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Characterization of the Pathogenicity of Members of the Newly Established H9N2 Influenza Virus Lineages in Asia

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The reported transmission of avian H9N2 influenza viruses to humans and the isolation of these viruses from Hong Kong poultry markets lend urgency to studies of their ecology and pathogenicity. We found that H9N2 viruses from North America differ from those of Asia. The North American viruses, which infect primarily domestic turkeys, replicated poorly in inoculated chickens. Phylogenetic analysis of the hemagglutinin and nucleoprotein genes indicated that the Asian H9N2 influenza viruses could be divided into three sublineages. Initial biological characterization of at least one virus from each lineage was done in animals. Early isolates of one lineage (A/Chicken/Beijing/1/94, H9N2) caused as high as 80% mortality rates in inoculated chickens, whereas all other strains were nonpathogenic. Sequence analysis showed that some isolates, including the pathogenic isolate, had one additional basic amino acid (A-R/K-S-S-R-) at the hemagglutinin cleavage site. Later isolates of the same lineage (A/Chicken/Hong Kong/G9/97, H9N2) that contains the PB1 and PB2 genes similar to Hong Kong/97 H5N1 viruses replicated in chickens, ducks, mice, and pigs but were pathogenic only in mice. A/Quail/Hong Kong/G1/97 (H9N2), from a second lineage that possesses the replicative complex similar to Hong Kong/97 H5N1 virus, replicated in chickens and ducks without producing disease signs, was pathogenic in mice, and spread to the brain without adaptation. Examples of the third Asian H9N2 sublineage (A/Chicken/Korea/323/96, Duck/Hong Kong/Y439/97) replicated in chickens, ducks, and mice without producing disease signs. The available evidence supports the notion of differences in pathogenicity of H9N2 viruses in the different lineages and suggests that viruses possessing genome segments similar to 1997 H5N1-like viruses are potentially pathogenic in mammals.

INTRODUCTION

In 1997, surveillance studies conducted in Hong Kong to trace the origin of the highly pathogenic H5N1 influenza isolates revealed that H5N1 and H9N2 influenza viruses were cocirculating in the poultry of the Hong Kong markets (Shortridge, 1999). Like the H5N1 influenza viruses, H9N2 viruses were isolated from various types of poultry, including chickens, ducks, quail, and pigeons. Molecular characterization and phylogenetic analysis of these H9N2 isolates revealed multiple lineages. One lineage represented in the poultry markets contain six internal genes that are closely related to those of highly pathogenic H5N1 viruses in Hong Kong. Another lineage is composed mainly of isolates from chickens and appears to have become stable and established because all tested H9N2 viruses isolated from chickens in southern China after 1994 were found to be phylogenetically clustered in this lineage (Guan *et al.,* 1999). The third lineage was from chickens in Korea with examples from ducks in the poultry markets in Hong Kong in 1997. These findings suggest that the H9N2 influenza viruses from Asia are genetically heterogeneous and broadly distributed among the poultry in the region.

Epidemiological data suggest that H9N2 influenza viruses are present on all continents. In North America, the H9N2 virus subtype was first isolated from turkeys in 1966 (Homme and Easterday, 1970). In field infections of turkeys, H9N2 influenza virus usually causes mild disease signs; however, some outbreaks are more severe, especially in mature birds, causing high morbidity rates, diarrhea, depression, and reduced egg production (McCapes *et al.,* 1986). In the United States, 16 outbreaks of H9N2-associated disease in turkeys were reported between 1981 and 1996 (Halvorson *et al.,* 1997). Surveillance studies in wild ducks in Alberta, Canada, from 1976 to 1990 found H9 influenza viruses (approximately 0.5% of the isolates) in apparently healthy wild ducks (Sharp *et*

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TABLE 1

Pathogenicity and Replication of H9N2 Influenza Viruses in Chickens after Intranasal, Tracheal, and Oral Inoculation

Virus	Age of chickens (weeks)	No. dead/total on day 5	No. infected/total on day 5		
			Trachea	Cloaca	*P*
Ty/WI/66	3	0/10	2/10	5/10	0.35
Ty/MN/38391/95	3	0/10	4/10	5/10	1.00
Sb/DE/9/96	3	0/10	2/10	5/10	0.35
Ck/Bei/1/94	3	1/9	9/9	0/9	<0.0001[a]
	6	8/20	20/20	2/20	<0.0001[a]
	12	8/10[b]	10/10	2/10	0.0007[a]
Ck/Kor/323/96	6	0/10	10/10	NT[c]	NT
	12	0/8	8/8	4/8	0.07
Ck/Kor/006/96	12	0/9	4/9	2/9	0.62

