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Avian influenza viruses in Korean live poultry markets and their pathogenic potential

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Abstract

We surveyed live-poultry markets in Korea in 2003 and isolated 9 H9N2, 6 H3N2, and 1 H6N1 influenza viruses. Antigenic and phylogenetic analyses showed that all 9 H9N2 isolates were of A/Chicken/Korea/25232-96006/96-like lineage (which caused disease in chickens in Korea in 1996) but were different from H9N2 viruses of southeastern China. They had at least 4 genotypes and replicated in chickens but not in mice. The H3N2 and H6N1 viruses were new to Korea and were probably reassortants of avian influenza viruses from southeastern China and recent Korean H9N2 viruses. All 8 segments of the H3N2 viruses formed a single phylogenetic cluster with 99.1 to 100% homology. The H3N2 viruses replicated in chickens and mice without preadaptation, but the H6N1 virus did not. Our results show an increasingly diverse pool of avian influenza viruses in Korea that are potential pandemic influenza agents.

Keywords: Avian influenza; Reassortment; Live-poultry markets; Korea

Introduction

Birds, particularly wild waterfowl, are the natural reservoir for all 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of influenza viruses (Alexander and Brown, 2000; Webster et al., 1992). In the last century, four influenza pandemics in humans caused significant mortality and morbidity. Genetic characterization revealed that the pandemic viruses were derived directly or indirectly from avian influenza viruses (Kawaoka et al., 1989; Taubenberger et al., 1997; Webster et al., 1992). The recent human infections with H5N1 (Chan, 2002; Peiris et al., 2004; Tam, 2002; Tran et al., 2004), H9N2 (Peiris et al., 1999), H7N3 (World Health Organization, 2004), and H7N7 (Koopmans et al., 2004) avian influenza viruses has highlighted the continuing threat of emergence of new pathogenic influenza viruses from the natural reservoir in birds.

Avian influenza viruses, especially H9N2 subtype, have been enzootic in Asia during the past decade and have been isolated from different types of terrestrial poultry worldwide (Cameron et al., 2000; Guo et al., 2000; Liu et al., 2003a, 2003b; Naeem et al., 1999; Saito et al., 2001). These facts raise the possibility that the H9N2 virus could be a threat to the human population. Because of its relatively low pathogenicity compared with the H5 and H7 subtypes of avian influenza viruses, the H9N2 subtype has not been studied intensively. However, infection with avian influenza viruses even of low pathogenicity can cause mortality and reduced egg production in chickens (Guo et al., 2000; Lee et al., 2000; Mo et al., 1997).

The internal genes of avian H9N2 (e.g., A/Quail/Hong Kong/G1/97) and H6N1 (e.g., A/Teal/Hong Kong/W318/

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97) viruses are closely related to those of the H5N1 viruses (Chin et al., 2002; Guan et al., 1999), which resulted in the death of 6 of 18 patients infected by these viruses in Hong Kong in 1997. Since 1997, H9N2 viruses have been isolated from humans (Lin et al., 2000; Peiris et al., 1999) and pigs (Peiris et al., 2001) in southern China. Theses studies suggested that some avian influenza viruses could be the donor of the replication gene complex to highly pathogenic viruses such as H5 and H7 subtypes, and have the potential for human pandemic.

The first avian influenza outbreak in Korea was reported in 1996. At that time, five H9N2 viruses were isolated from infected broiler breeder flocks with clinical signs of infection (Mo et al., 1997). Sequence analysis reveled that these viruses were genetically closely related to A/Duck/Hong Kong/Y437/97-like (H9N2) viruses from aquatic birds (Lee et al., 2000) but different from those of terrestrial poultry in China. However, there were no surveillance studies of avian influenza viruses in live-bird markets in Korea. Live-bird markets are a highly productive source of avian influenza viruses because they bring together numerous hosts (e.g., broiler chickens, ducks, turkeys, geese, and sometimes even doves) in a high-density setting, an ideal environment for viral reassortment and interspecies transfer (Liu et al., 2003a, 2003b; Shortridge, 1992). Live-bird markets are therefore hypothesized to be "a missing link in the epidemiology of avian influenza viruses" (Senne et al., 1993). Despite the role of live-bird markets in the ecology of influenza viruses and the hypothesis that Asia is an influenza epicenter (Shortridge and Stuart-Harris, 1982), little is known about the influenza virus gene pool in live-bird markets outside southern China. We therefore investigated the epidemiology of influenza viruses in Korea, and here we describe the results of the first surveillance of influenza viruses in live-poultry markets in Korea in 2003. This report establishes the gene pool of influenza viruses in the markets and discusses the continual evolution of influenza viruses in domestic birds, the extent of reassortment of isolates, and the pathogenicity of the isolated viruses in experimentally infected chickens, quail, and mice.

