea, lung, cecal tonsil, kidney, and spleen on day 5 p.i. (Table 3). Most of the inoculated viruses were detected in trachea and cecal tonsil, but those of the AB/Kor/W02/04 isolate was not. Interestingly, most of the 2004 chicken isolates, including Ck/Kor/164/04 (H9N8), were recovered from all organs at titers higher than those of Ck/Kor/MS/96. Ck/Kor/MS/96 was found at a low viral titer ( $10^{0.5}$  EID<sub>50</sub>/ml) in the kidneys from 1 of the 8 inoculated chickens but was not detected in the spleen. These results suggest that the viruses isolated in 2004 from chicken farms in Korea had gained the ability to replicate throughout the internal organs. Disease signs were apparent only in chickens inoculated with Ck/Kor/116/04 and Ck/Kor/Q30/04 isolates.

The H9N8 virus, Ck/Kor/164/04, in domestic chicken is the completely new subtype to our knowledge. Therefore, we investigated the ability of transmission of H9N2, H9N8, and H3N8 subtypes in chickens. Although most of the H9N2 viruses isolated from chickens were transmitted to contact chickens after 3 days, AB/Kor/W02/04 (H9N2) and AB/Kor/W04/04 (H3N8) isolates did not (data not shown). All birds contacted with Ck/Kor/164/04 (H9N8) infected chickens were positive for the virus in their trachea and cloacal swabs on 4 days of post contact. In the contact group of AB/Kor/W04/04 (H3N8) infected chickens, only two out of eight chickens were positive for the virus at 4 days of post contact. None of the

contact group of AB/Kor/W02/04 (H9N2) infected chicken was positive for the virus until the end of the experiment (12 days). No chicken died during the course of the experiment. These results suggest that the chicken H9N8 isolate is relatively well adapted to chickens but isolates from wild aquatic birds are not.

#### *Pathogenicity of H9N2 influenza viruses in mature chickens and breeders*

The Ck/Kor/116/04 and Ck/Kor/Q30/04 isolates caused severe signs of clinical disease and mortality rates (>30%) at both affected farms during outbreaks. However, we observed no deaths among 5-week-old chickens during our experiments. We hypothesized that this disparity reflected the different ages of the chickens and breeders. To test our hypothesis, we inoculated the viruses into 35-week-old commercial Hy-Line white and brown chickens that were seronegative for avian influenza viruses. Hy-Line white chickens inoculated with Ck/Kor/116/04 and Ck/Kor/Q30/04 viruses showed severe clinical signs (depression, diarrhea, and fecal edema), and one chicken inoculated with Ck/Kor/Q30/04 was dead on day 5 p.i. We randomly selected two breeder chickens of each type for titration of virus in trachea, lung, cecal tonsil, kidney, and spleen on day 5 p.i. (Table 4). Although the titers differed, all tracheas, lungs, and cecal tonsils were positive for virus in both breeds. Interestingly, Ck/Kor/150/03 virus (genotype C) was not recovered from kidney or spleen, but the other viruses were recovered from all organs tested (Table 4, Fig. 3). In both breeds, tissue titers of the Ck/Kor/Q30/04 isolate were higher than those of other isolates. Furthermore, 7 (43.7%), 3 (18.7%), and 2 (12.5%) of the 16 Hy-Line white chickens inoculated with Ck/Kor/Q30/04, Ck/Kor/Q19/04, and Ck/Kor/116/04, respectively, were dead by day 14 p.i. (Table 5). Three and one of the Hy-Line brown chickens inoculated with Ck/Kor/Q30/04 and Ck/Kor/Q19/04 died by day 6 p.i., respectively. Despite the small number of chickens that we were able to test, statistical

Table 4  
Replication in 35-week-old chickens<sup>a</sup> on day 5 after inoculation of 2004 Korean isolates