[a] Infection rate of the trachea is significantly higher than that of the cloaca ($P < 0.05$).
[b] Mortality rate of Ck/Bei/1/94 in 12-week-old birds is significantly higher than that in 3- or 6-week birds ($P < 0.05$).
[c] NT, not tested.

al., 1993, 1997). H9 influenza viruses were more prevalent in sea birds than in wild ducks in the Americas (Kawaoka *et al.,* 1988), representing 8.3% of isolates between 1985 to 1990; in recent years (1995 to 1998), 16% of isolates from shore birds have been H9 viruses (unpublished data). In Europe, H9N2 influenza viruses were isolated from apparently healthy aquatic birds at relatively low prevalence rates (fewer than 1% of isolates) between 1977 and 1989 (Suss *et al.,* 1994) and were isolated from domestic poultry, including turkeys, chickens, pheasants, and domestic ducks, between 1995 and 1997 (Alexander, 1997). In Asia, surveillance of live poultry markets in Hong Kong from 1975 to 1985 detected H9N2 influenza viruses only in apparently healthy ducks (Shortridge, 1992).

Recent information suggests that H9N2 influenza viruses remain prevalent in poultry in southern China and that avian-to-mammalian transmission of these viruses has already occurred in this region. In April 1998, two H9N2 influenza viruses were isolated from domestic pigs in Hong Kong (Markwell *et al.,* unpublished information). During the same period (July to August 1998), H9N2 influenza viruses were isolated from five humans with influenza in Guangdong Province (Guo *et al.,* 1999). All five patients had typical clinical signs of influenza, and all recovered from the disease. Serological studies indicated antibodies to H9 influenza viruses in the human population of southern and northern China (Guo *et al.,* 1999), which is in keeping with observations made in the mid-1980s (Shortridge, 1992). In March 1999, H9N2 viruses were isolated from two patients with influenza-like illness in Hong Kong (Lin *et al.,* personal communication). These events show that H9N2 influenza viruses are capable of infecting mammals, including humans. Thus the pathogenicity of these viruses in avian and mammalian species should be ascertained.

We provide preliminary characterization of the pathogenicity of H9N2 viruses from different poultry and geographical locations, including samples from the three different sublineages in Asia. Phylogenetic analysis also suggests that a stable lineage of H9N2 influenza viruses has become established in chickens in southern China.

RESULTS

Viruses used in the studies and their phylogenetic relationship

Background information. The H9N2 influenza viruses from North America include isolates from shore bird and from domestic avian species, including turkeys, quail, and geese. The shore birds were apparently healthy, whereas H9N2 in domestic avian species have been associated with outbreaks of moderate to severe disease (Homme *et al.,* 1970; Halvorson *et al.,* 1983). The isolates from Asia were associated with outbreaks that ranged from inapparent infections from Hong Kong poultry markets to serious losses in poultry production (Tables 1 and 5). The outbreak of disease in chickens in Hong Kong (Ck/Hong Kong/739/94) was associated with coughing and respiratory distress in 75% of birds, with a mortality rate near 10%. Treatment with antibiotics (tetracycline) reduced the mortality rate to background levels, suggesting that bacteria played a significant role in the clinical syndrome (Guan, 1997). The outbreak of disease in chickens from which Ck/Beijing/1/94 (H9N2) was isolated caused severe respiratory distress and 10–40% mortality rates. The H9N2 viruses isolated from chickens in Korea were associated with disease that caused 20% mortality rates and a severe (98.1%) drop in egg production (Mo *et al.,* 1997).