Results

We collected a total of 281 tracheal swabs and fecal specimens from clinically healthy chickens (*Gallus gallus*,

Table 1
Antigenic analysis[a] of avian influenza viruses isolated from birds in Korean live-poultry markets in 2003

	H9N2 antisera			
H9N2 viruses	Qa/HK/G1/97	Dk/HK/Y280/97	Ck/Kor/25232-96006/96	Ck/Kor/S4/03
Silkie Ck/Kor/S3/03	40	20	640	≥1280
Ck/Kor/S4/03	40	20	640	≥1280
Ck/Kor/S5/03	40	20	320	640
Ck/Kor/S12/03	80	40	320	≥1280
Dk/Kor/S13/03	80	40	≥1280	≥1280
Dv/Kor/S14/03	40	20	640	≥1280
Ck/Kor/S15/03	40	20	640	≥1280
Ck/Kor/S16/03	40	20	≥1280	≥1280
Ck/Kor/S18/03	40	20	≥1280	640
Ck/Kor/25232-96006/96	40	40	≥1280	640
Qa/HK/G1/97	1280	80	40	40
Dk/HK/Y280/97	160	≥1280	40	40
	H3N2 and H3N8 antisera			
H3N2 viruses	SB/HK/SB24/01	Dk/ST/2183/01	Sw/TX/4199-2/98	Dk/Kor/S8/03
Ck/Kor/S6/03	80	1280	40	≥1280
Dk/Kor/S7/03	40	640	≤20	≥1280
Dk/Kor/S8/03	40	640	≤20	≥1280
Dk/Kor/S9/03	40	640	≤20	640
Dk/Kor/S10/03	40	640	≤20	≥1280
Dv/Kor/S11/03	40	640	40	640
Dk/ST/1283/01	80	1280	40	640
Sw/TX/4199-2/98	≤20	20	640	≤20
	H6N1 antisera			
H6N1 virus	Ty/MA/65	Tl/HK/W318/97		
Dk/Kor/S17/03	40	80		

Ck, chicken; Dk, duck; Dv, dove; Qa, quail; SB, song bird; Sw, swine; Tl, teal; Ty, turkey; ST, Shantou.
[a] HI titer.

n = 211), ducks (*Anas domesticus*, *n* = 51), doves (*Streptopelia orientalis*, *n* = 11), turkeys (*Meleagris gallopavo gallopavo*, *n* = 6), and geese (*Anser cinereus domestica*, *n* = 2) in four different live-poultry markets in Korea in 2003. Influenza viruses were isolated, in 10-day-old embryonated chicken eggs, from 16 (6%) of the 281 specimens. The viruses were isolated from chickens (*n* = 8), ducks (*n* = 6), and doves (*n* = 2) but not from turkeys or geese. Serologic analysis using a panel of reference antisera revealed the viral subtypes as H9N2 (9 isolates), H3N2 (6 isolates), and H6N1 (1 isolate). We genetically characterized all 16 isolates and determined their pathogenicity in chickens, quail, and mice.

Antigenic analysis

H9N2 viruses

H9N2 viruses were the most abundant influenza virus isolated from chickens, ducks, and doves in the South Korean live-poultry markets and accounted for approximately 56% (9 of 16 isolates) of the influenza viruses isolated during this study. Genetically distinct lineages of H9N2 viruses have been reported to be circulating in Asia (Guan et al., 2000; Li et al., 2003; Choi et al., 2004). To investigate the antigenic properties of our H9N2 viruses, we performed hemagglutination inhibition (HI) assays with polyclonal antisera to each subtype known to be circulating in Asia and US (Table 1). Most of the 2003 H9N2 viruses reacted poorly with antisera to Dk/HK/Y280/97 and Qa/HK/G1/97 (H9N2 viruses circulating in southeastern China). However, all isolates reacted well with antisera to Ck/Kor/S4/03 (this study) and the old Korean isolate Ck/Kor/25232-96006/96. These results demonstrate that all of the H9N2 viruses circulating in the live-poultry markets of Korea in 2003 were antigenically similar to the previous Korean lineage circulating in 1996 and different from those of southeastern China. Therefore, the H9N2 viruses of Korea probably evolved separately from those of southeastern China.

H3N2 viruses

Six H3N2 viruses were isolated from ducks (4 isolates), doves (1 isolate), and chickens (1 isolate) in this study. To investigate the antigenic relations among the HAs of these avian viruses and of previously circulating avian and swine H3 influenza viruses, we tested the viruses in HI assays using a panel of polyclonal antisera specific for a swine H3N2 influenza virus (Sw/TX/4199-2/98) and avian H3N8 influenza viruses circulating in song birds and wild ducks in southeastern China. Antisera to avian H3N2 viruses were not available, so we also included an antiserum to one of the isolated viruses identified as an H3N2 subtype (Dk/Kor/S8/03) in the HI assay. All isolates from 2003 reacted poorly with swine H3N2 antiserum (HI titer ≤ 40), but they showed moderate HI titers with H3N8 antiserum to the song bird virus SB/HK/SB24/01 (HI titer, 40 to 80) and high titers with a duck H3N8 (A/Dk/ST/1283/01) antiserum (HI titer, 640 to 1280). These data suggest that the Korean H3N2 viruses are antigenically more similar to avian H3N8 viruses circulating in ducks in southeastern China than to swine H3N2 viruses circulating in pigs and responsible for an influenza outbreak in pigs in South Korea in 1998 (Song et al., 2003).

H6N1 virus

Only one H6N1 virus was isolated during this study, from a duck. Antisera against two H6 viruses (Turkey/MA/65, an old American isolate, and Teal/HK/W318/97) were used for antigenic analysis. Our H6N1 isolate, Duck/Korea/S17/03, reacted moderately (HI titer, 40) with antiserum to Turkey/MA/65 and (HI titer, 80) with antiserum to Teal/HK/W318/97.