Virus	Trachea	Lung	Cecal tonsil	Kidney	Spleen	Brain	Disease signs
<i>Hy-Line white (W-36)</i>							
Ck/Kor/150/03	2/2 (1.5) <sup>b</sup>	2/2 (2.5)	2/2 (5.5)	0/2	0/2	0/2	Depression
Ck/Kor/116/04	2/2 (3.5)	2/2 (2.5)	2/2 (5.5)	1/2 (2.0)	2/2 (<1)	0/2	Depression, diarrhea, fecal edema
Ck/Kor/Q30/04	2/2 (3.5)	2/2 (3.6)	2/2 (6.0)	1/2 (4.5)	1/2 (2.3)	0/2	Cyanoses, diarrhea, fecal edema, 1 death among 8 birds
Ck/Kor/Q19/04	2/2 (2.8)	2/2 (3.6)	2/2 (4.5)	2/2 (2.5)	1/2 (<1)	0/2	Depression, diarrhea
Control (PBS)	0/2	0/2	0/2	0/2	0/2	0/2	None
<i>Hy-Line brown plus</i>							
Ck/Kor/150/03	1/2 (1.5)	2/2 (<1)	2/2 (3.5)	0/2	0/2	0/2	None
Ck/Kor/116/04	2/2 (2.5)	2/2 (1.5)	2/2 (3.8)	1/2 (<1)	2/2 (<1)	0/2	Depression
Ck/Kor/Q30/04	2/2 (3.5)	2/2 (2.5)	2/2 (4.5)	1/2 (1.8)	1/2 (1.3)	0/2	Depression, diarrhea
Ck/Kor/Q19/04	2/2 (2.5)	2/2 (1.5)	2/2 (3.5)	2/2 (1.5)	1/2 (<1)	0/2	Depression, diarrhea
Control (PBS)	0/2	0/2	0/2	0/2	0/2	0/2	None

Ck, chicken; AB, aquatic bird; Kor, Korea.

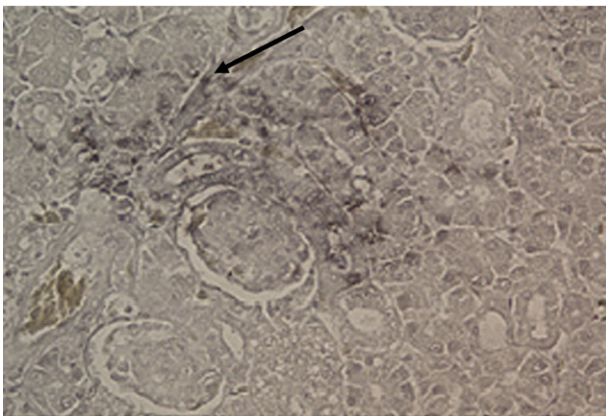
<sup>a</sup> The dose of inoculum was  $10^{5.5}$  EID<sub>50</sub>/0.1 ml.

<sup>b</sup> No. of animals infected/no. inoculated by intranasal and tracheal route and (in parentheses) the mean virus titer ( $\log_{10}$  EID/0.1 ml) in samples taken on day 5 post-inoculation.

analysis suggested that age could be a factor in susceptibility ( $P < 0.05$ ,  $\chi^2$ -test) to some influenza viruses isolated in 2004 from Korean chicken farms. Although a high number of dead

chickens was observed in Hy-Line white chickens compared to brown chickens, significant differences in susceptibility were not observed between breeders ( $P < 0.25$ ,  $\chi^2$ -test).

(a)



(b)

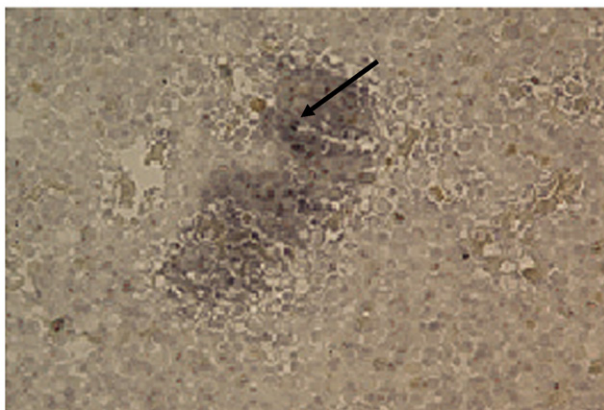


Fig. 3. Sections of kidney (a) and spleen (b) from a chicken infected with Ck/Kor/Q30/04 virus were stained with monoclonal antisera to NP. Kidney and spleen tissues were collected 5 days after inoculation with  $10^{5.5}$  EID<sub>50</sub> of infective virus. Dark staining indicates replication of Ck/Kor/Q30/04 virus. Magnification,  $\times 400$ .