Phylogenetic analysis. Phylogenetic analysis of the nucleotide sequences of the HA1 of the H9 viruses examined is shown in Fig. 1A. The viruses separate into American and Eurasian lineages. The Sb/DE/9/96 isolate

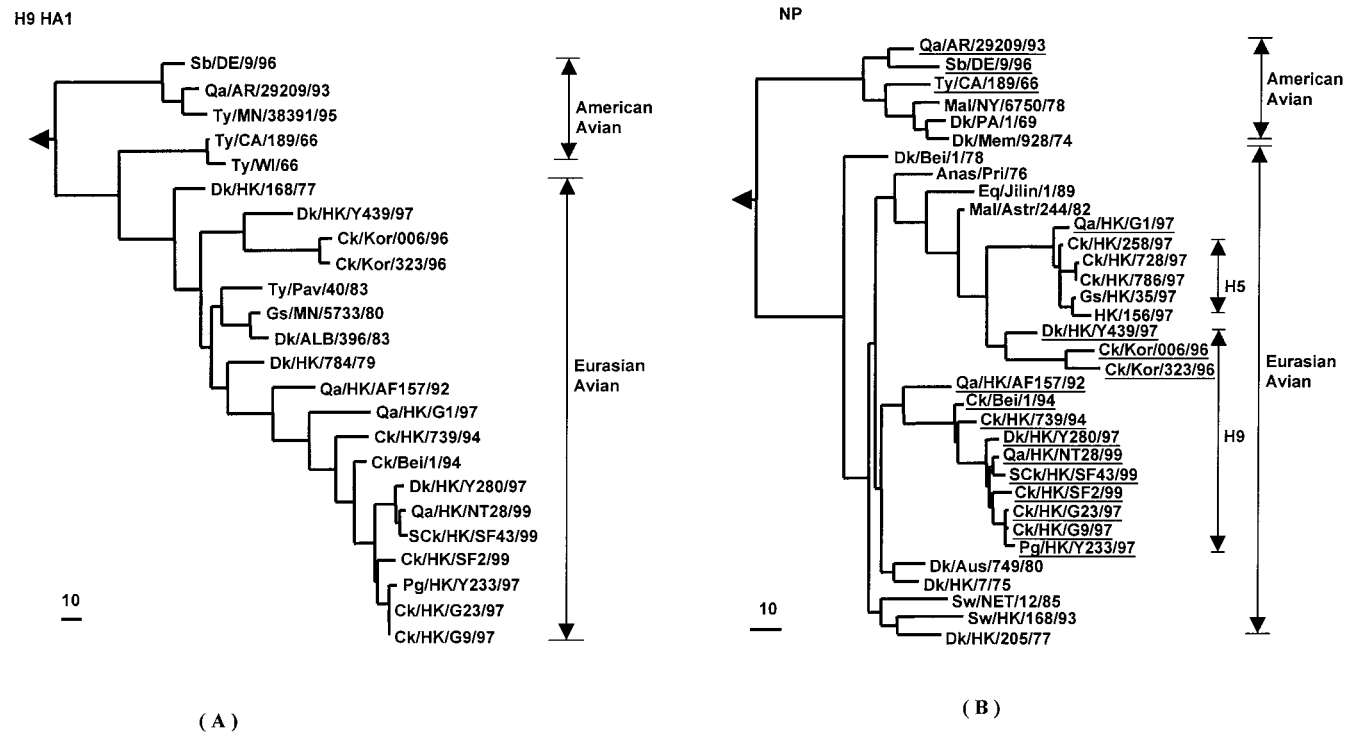

FIG. 1. Phylogenetic trees for the H9 HA1 (A) and NP (B) genes of influenza A viruses. The nucleotide sequences of the HA1 and NP genes were analyzed with PAUP by using a maximum-parsimony algorithm. Nucleotides 47–698 (652 bp) of H9 HAs and nucleotides 741-1398 (658 bp) of NPs were used for the phylogenetic analysis. The HA1 phylogenetic tree is rooted to A/Duck/Alberta/60/76 (H12N5). The NP phylogenetic tree is rooted to A/Equine/Prague/1/56 (H7N7). The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join nodes. Vertical lines are for spacing branches and labels. Virus names and abbreviations are listed in Table 5 or can be found in Guan *et al.* (1999) or GenBank by using the following accession numbers: D00050, M63775, M63776, M63782, M36812, M63786, M30764, M63783, M22573, and M30749. All viruses underlined in the NP tree are H9N2 influenza viruses.

is at the root of the Ty/MN/95 and Qa/AR/93 branches, and these are in a separate subgroup from the Ty/CA/66 and Ty/WI/66 viruses. The hemagglutinin (HA) of Gs/MN/5733/80 and Dk/Alb/396/83 form a sister-group relationship with the Eurasian H9 viruses, raising the possibility of interclade transmission of these viruses.