Genetic and phylogenetic analyses

To determine the genetic diversity of these viruses, we compared partial sequences of the 8 gene segments of each virus.

H9N2 viruses

We aligned the deduced amino acid sequences of the H9 HA genes and compared them with those of H9 viruses listed in GenBank. The HA genes of all of the 2003 H9N2 isolates encoded a glutamine residue at position 226 of the receptor binding site, as did previous Korean H9N2 isolates (Matrosovich et al., 2001), and were 95.7% to 100% homologous. Gln226 in the receptor binding site of the HA1 region confers the HA with a high binding affinity to 2,3-linked sialic acid (SA) moieties but low binding affinity to the 2,6-linked SA moieties found in mammals (including humans) (Matrosovich et al., 2001). Receptor affinity was confirmed by hemagglutination tests with horse red blood cells (which exclusively display 2,3-linked SA) and guinea pig red blood cells (which exclusively display 2,6-linked SA). The hemagglutination titers with guinea pig red blood cells were lower than those obtained with horse red blood cells by a factor of at least 4 times.

Compared with the first H9N2 virus isolated in Korea in 1996, whose HA has the connecting peptide sequence Ala-Ser-Tyr-Arg (ASYR), all of our 2003 H9N2 isolates had a mutation in this peptide (Fig. 1). Three (Ck/Kor/S5/03, Dv/Kor/S14/03, and Ck/Kor/S15/03) had a glycine in the tyrosine position (Ala-Ser-Gly-Arg), and the other 6 isolates also had a threonine in place of the alanine (Thr-Ser-Gly-Arg). However, none of the 2003 isolates had the series of basic amino acids at the connecting peptide that are seen in HA of highly pathogenic avian influenza viruses (Xu et al., 1999), and there were no changes in potential glycosylation sites (Fig. 1).

Phylogenetic analysis of the HA1 region of the H9 gene showed that, as predicted by antigenic comparison, all of the

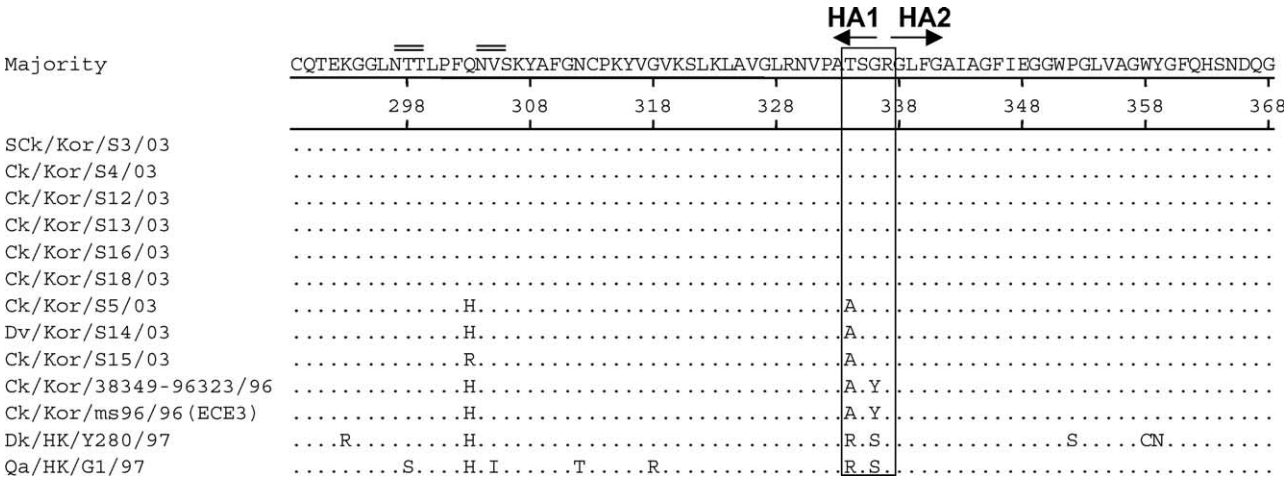

Fig. 1. Comparison of amino acid sequences near the cleavage site of the HA protein among Korean H9N2 isolates and published sequences of H9 subtype viruses. The proteolytic site that divides HA into HA1 and HA2 subunits is indicated by the tinted box. The positions of potential glycosylation sites are indicated by double horizontal lines above the sequence.