#### Chicken intravenous pathogenicity test

Although all highly virulent avian influenza strains isolated to date have been of either the H5 or H7 subtype, we performed pathogenicity testing of Korean isolates in accordance with instructions in the OIE manual. By definition, avian influenza viruses that kill 75% or more of 8 chickens inoculated intravenously within 10 days of inoculation are classified as highly pathogenic (Swayne and Halvorson, 2003). No virus killed the inoculated chickens within 10 days in this study. These data suggest that none of the 12 H9 viruses isolated in Korea during 2002–2004 that we tested are highly pathogenic strains of avian influenza virus.

#### Discussion

Here we have shown that H9 influenza A viruses isolated during the period 2002 through 2004 in Korea have evolved continuously by antigenic drift and have undergone reassortment with aquatic avian influenza A viruses. The first avian influenza outbreak in Korea was reported in 1996. At that time, five H9N2 viruses were isolated from several broiler breeder flocks in the country. Most of the affected birds showed signs typical of influenza, such as a pronounced drop in egg production and occurrence of mortality. However, the isolates produced no detectable signs of disease in experimentally inoculated chickens (Mo et al., 1997). Similarly, as we previously reported, chickens, quails, and mice inoculated with H9N2 viruses isolated from Korean live-bird markets in 2003 showed no clinically significant signs of disease (Choi et al., 2005). In late 2003 to 2004, several Korean chicken farms suffered serious economic damage due to avian influenza viruses. Most of the affected birds showed clinical signs typical of influenza infection, such as a pronounced drop in egg

Table 5  
Mortality of 35-week-old chickens inoculated with H9N2 viruses

Virus	Hy-Line white (W-36)				Hy-Line brown plus			
	5 d.p.i.	6 d.p.i.	7 d.p.i.	Total (14 d.p.i.) <sup>a</sup>	5 d.p.i.	6 d.p.i.	7 d.p.i.	Total (14 d.p.i.) <sup>a</sup>
Ck/Kor/150/03	1/16	0/13	0/13	1 (6.25%)	0/16	0/14	0/14	0 (0%)
Ck/Kor/116/04	0/16	2/14	0/12	2 (12.5%)	0/16	1/14	0/13	1 (6.25%)
Ck/Kor/Q19/04	0/16	2/14	1/12	3 (18.7%)	0/16	1/14	0/13	1 (6.25%)
Ck/Kor/Q30/04	2/16	4/12	1/8	7 (43.7%)	1/16	2/13	0/11	1 (18.7%)

d.p.i., days post-inoculation.

<sup>a</sup> No. of dead animals/no. of inoculated animals by intranasal and tracheal route; mortality rate in parentheses.

production, diarrhea, and moderate to high mortality rates, compared with those of 2002. Antigenic and genetic analyses show that two antigenically distinguishable H9 viruses are circulating in Korea. Whereas the H9N2 viruses isolated from 1996 to mid-2003 caused mild disease signs, those isolated in late 2003 and 2004 associated with relatively high mortality in experimentally inoculated chickens. Therefore, the H9N2 viruses circulating in Korea appear to have undergone antigenic and pathogenic changes.