The Eurasian lineage of H9 HAs also has an aquatic bird isolate at the base (Dk/HK/168/77), and the Eurasian H9 HAs are separated into two sublineages. One sublineage contains recent chicken H9 strains from Korea (Ck/Kor/006/96, Ck/Kor/323/96) that are most closely related to a duck isolate (Dk/HK/Y439/97) obtained from a live poultry market in Hong Kong in 1997. The second sublineage contains isolates obtained from different types of poultry (duck, quail, pigeon, and chicken) from China between 1979 and 1999, supporting the inference that H9 influenza viruses have established a stable lineage in domestic poultry in Asia.

Phylogenetic analysis of the nucleotide sequence of the nucleoprotein (NP) gene of H9N2 and other avian influenza viruses again reveals separation into American and Eurasian lineages (Fig. 1B). The Eurasian lineage is separable into a number of sublineages. Most noteworthy is the finding that the NP of the H5N1 influenza viruses isolated from poultry and humans in Hong Kong in 1997 forms a major part of one sublineage with Qa/HK/G1/97 at its root (Guan *et al.,* 1999). As with the HA, the NP of chicken isolates from Korea (Ck/Kor/006/96, Ck/Kor/323/96) forms a separate sublineage. The NP genes of the H9N2 viruses isolated from different avian hosts (mainly from chickens) from China between 1992 and 1999 form a separate sublineage, again supporting the establishment of a stable H9N2 lineage in Asia. Thus there are multiple sublineages of H9N2 influenza viruses circulating in Asia.

Pathogenicity testing

Replication of H9N2 influenza viruses in chickens. Because little information is available about pathogenicity of H9N2 influenza viruses in chickens, we examined viruses near the root of the HA phylogenetic tree from North America and from Asia. The reference H9N2 strain (Ty/WI/66), a recent turkey isolate (Ty/MN/38391/95), and shore bird virus (Sb/DE/9/96) had similar patterns of replication in chickens (Table 1). Virus replicated at low levels in the tracheas of less than 40% of the chickens but replicated in the intestinal tracts of half of the chickens. None of the chickens infected with the North American H9N2 strains showed disease signs. In contrast, the

TABLE 2

Replication in Chickens of H9N2 Influenza Viruses Isolated from Different Hosts in Hong Kong

	Virus shedding and mean infectivity titer on the specified days after inoculation[a]									
	2		3		4		5		7	
Virus	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca
Ck/HK/G9/97	5/5[b] (3.4)[c]	2/5 (1.2)	5/5 (6.0)	1/5 (0.4)	5/5 (4.9)	4/5 (2.6)	3/5 (0.9)	1/5 (Tr)[d]	0/5	0/5
Qa/HK/G1/97	4/5 (2.6)	4/5 (1.7)	5/5 (4.4)	1/5 (0.4)	5/5 (2.8)	2/5 (1.0)	1/5 (Tr)	1/5 (0.9)	0/5	0/5

[a] Infection rates of the trachea are significantly higher than that of the cloaca ($P < 0.05$).
[b] Number of chickens shedding virus/number of chickens inoculated.
[c] Infectivity titer $\log_{10}$/ml.
[d] Tr, no virus detectable at dilution of $\geq 10^{-1}$.

Asian H9N2 influenza viruses were isolated from the tracheas of all chickens infected with Ck/Bei/1/94 and Ck/Kor/323/96 and from the tracheas of four of the nine birds infected with Ck/Kor/006/96. Although a smaller proportion of chickens infected with the Asian H9N2 viruses also shed virus in the feces, statistical analysis suggests that except for Ck/Kor/006/96, the infection rates of the trachea in most Asian viruses are significantly higher than the cloaca (Table 1). This difference was most pronounced with Ck/Bei/1/94, which was detectable in the feces of only 4 of 39 chickens ($P < 0.001$). These findings indicate that current H9N2 influenza viruses in southern China are mainly spread via respiratory route.