2003 H9N2 viruses belonged to the Ck/Kor/25232-96006/96-like lineage (Fig. 2a), which caused an outbreak of disease in chickens in Korea in 1996 (Lee et al., 2000). The 3 viral polymerase (PB1, PB2, and PA) and NA genes were also clustered with Ck/Kor/25232-96006/96-like viruses. However, phylogenetic analysis of the NP and M genes revealed at least 4 viral genotypes and showed that reassortment had occurred. The M genes of the 2003 H9N2 viruses separated into 3 groups: the Ck/Kor/25232-96006/96-like group, a group of unknown avian origin, and Dk/HK/Y280/97-like lineages circulating in southeastern China (data not shown). Two groups were based on NP gene sequence: most viruses were similar to Ck/Kor/25232-96006/96-like H9N2 viruses or to the Goose/Guangdong/1/96-like H5N1 viruses responsible for influenza outbreaks in chickens and humans in Hong Kong in 1997. NP gene of Ck/Kor/S18/03 was more similar to the H3N8 viruses circulating in pet birds and aquatic birds. The NS genes of the 2003 H9N2 viruses formed a single group, being similar to those of the Ck/Kor/25232-96006/96-like viruses. However, 3 isolates (Ck/Kor/S4/03, Ck/Kor/S16/03, and Ck/Kor/S18/03) had a single amino acid deletion at position 76 of

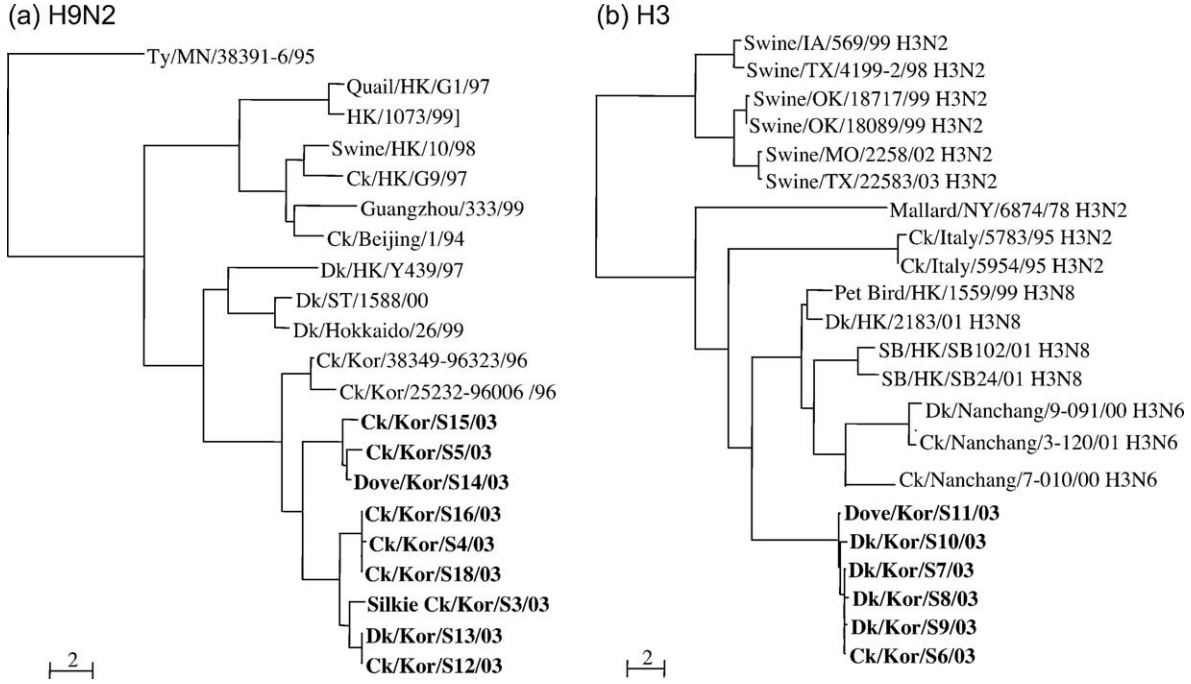

Fig. 2. Phylogenetic analysis of (a) the HA1 region of the HA genes of the nine 2003 H9N2 isolates and (b) the HA genes of the six H3N2 isolates. As predicted by antigenic comparison, the HA1 of all H9N2 isolates was of Ck/Kor/25232-96006/96-like lineage. The HA genes of the H3N2 isolates were clustered in one of the main H3N8 lineages from China and differed from the HA genes of H3N2 lineages from chickens in Italy and pigs in the United States. Ck = chicken, Dk = duck, SB = song bird, and Ty = turkey; standard abbreviations are used for state names in the United States.

the NS1 protein. On the basis of these data, we identified at least 4 different genotypes, which we designated A, B, C, and D, among the 9 viruses (Fig. 3). These data suggest that most of the H9N2 viruses in Korea are of the same lineage as Ck/Kor/25232-96006/96-like viruses, but some viruses have continued to evolve and reassort with cocirculating influenza viruses.

H3N2 viruses

The amino acid sequences deduced from the 2003 H3 HA genes were aligned and compared with those of H3 viruses available in GenBank. All 2003 H3N2 isolates had the same amino acid residues at the receptor binding site of HA as did the H3N8 viruses from southeastern China. In contrast to the 2003 H9N2 viruses, all 8 segments of the H3N2 viruses formed a single cluster within the phylogenetic tree of H3 subtypes of influenza viruses (Fig. 2b). The HA and NP genes were clustered with those of duck H3N8 viruses (Dk/ST/2183/01), as predicted by antigenic analysis. Most of the internal genes (i.e., M, NS, PA, PB1, and PB2, but not NP) were clustered with those of the Ck/Kor/25232-96006/96-like lineages (H9N2). All of the H3N2 viruses also had the same NA genes as Ck/Kor/25232-96006/96-like viruses (H9N2), whose NA genes differ from those of the predominant H9N2 viruses circulating in southeastern China, such as Dk/HK/Y280/97-like and Ck/HK/G9/97-like viruses. These data show that the H3N2 viruses circulating in Korea in 2003 are reassortants of viruses circulating in aquatic birds in southeastern China and viruses previously circulating in chickens in Korea.