Most of the H9N2 viruses recently isolated in Hong Kong that show preferential affinity for sialic acid with a 2,6-Gal linkage, have leucine at position 226 of HA-a feature characteristic of mammalian viruses (Matrosovich et al., 2001). However, all of the 12 H9 viruses tested in this study have glutamine at position 226 and high binding affinity to 2,3-linked sialic acid (SA) moieties but a low binding affinity for the 2,6-linked SA moieties found in mammals, as did the earlier Korea group viruses and avian H9 viruses isolated from ducks in Asia in the 1970s. Although the relative importance of this receptor trait and the potential for transmission are unknown, some Korean H9N2 viruses replicated well in mice. These observations suggest that the H9 viruses of Korea have not acquired human virus-like receptor specificity, as did Hong Kong H9N2 viruses. Furthermore, all of the Korean H9 isolates had HA cleavage site peptides (Ala-Ser-Gly-Arg, Thr-Ser-Gly-Arg, or Val-Ser-Gly-Arg) that differed from those of recent southeastern China H9 viruses (RSSR) and only variation at position -4 (T, V or A). The Korean HA connecting peptide sequences were not observed in any other H9 isolates described in GenBank. These findings suggest that the Korean H9 viruses evolved separately from those of southeastern China.

Interestingly, one isolate that we tested, Ck/Kor/164/04, was of the H9N8 subtype, which is a novel subtype in domestic chickens. Genetic characterization revealed that the *HA* and *PB1* genes of A/Ck/Kor/164/04 belong to the Korea group-like H9N2 viruses, whereas its other genes are closely related to those of an H3N8 avian influenza virus (AB/Kor/W04/04) isolated in 2004 from a wild aquatic bird. Our previous genetic study of influenza viruses isolated from live-bird markets in Korea showed the H3N2 subtype to be one of the main subtypes in chickens and ducks (Choi et al., 2005). Although avian H3N2 viruses consistently are isolated from live-bird markets and can infect experimentally inoculated chickens, no H3N2 virus was detected in chicken farms during this study.

Although infection of chickens with various avian influenza viruses has been documented, this report is the first (to our

knowledge) of H9N8 infection in this host. Our findings suggest that Ck/Korea/164/04 resulted from reassortment of an H9N2 (Korea-lineage) virus and an H3N8 wild aquatic bird virus, such as AB/Kor/W04/04. In this situation, chickens can serve as a host for viruses that infect terrestrial poultry and those that infect wild aquatic birds. Our findings have implications for influenza ecology and the possibility of further evolution of these viruses.

In our previous study, H9N2 influenza viruses isolated from live-bird markets in Korea did not replicate in mice (Choi et al., 2005), unlike viruses isolated in Hong Kong. However, most of the Korean genotype A viruses isolated in 2004 replicate in mice without pre-adaptation (4 out of 5 isolates), except the Ck/Kor/Q19/04 (H9N2) virus (data not shown). All isolates of genotype C and B were not recovered from mice lungs during 7 days of post inoculation. The *NS* and *M* genes of genotype A isolates are of the lineages as unknown aquatic bird viruses (Fig. 2). The factors that control host range restriction of influenza viruses remain unknown. However, the *NP*, *NS*, and *M* genes are responsible for the replication of avian influenza viruses in mammalian hosts and of mammalian influenza viruses in avian hosts (Buckler-White et al., 1986; Murphy et al., 1989; Hatta et al., 2002). Therefore, the *NS* and *M* genes of genotype A may contribute to the replication of these viruses in mice. However, further studies are needed to understand the role of these genes in restricting the host ranges of these viruses to chickens and mice.

Another difference among the Korean virus isolates is the tissue distribution of genotype A influenza viruses in chickens. The genotype A isolates (Ck/Kor/116/04 and Ck/Kor/Q19/04), including Ck/Kor/164/04 (H9N8), were recovered from all organs tested and at higher titers at 5 days p.i. than those of Ck/Kor/MS/96. However, Ck/Kor/MS/96 was recovered from the kidneys at a low titer ( $10^{0.5}$  EID<sub>50</sub>/ml) in only 1 of 8 infected chickens and was not recovered from the spleen. These results suggest that some of the viruses isolated in 2004 from chicken farms in Korea had gained the ability to replicate in the organs, such as kidney and spleen. Furthermore, the Ck/Kor/116/04 and Ck/Kor/Q30/04 isolates caused more severe clinical signs than did the genotype C isolate (A/Ck/Kor/150/03) in mature breeder chickens (Table 4). Specifically, the Ck/Kor/Q30/04 isolate showed higher viral titer and mortality than did other isolates in tissues of Hy-Line white (43.7%) and brown (18.7%) breeder chickens at 7 days p.i. Despite the small number of animals that we were able to test, statistical analysis suggests that age was a factor in susceptibility rather



than the breeder to some influenza viruses isolated in 2004 from Korean chicken farms.