The two Korean H9N2 isolates from flocks with a nearly 100% drop in egg production and a 40% mortality rate (Mo *et al.,* 1997) produced no detectable signs of disease after experimental infection. These viruses replicated in the trachea a little more efficiently than in the intestinal tract (Table 1). However, the Ck/Bei/1/94 isolate did cause disease, especially in older birds; 80% of the 12-week-old birds inoculated intratracheally died by the fifth day (Table 1), whereas 40% of the 6-week-old birds and about 10% of the 3-week-old birds died. The only pathological signs of disease were pulmonary consolidation and hemorrhage. Statistical analysis shows that the mortality rates of Ck/Bei/1/94 of the 12-week-old group compared with the 3-week-old or 6-week-old groups of chickens are significantly different (Table 1). Thus H9N2 influenza viruses can show different pathogenic properties in chickens of different ages.

Replication of Hong Kong H9N2 viruses in chickens. During surveillance of live poultry markets in Hong Kong in 1997, H9N2 influenza viruses were isolated from about 5% of poultry tested (Shortridge, 1999). These viruses were typified by Ck/HK/G9/97 with a single isolate identified as Qa/HK/G1/97 that was phylogenetically distinct (Guan *et al.,* 1999). Ck/HK/G9/97 replicated in all birds tested (Table 2). High titers of virus were detected in the tracheas of all birds on days 3 (6.0 $\log_{10}$/ml) and 4 (4.9 $\log_{10}$/ml), whereas virus shedding in feces was less and titers were consistently lower (0.4–2.6 $\log_{10}$/ml). Qa/HK/G1/97 replicated in all birds, but compared with Ck/HK/G9/97, lower titers were detected in the trachea (4.4 $\log_{10}$/ml) with fewer birds shedding virus in their feces on days 4 and 5. Statistical analysis suggests that infection of the trachea was significantly higher than for the cloaca ($P < 0.05$). None of the inoculated birds showed signs of disease, and the birds continued to gain weight during the experiment (data not shown). This result indicates that Qa/HK/G1/97 can also replicate efficiently in chickens and, like Ck/HK/G9/97, is generally shed in higher titers from the respiratory tract than from the intestinal tract.

After oral inoculation of chickens with H9N2 influenza viruses, only the Ck/Bei/1/94 strain produced disease signs (Tables 1 and 5). However, some of the H9N2 influenza viruses examined had been isolated from chicken or quail flocks that experienced a severe drop in egg production and mortality rates as high as 40%. To further determine pathogenicity, groups of chickens were inoculated intravenously, and we sought to correlate their pathogenicity with the sequence of the connecting peptide of the HA molecule (see later).

Replication of H9N2 viruses in ducks. Because aquatic birds are considered to be the source of all influenza viruses, we inoculated ducks orally and intratracheally with representatives of three lineages of H9N2 viruses (Guan *et al.,* 1999) (Table 3). Ck/HK/G9/97 replicated in three of four birds and Qa/HK/G1/97 replicated in two of five birds; viruses were shed in low titers in the trachea and cloaca (approximately trace to 1.3 $\log_{10}$/ml), and no virus was detected on day 5. Dk/HK/Y439/97 infected two of four inoculated birds and was shed predominantly in the feces (2.1 $\log_{10}$/ml). However, statistical analysis suggests that there are no significant differences in the levels of replication between the trachea and cloaca for the viruses tested in ducks ($P > 0.05$). None of the ducks showed any signs of disease, and they gained weight throughout the experiment (data not shown).

TABLE 3

Replication of H9N2 Influenza Viruses in Ducks

Virus	Infection rate (no. shedding virus/no. inoculated) and infectivity titer (log_{10}/ml) on the specified days after inoculation[a]			
	Day 3		Day 5	
	Trachea	Cloaca	Trachea	Cloaca
Ck/HK/G9/97	3/4 (0.6)	2/4 (Tr)[b]	0/4 (<)[c]	0/4 (<)
Qa/HK/G1/97	2/5 (1.3)	1/5 (Tr)	0/5 (<)	0/5 (<)
Dk/HK/Y439/97	0/4 (<)	2/4 (2.1)	1/4 (Tr)	2/4 (Tr)

[a] No significant difference between infection rates in the trachea and cloaca ($P > 0.05$).

[b] Tr, virus detected in undiluted sample.

[c] <, No detectable virus.