H6N1 virus

The HA and PA genes of Dk/Kor/S17/03 were closely related to those of H6 viruses circulating in Hong Kong in 1997 and in Taiwan in 2001. However, the other genes of Dk/Kor/S17/03 were more closely related to those of influenza viruses from Eurasian aquatic birds rather than H6N1 viruses circulating in Hong Kong: the NA gene was similar to that of Dk/HK/380.5/02 (H5N1), the M gene to Dk/HK/P54/97 (H11N9), the NS gene to Dk/Nanchang/1944/93 (H7N4), the NP gene to Aquatic bird/HK/399/99 (H3N8), the PB1 gene to Dk/ST/2144/00 (H9N2), and the PB2 gene to Pheasant/Ireland/PV18/97 (H9N2).

Replication of the 2003 isolates in animals

To determine whether the genetic heterogeneity is accompanied by biologic heterogeneity, we compared the replication capacity of representatives of each subtype of the 2003 viruses in chickens, quail, and mice after intranasal inoculation with 10^5 EID_{50} of each virus. The H9N2 viruses we tested were Silkie Ck/Kor/S3/03, Ck/Kor/S4/03, Dv/Kor/S14/03, Ck/Kor/S16/03, and Ck/Kor/S18/03; H3N2 viruses were Ck/Kor/S6/03, Dk/Kor/S7/03, Dk/Kor/S8/03, and Dv/Kor/S11/03; and the H6N1 virus was Dk/Kor/S17/03. We included the viruses Ck/Kor/25232-96006/96 (H9N2) and Dk/ST/2183/01 (H3N8) for comparison.

Growth in chickens and quail

All of the 2003 H9N2 viruses we tested were recovered from tracheal swabs from infected chickens and quail at titers $\geq$2.3 $\log_{10}$ EID_{50}/0.1 ml on day 3 after inoculation

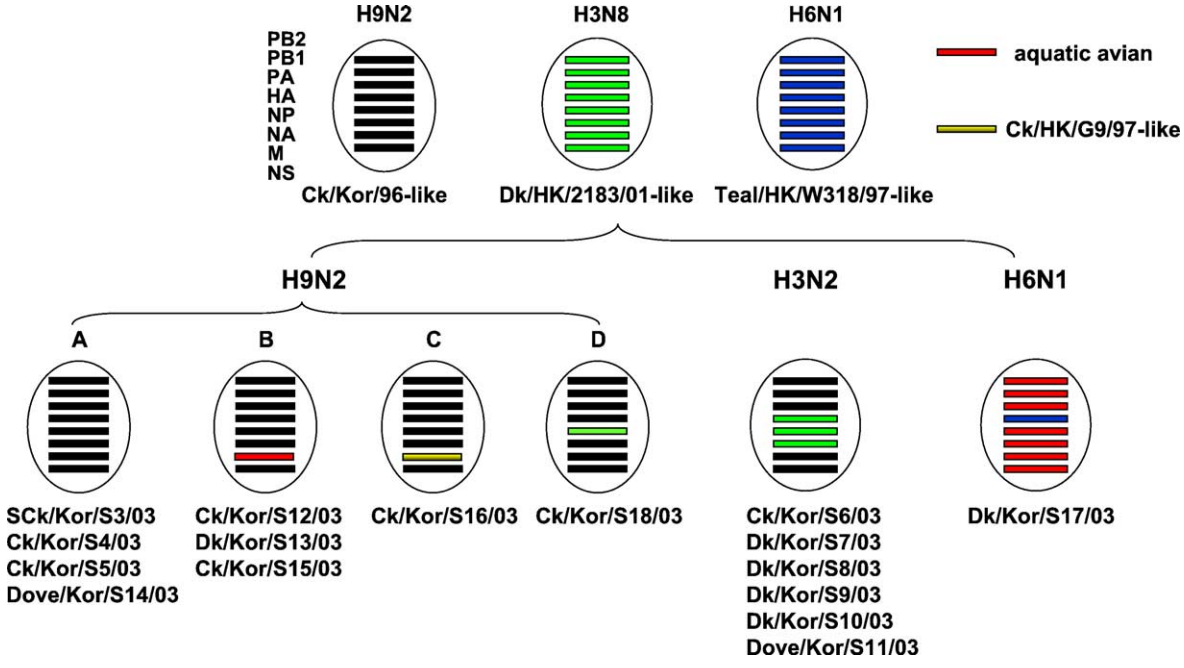

Fig. 3. To determine the genetic diversity of the 2003 avian influenza virus isolates, we compared partial sequences of their 8 gene segments. We identified 4 different genotypes, designated A, B, C, and D, of H9N2 viruses and 1 genotype of H3N2 virus.