At least 2 subtypes of avian influenza virus, including the novel reassortant H9N8 subtype, were co-circulating in Korean chicken farms during 2002–2004. These H9 viruses had evolved continuously by antigenic drift and had undergone reassortment with aquatic avian influenza A viruses, and their pathogenicity in animals had changed. New influenza virus genes can easily be introduced into this area by migrating birds, as occurred nearby in China (Liu et al., 2003). In light of this situation, we would expect to isolate additional reassortant viruses with unique combinations of genes. Therefore, continued monitoring of the domestic and wild bird populations is needed to better understand interspecies transmission, including that which has resulted in infection of humans with H5N1 (Tran et al., 2004; Tam, 2002; Chan, 2002; Peiris et al., 2004), H9N2 (Peiris et al., 1999), and H7N7 (Koopmans et al., 2004) avian influenza viruses, and to clarify the importance of avian hosts in the ecology of influenza viruses.

## Materials and methods

### *Virus sampling and isolation*

The Korean National Veterinary Research and Quarantine Service (NVRQS) conducts routine avian influenza virus surveillance and diagnostic services for poultry farms in Korea. Most of the viruses used in this study were isolated by NVRQS in embryonated eggs from swab specimens obtained from the tracheas and cecal tonsils of affected chickens from January 2002 through December 2004. Two avian influenza viruses (A/aquatic bird/Kor/W02/04 [H9N2] and A/aquatic bird/Kor/W04/04 [H3N8]) were isolated in embryonated eggs from fecal specimens obtained from wild birds during the period October 2004 through December 2004.

### *Antigenic analysis*

We investigated the cross-reactivity of the isolated viruses by hemagglutinin inhibition (HI) assay, as previously described (Palmer et al., 1975), using polyclonal antibodies to seven H9N2 viruses that recently circulated in southeastern China and Korea: A/Quail/Hong Kong/G1/97 (Qa/HK/G1/97); A/Chicken/Hong Kong/Y280/97 (Ck/HK/Y280/97); A/Chicken/Korea/MS96/96 (Ck/Kor/MS/96); A/Chicken/Korea/311/02 (Ck/Kor/311/02); A/Chicken/Korea/150/03 (Ck/Kor/150/03); A/Chicken/Korea/164/04 (Ck/Kor/164/04); and A/Chicken/Korea/Q19/04 (Ck/Kor/Q19/04). Because no antiserum to H9N2 from the period 2002–2004 was available, we obtained antisera by vaccinating chickens with the Ck/Kor/311/02, Ck/Kor/150/03, Ck/Kor/164/04, and Ck/Kor/Q19/04 viruses.

### *Neuraminidase inhibition (NI) test*

Assay for NA inhibition antibodies was performed according to the procedure recommended by the World Health Organization (Aymard-Henry et al., 1973).