Replication of H9N2 influenza viruses in mice. Because some H9N2 viruses were considered the possible donors of the internal genes of H5N1 viruses in Hong Kong (Guan *et al.,* 1999), a representative strain of each of the predominant lineages of H9N2 influenza viruses circulating in Asia was inoculated intranasally into mice to observe their potential pathogenicity for mammals (Table 4). Qa/HK/G1/97 virus replicated to high titers in the lungs on initial passage, with infectivity titers of 6.4 log_{10}/ml by day 5 after infection; the virus also spread to the brain. Three of the eight mice died with lung collapse and hemorrhage. On the second passage of Qa/HK/G1/97 in mice, all of the animals died with high titers of virus in the lungs and the brain (data not shown). The Ck/HK/G9/97 strain was also pathogenic in mice, causing the deaths of two of the eight tested animals on initial passage, with high virus titers (5.7 log_{10}/ml) in the lungs. This virus adapted quickly to mice and caused death but did not spread to the brain. The Dk/HK/Y439/97 strain of H9N2 also replicated in the lung with a relatively lower titer but did not cause death and spread to the brain. As shown in Table 4, only Qa/HK/G1/97 caused significant weight loss in mice ($P < 0.05$) (Table 4). It is noteworthy that the Qa/HK/G1/97 and the H5N1 influenza virus isolated from humans and poultry in Hong Kong in 1997 contained nearly identical internal genes making up the replicative complex (Guan *et al.,* 1999).

Replication and transmission of Ck/HK/G9/97 in pigs. Because surveillance studies of pigs in Hong Kong in 1998 revealed the presence of H9N2 influenza viruses (Markwell *et al.,* unpublished information), we tested the ability of the most prevalent H9N2 influenza virus in the live poultry market in 1997 to replicate in pigs. Ck/HK/G9/97 was inoculated into two minipigs, and one uninoculated animal was kept in contact with them. Each of the inoculated pigs shed the virus from the nasal tract for more than 5 days, with modest levels of virus from days 2 to 5 (virus titers, 2.5–4.5 log_{10}/ml). The pigs showed no signs of disease, and the virus did not spread to the contact animal (results not shown).

Genetic analysis of H9N2 influenza viruses

Although the virulence of avian influenza viruses is a polygenic property, the amino acid sequence of the connecting peptide of HA is considered its major determinant (Kawaoka and Webster, 1988). To understand the molecular basis of the pathogenicity of H9N2 viruses, we analyzed the sequences of the HAs of these viruses.

Connecting peptide of H9 HA. Translation of the nucleotide sequences of the HAs of the H9 viruses revealed two different structural motifs at the carboxyl-terminus of HA1: one motif was typical of the nonpathogenic avian influenza viruses (X-X-X-R, where X and R represent nonbasic and basic amino acids, respectively), whereas the other was R-S-S-R (Table 5), which is similar to the motif (R-X-R/K-R) required for highly pathogenic viruses of the H5 and H7 subtypes (Kawaoka and Web-

TABLE 4

Replication of H9N2 Influenza Viruses in Mice

Virus	Weight change of infected mice (n = 5)[a]			Virus titer (infectivity titer, log_{10}/ml)				Mortality rate (no. dead/no. tested)
				Day 3		Day 5		
	Weight (g) on day 0	Change (g/day)	*P*	Lung	Brain	Lung	Brain	
CK/HK/G9/97	25.1	−0.06	0.64	5.7	<[b]	5.2	<	2/8
Qa/HK/G1/97	26.6	−1.24	0.006[c]	6.2	Tr[d]	6.4	2.2	3/8
Dk/HK/Y439/97	23.3	−0.18	0.13	2.6	<	NT[e]	NT	0/3
Control	26.1	0.02	0.78	<	<	<	<	0/3

[a] N, number of mice tested in weight change study.

[b] <, No detectable virus.

[c] Weight change is significant ($P < 0.05$).

[d] Tr, virus detected in undiluted sample only.

[e] NT, not tested.