(Table 2). Virus was detected in tracheal swabs for at least 5 days after inoculation, and traces of virus (1.3 to 0.33 $\log_{10}$ EID_{50}/0.1 ml) were found in birds inoculated with Silkie Ck/Kor/S3/03 or Ck/Kor/S4/03 on day 7 after inoculation. Viral titers in the chickens' lungs, however, were low (0.3 to 1.3 $\log_{10}$ EID_{50}/0.1 ml) on day 3 after inoculation with the H9N2 viruses, and no virus was detected in any of the chickens inoculated with 3 of the 5 Korean H9N2 isolates (Dv/Kor/S14/03, Ck/Kor/S16/03, and Ck/Kor/S18/03). None of the H9N2 viruses tested induced disease signs in inoculated chickens.

Two of the 4 H3N2 viruses tested (Ck/Kor/S6/03 and Dk/Kor/S8/03) were recovered from tracheal swabs at moderate titers (chickens, 1.3 or 1.7; quail, 2.3 or 2.7 $\log_{10}$ EID_{50}/0.1 ml), but none was recovered from lung tissues of the inoculated chickens or quail. Because phylogenetic analysis revealed that the 2003 Korean H3N2 viruses have the HA genes of Dk/HK/2183/01-like viruses, we also tested the replicative capacity of Dk/HK/2183/01 (H3N8) virus in chickens and quail to determine whether the ability of H3N2 viruses to replicate in chickens was attributable to the HA of these viruses. We recovered virus from tracheal swabs taken on day 3 after inoculation from 2 of 4 chickens and 3 of 4 quail, albeit at lower titer (0.33 to 0.75 EID_{50}/0.1 ml) than was obtained after inoculation with the Korean H3N2 isolates.

Although we detected virus in tracheal swabs and lung tissues from all of the 4 quail inoculated with the H6N1 isolate, no virus was detected in either trachea or lung tissues from chickens inoculated with this virus for the duration of the experiment (9 days).

Growth in mice

All of the 2003 Korean H3N2 isolates and the H3N8 (Dk/HK/2183/01) virus replicated well in mice without prior adaptation. Viral titers in the lungs ranged from 2.3 to 3.7 $\log_{10}$ EID_{50}/0.1 ml on day 3 after inoculation. Although mice inoculated with the H3N2 viruses showed slight loss of body weight, no deaths occurred during the 14 days of the experiment. To identify changes in the HA gene of the H3N2 viruses after their inoculation into mice, we extracted and sequenced the HA RNA from infected mouse lungs. All H3N2 viruses isolated from the infected mice had a unique mutation (leucine to tryptophan) at position 222 of HA. Tryptophan in this position is usually observed in mammalian (e.g., swine and human) H3 viruses.

Mice inoculated with the H9N2 and H6N1 viruses showed no clinical signs of disease, and neither virus replicated in any organs, including lung tissues (Table 2), for the duration of the experiment.

Discussion

Studies since the early 1970s have shown the live-poultry markets of Hong Kong to be a highly productive source of avian influenza virus (Shortridge, 1992). Similar studies of live-poultry markets in the United States have shown that

Table 2
Replication of the 2003 Korean avian influenza viruses and a Hong Kong virus in chickens, quails, and mice[a] on day 3 after inoculation

Virus	Chickens				Quails			Mice	
	Trachea	Lung	Cloaca	Disease signs	Trachea	Lung	Disease signs	Lung	Disease signs
H9N2									
Silkie Ck/Kor/S3/03	4/4 (4.5)[b]	2/4 (1.3)	2/4	No	2/4 (3.2)	1/4 (2.3)	No	0/3	No
Ck/Kor/S4/03	4/4 (3.7)	1/4 (0.7)	0/4	No	NT	NT	NT	0/3	No
Dv/Kor/S14/03	4/4 (2.7)	0/4	1/4	No	3/4 (3.2)	3/4 (1.7)	REP	0/3	No
Ck/Kor/S16/03	4/4 (3.3)	0/4	2/4	No	NT	NT	NT	0/3	No
Ck/Kor/S18/03	4/4 (2.3)	0/4	0/4	No	3/4 (3.7)	3/4 (3.7)	REP	0/3	No
Ck/Kor/25232-96006/96	4/4 (3.5)	1/4 (0.3)	1/4	No	0/4	0/4	No	0/3	No
H3N2									
Ck/Kor/S6/03	2/4 (1.7)	0/4	0/4	No	2/4 (2.3)	0/4	No	3/3 (3.7)	WL
Dk/Kor/S7/03	0/4	0/4	0/4	No	NT	NT	NT	2/3 (2.3)	No
Dk/Kor/S8/03	3/4 (1.3)	0/4	1/4	No	3/4 (2.7)	0/4	No	3/3 (3.3)	No
Dv/Kor/S11/03	0/4	0/4	0/4	No	2/4 (2.3)	0/4	No	3/3 (3.7)	WL
H3N8									
Dk/HK/2183/01	2/4 (0.3)	0/4	0/4	No	3/4 (0.75)	0/4	No	3/3 (2.7)	WL
H6N1									
Dk/Kor/S17/03	0/4	0/4	0/4	No	4/4 (3.7)	4/4 (2.3)	No	0/3	No

Ck, chicken; Dk, duck; Dv, dove; Kor, Korea; HK, Hong Kong; NT, not tested; REP, reduced egg production; WL, weight loss.

[a] The dose of inoculum was 10^5 EID_{50}.