### *DNA sequencing and phylogenetic analysis*

Viral genes were sequenced and analyzed as described previously (Choi et al., 2005). Briefly, viral RNA was extracted from the allantoic fluid of embryonated eggs by using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Reverse transcription and PCR amplification were carried out under standard conditions by using influenza-specific primers (Hoffmann et al., 2001). PCR products were purified by using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA). Nucleotide sequences were analyzed by directly sequencing the PCR products on an automated 3700 DNA sequencer (Applied Biosystems, Foster City, CA). The sequences were resolved using the ABI PRISM collection program (Perkin-Elmer, Foster City, CA). The DNA sequences were compiled and edited using Lasergene sequence analysis software package (DNASTAR, Madison, WI). Multiple sequence alignments were made using Clustal\_X (Aiyar, 2000; Thompson et al., 1997). The rooted phylograms were prepared using the neighbor-joining algorithm, and then plotted using NJ plot (Perriere and Gouy, 1996). For comparison, the phylogenetic analysis included sequences from avian influenza viruses established in southern China since the mid-1990s (Dk/HK/Y280/97-like and Qa/HK/G1/97-like H9N2 viruses), sequences from A/Goose/Guangdong/1/96 (Gs/Gd/1/96)-like viruses (H5N1) isolated in Hong Kong, two Korean H9N2 viruses (Ck/Kor/96006/96 and Ck/Kor/99029/99), two Korean H5N1 viruses (Ck/Kor/ES/03 and Dk/Kor/ESD1/03), and avian influenza viruses recently isolated from live-bird markets and wild birds in Korea. The full-length nucleotides of each gene were used in the phylogenetic analyses. Gene sequences determined in this study have been deposited in GenBank (sequence data will be provided upon request).

### *Measurement of pathogenicity in chickens and mice*

Chickens (specific pathogen-free [SPF] White Plymouth Rock chickens [*Gallus gallus domesticus*]) and mice (Balb/C, *Mus musculus*) were inoculated intranasally with allantoic fluid containing  $10^{5.5}$  50% egg-infective doses [EID<sub>50</sub>] of each genotype virus, as previously described (Li et al., 2003). Tracheal, cloacal swab, and lung tissue specimens were collected from chickens on days 3, 5, and 7 post-inoculation (p.i.), and virus was titrated in 10-day-old embryonated chicken eggs. Eight 5-week-old chickens and twelve 5-week-old mice were inoculated with each virus. HI testing before inoculation confirmed the absence of antibody to the inoculated influenza viruses. The body weight of mice was measured daily before inoculation and for 14 days after inoculation. Animals were killed on days 3, 5, 7, and 14 p.i., and virus in lung tissues was titrated in embryonated chicken eggs. To investigate the tissue distribution of virus in mice and chickens, we collected samples of trachea, lung, liver, intestine, spleen, and kidney for virus titration on day 5 p.i. All tissues were collected with separate scissors to prevent cross-contamination.

### *Transmission of H9N8 virus from chickens to chickens*

Three- to 4-week-old chickens (SPF White Plymouth Rock chickens [*G. gallus domesticus*]) were housed in wire mesh

cages. Three birds were inoculated with  $10^{5.75}$  EID<sub>50</sub> of each virus (Table 3) in a total volume of 1.0 ml by the tracheal and oral routes. The infected birds were put into contact with eight chickens of the same age. Tracheal and cloacal swab specimens were collected from contact chickens on every day and transmission from chicken to chickens was tested in 10-day-old embryonated chicken eggs.

#### *Pathogenicity of H9N2 influenza viruses in mature chickens and breeders*

Thirty-five-week-old commercial Hy-Line white (W-36) and brown chickens were purchased from commercial chicken farms and their sera were tested by HI to ensure that none had been exposed to our virus isolates. Sixteen breeders of each type were inoculated intranasally with  $10^{5.5}$  EID<sub>50</sub> of Ck/Kor/150/03, Ck/Kor/116/04, Ck/Kor/Q19/04, and Ck/Kor/Q30/04 isolates and were maintained in separate incubators in the NVRQS animal facility. We euthanized two chickens on day 5 p.i. to titrate virus in the organs; the remaining chickens were observed for clinical signs for 14 days p.i. Statistical analysis was conducted by  $\chi^2$ -test with SPSS Ver 10.1.

#### *Chicken intravenous pathogenicity test*

Pathogenicity tests were performed in accordance with instructions in the Office International des Epizooties (OIE) manual (22). Briefly, eight 4-week-old SPF White Plymouth Rock chickens were used for intravenous pathogenicity studies (using 0.2 ml of a 1:10 dilution of bacteria-free allantoic fluid containing  $10^{7.4}$  EID<sub>50</sub> of each genotype virus) to establish the virulence of the virus strain for international regulatory purposes.

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