[b] Values shown are the number of animals infected per number inoculated and (in parentheses) the average virus titer $\log_{10}$ EID_{50}/0.1 ml in samples taken on day 3 after inoculation.

these markets play a pivotal role in the distribution and genetic interaction of influenza viruses (Senne et al., 1993). Here, we document the avian influenza viruses present in live-poultry markets in Korea in 2003. We isolated 3 different subtypes (H9N2, H3N2, and H6N1) of avian influenza viruses. H9N2 isolates predominated (56%), followed by H3N2 (37.5%) and H6N1 (6%) isolates.

In Asia, H9N2 influenza viruses were isolated only from ducks before 1992 (Shortridge, 1992), but subsequent surveillance studies showed H9N2 viruses to be prevalent in domestic poultry in that region (Cameron et al., 2000; Naeem et al., 1999; Mo et al., 1997). Most of our Korean 2003 H9N2 viruses were isolated from chickens. The first avian influenza outbreak in Korea was reported in 1996. At that time, 5 H9N2 viruses were isolated from several broiler breeder flocks throughout the country. Most of the affected birds showed typical clinical signs of influenza, such as a significant drop in egg production, and up to 40% mortality was observed, but the isolates produced no detectable signs of disease after experimental infection (Mo et al., 1997), a finding that is consistent with those of the present study. However, in the field, clinical signs of disease are usually associated with coinfection with other viruses or bacteria. Sequence analysis of these 1996 H9N2 viruses revealed that they were genetically closely related and possibly came from the same source (Lee et al., 2000).

Phylogenetic analysis of all 8 gene segments revealed that 4 of the 9 Korean 2003 H9N2 isolates were closely related to Korean 1996 H9N2 isolates. However, the remaining isolates had a different NP gene (Ck/Kor/S18/03) or different M genes (Ck/Kor/S12/03, Dk/Kor/S13/03, Ck/Kor/S15/03, and Ck/Kor/S16/03) from those of the 1996 isolates (Fig. 3). These data suggest that most of the H9N2 viruses circulating in Korea were derived from Ck/Kor/25232-96006/96-like viruses, but that some are the result of reassortment of Ck/Kor/25232-96006/96-like and aquatic bird influenza viruses. Such reassortment would have generated multiple genotypes, as occurred in Hong Kong (Li et al., 2003).

In Italy, multiple H3N2 influenza viruses isolated in 1995 from chickens with mild respiratory disease were shown to replicate in the respiratory tracts of experimentally infected chickens. This finding was the first to show that avian-like H3N2 influenza viruses could replicate and cause disease in chickens (Campitelli et al., 2002). The Korean H3N2 viruses of the present study were isolated mostly from ducks; only Ck/Kor/S6/03 was isolated from a tracheal swab from a healthy chicken. However, our experiments clearly showed that the H3N2 viruses can replicate in chicken and even mice, without preadaptation. This finding raises concern that transmission may be possible into other mammalian species such as pigs, or even humans, especially as these viruses were nonpathogenic in commercial poultry. Interestingly, all mouse-passaged H3N2 viruses had the unique leucine to tryptophan mutation at position 222 of HA that is usually observed in mammalian H3 viruses. Although this position is not in a receptor binding site, it is close to the receptor binding site comprising residues 224 to 228. Therefore, this mutation could be crucial for the adaptation of avian H3N2 viruses to mice. Further studies are needed to understand the role of this position in the adaptation of avian H3N2 viruses to mammalian hosts.

Antigenic and phylogenetic analyses revealed that the HA genes of the 2003 Korean H3N2 isolates were different from those of isolates from swine in Korea (Song et al., 2003) and from chickens in Italy and were more closely related to those of aquatic bird H3N8 viruses circulating in southeastern China in 2001. This result suggests that multiple variants of H3 influenza viruses are circulating in these regions and causing disease in domestic animals. Furthermore, analyses of the other genes of the 2003 Korean H3N2 isolates showed that all except the NP gene were closely related to those of the Ck/Kor/25232-96006/96 (H9N2)-like lineage. These data suggest that the H3N2 viruses were the result of reassortment of Ck/Kor/25232-96006/96 (H9N2)-like and Dk/HK/2183/01 (H3N8)-like avian influenza viruses. This reassortment may have established a H3N2 lineage of influenza virus in domestic poultry. The H3N2 viruses from Italy, which caused mild respiratory disease in chickens and pigs, did not establish a stable lineage and have since disappeared (Campitelli et al., 2002). Whether the H3N2 virus will spread within the regional or national poultry population of Korea and establish a stable lineage remains to be determined.

Only one H6N1 virus was isolated in the present study. This may have been due to a limited sample size or the collection of specimens from birds that serve as inadequate avian hosts for H6 viruses. Until the proposal of the theory that the internal genes of an avian H6N1 virus (Teal/HK/W318/97) are closely related to those of the highly pathogenic H5N1 viruses, influenza viruses of the H6 subtype received little attention in Asia because of their low virulence. In contrast, in California H6 viruses have commonly been isolated from chickens with clinical signs of disease and have multiple genotypes (Webby et al., 2002). The H6N1 virus isolated in Korea was a multiple reassortant of H6 viruses and influenza viruses from aquatic birds. These findings suggest that more extensive studies including minor domestic and wild birds are needed to shed further light on the H6 viruses circulating in Korea.

In summary, at least 3 subtypes of avian influenza viruses were cocirculating in Korea in 2003, including novel reassortant H3N2 and H6N1 viruses. New influenza virus genes might easily be introduced into this region by migrating birds, as occurred in Japan (Liu et al., 2003a, 2003b), given the proximity of Korea to southern China. Furthermore, if H9N2, H3N2, and H6N1 viruses continue to cocirculate within the live poultry markets of Korea, we should expect to isolate additional reassortant viruses with unique combinations of genes encoding surface proteins,

internal proteins, or both. Our findings also indicate the existence of an extensive gene pool for influenza viruses. Therefore, we emphasize the continued need to monitor domestic and wild bird populations to better understand interspecies transmission, such as that which resulted in recent human infections with H5N1 (Peiris et al., 2004; Tran et al., 2004), H9N2 (Peiris et al., 1999), H7N3 (World Health Organization, 2004), and H7N7 (Koopmans et al., 2004) avian influenza viruses from the natural reservoir in birds. Such monitoring would also lead to a better understanding of the importance of avian hosts in the ecology of influenza viruses.

Materials and methods

Sampling and virus isolation

It is estimated that there are approximately 200 live bird markets in Korea where aquatic and terrestrial poultry are housed in adjacent cages. These 200 markets can be separated into 2 distinct types, sentinel and 5-day. Sentinel markets sell live birds every day, whereas, 5-day markets are open once every 5 days. The birds sold in the markets are brought in from local farms, usually within 20 km of the city. In this study, we investigated the influenza virus burden in 4 sentinel markets which were located in Kyunggi province (Northern area), Chungbuk province (Western area), Chungnam province (central area) and Kyungpook province (Southern area) of Korea.

We surveyed influenza activity in 4 different live-poultry markets in Korea in 2003. We collected a total of 281 fecal and tracheal samples from clinically healthy live chickens, ducks, doves, turkeys, and geese and inoculated embryonated chicken eggs with the samples, as described previously (Shortridge et al., 1998). Virus isolates were subtyped by using a panel of reference antisera recommended by the World Health Organization (WHO Animal Influenza Network, 2002). We characterized and analyzed a total of 16 avian influenza viruses (9 H9N2, 6 H3N2, and 1 H6N1), of which 8 were isolated from chickens, 6 from ducks, and 2 from doves.

Antigenic analysis

Polyclonal antibodies to 4 H9N2 viruses recently circulating 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/25232-96006/96 (Ck/Kor/25232-96006/96), and A/Chicken/Korea/S4/03 (Ck/Kor/S4/03) were used to investigate the cross reactivity of the isolated viruses by HI assay, as previously described (Palmer et al., 1975). Polyclonal antibodies to 2 H3N8 viruses, A/Song bird/Hong Kong/SB24/01 (SB/HK/SB24/01) and A/Duck/Shantou/1283/01 (Dk/ST/2183/01), a swine H3N2 virus, A/Swine/Texas/4199-2/98 (Sw/TX/4199-2/98), and a avian H3N2 virus of this study, A/Duck/Korea/S8/03 (Dk/Kor/S8/03), were used for antigenic analysis of the isolated H3N2 viruses. Antibodies to A/Teal/Hong Kong/W318/97 (Teal/HK/W318/97) and A/Turkey/Massachusetts/65 (Turkey/MS/65) were used for antigenic testing of H6N1 virus.

Genetic and phylogenetic analyses

Viral gene sequencing and analysis were carried out as described previously (Choi et al., 2002). For comparison, the phylogenetic analysis included sequences from avian influenza viruses established in southern China since the mid-1990s (including Dk/HK/Y280/97-like and Qa/HK/G1/97-like H9N2 viruses), sequences from A/Goose/Guangdong/1/96-like viruses (Gs/Gd/96, H5N1), sequences from H3 viruses, and sequences from H6N1 viruses isolated in Hong Kong. Two previous Korean H9N2 viruses (Ck/Kor/38349-p96323/96 and Ck/Kor/25232-96006/96 were also included in the analysis. The nucleotide regions used in the phylogenetic analyses were PB2: 1-1262, PB1: 66-1368, PA: 8-1290, HA: 1-1680, NP: 55-962, NA: 41-1393, M: 1-889, and NS: 1-842.

Gene sequences determined in this study have been deposited in GenBank.

In vivo growth characteristics

All animal experiments were approved by the Animal Care and Use Committee of St Jude Children's Research Hospital and performed in USDA inspected and AAALAC accredited facilities. Viral replication in chickens (specific pathogen−free white leghorn chicken), quail (*Coturnix coturnix*), and mice (Balb/C) was measured after intranasal inoculation with virus-infected allantoic fluid containing 10^5 EID_{50} of virus, as previously described (Li et al., 2003). Tracheal swabs and lung tissues were collected from chickens on days 3 and 7 after inoculation and virus was titrated in 10-day-old embryonated chicken eggs. Eight chickens, 8 quail, and 12 mice were inoculated with each virus. The body weight of the inoculated mice was measured daily on days 0 through 14 after inoculation, and the animals were killed on days 3, 5, 7, and 14 after inoculation, at which time virus in lung tissue was titrated.

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