TABLE 5

H9N2 Influenza Virus HA Connecting Peptide Sequence and Pathogenicity in Chickens

Virus[a]	HA connecting peptide amino acid sequence ← HA1-5-4-3-2-1	Pathogenicity: Intravenous inoculation	Pathogenicity: Oral inoculation
Ty/CA/189/66	A-V-S-S-**R**	ND[b]	ND
Ty/WI/66	A-V-S-S-**R**	ND	−[c]
Dk/HK/169/77	A-A-S-G-**R**	ND	ND
Dk/HK/794/79	A-A-S-D-**R**	ND	ND
Gs/MN/5733/80	A-V-S-D-**R**	ND	ND
Qa/HK/AF157/92	A-**K**-S-S-**R**	ND	ND
Qa/AR/29209/93	A-A-S-N-**R**	ND	ND
Ck/HK/739/94	A-**R**-S-S-**R**	ND	ND
Ck/Bei/1/94	A-**R**-S-S-**R**	5/8	+[d]
Ck/Kor/323/96	A-A-S-Y-**R**	−	−
Ck/Kor/006/96	A-A-S-V-**R**	−	−
Sb/DE/9/96	A-A-S-N-**R**	ND	−
Qa/HK/G1/97	A-**R**-S-S-**R**	−	−
Ck/HK/G9/97	A-**R**-S-S-**R**	−	−
Ck/HK/G23/97	A-**R**-S-S-**R**	−	−
Pg/HK/Y233/97	A-**R**-S-S-**R**	ND	−
Dk/HK/Y280/97	A-**R**-S-S-**R**	−	−
Dk/HK/Y439/97	A-A-S-N-**R**	−	−
Ck/HK/SF2/99	A-**R**-S-S-**R**	ND	ND
Qa/HK/NT28/99	A-**R**-S-S-**R**	ND	ND

[a] Virus abbreviation: Animals: Ty, turkey; Gs, goose; Qa, quail; Ck, chicken; Sb, Shorebird; Pg, pigeon; Dk, Duck. Place: AR, Arkansas; CA, California; DE, Delaware; WI, Wisconsin; MN, Minnesota; Bei, Beijing; Kor, Korea; HK, Hong Kong.

[b] ND, not done.

[c] −, Did not cause any disease signs in chickens.

[d] +, Killed chickens (8 of 10; see Table 1).

ster, 1988). Most of the H9 viruses isolated from poultry markets in Hong Kong and the early H9N2 chicken viruses isolated in Guangdong Province and Hong Kong (Ck/Bei/1/94 and Ck/HK/739/94) possessed the second connecting peptide motif (R-S-S-R), whereas Dk/HK/Y439/97, the Korean chicken viruses, and all North American H9 viruses had the nonpathogenic motif in their connecting peptides.

The nucleotide sequence of the HA connecting peptides of the H9N2 viruses shows that only one additional nucleotide substitution (C → A or G) is needed at the −2 position to change serine to arginine and produce the basic motif required for highly pathogenic viruses. This finding suggests that some H9N2 influenza viruses circulating in southern China are potentially capable of becoming highly pathogenic viruses.

Potential glycosylation sites. It has been postulated that additional carbohydrate residues on the HA accompanied by shortening of the neuraminidase (NA) stalk by deletion of amino acids is characteristic of highly pathogenic chicken H5 and H7 influenza viruses (Matrosovich *et al.,* 1999). We therefore analyzed these characteristics of the HA and NA of H9N2 viruses.

Analysis of the potential glycosylation sites in the HA of the H9N2 viruses revealed six sites with the N-X-T/S motif (in which X may be any amino acid except proline); five were located in the HA1 portion and one in the HA2 portion of the molecule (Fig. 2A) (Kornfeld and Kornfeld, 1985). Like other HA subtypes, the HA of H9N2 viruses contains potential glycosylation sites at Asn11 and Asn474, which are conserved in all H9 HAs. However, the H9 HAs lack a potential glycosylation site, Asn18, which is conserved in most other HA subtypes (Nobusawa *et al.,* 1991). One virus (Qa/HK/G1/97) has an additional potential glycosylation site at residue 87 due to a substitution (Met → Thr) at residue 89 that was not found in the other H9 HAs examined.

Analysis of the N2 NA sequence of the H9N2 viruses showed the presence of at least seven potential glycosylation sites (Fig. 2B). The site at Asn146 that has been demonstrated to be glycosylated (Ward *et al.,* 1983) was conserved in each of the viruses examined. Similarly, in keeping with all analyzed N2 NAs, the potential glycosylation sites at Asn86, Asn200, Asn234, and Asn311 were conserved in the H9N2 NAs examined. There was more variability in the extent of glycosylation of the NAs than of the HAs among the H9N2 viruses examined. Three of the H9N2 viruses (Ck/HK/G9/97, Ck/HK/G23/97,

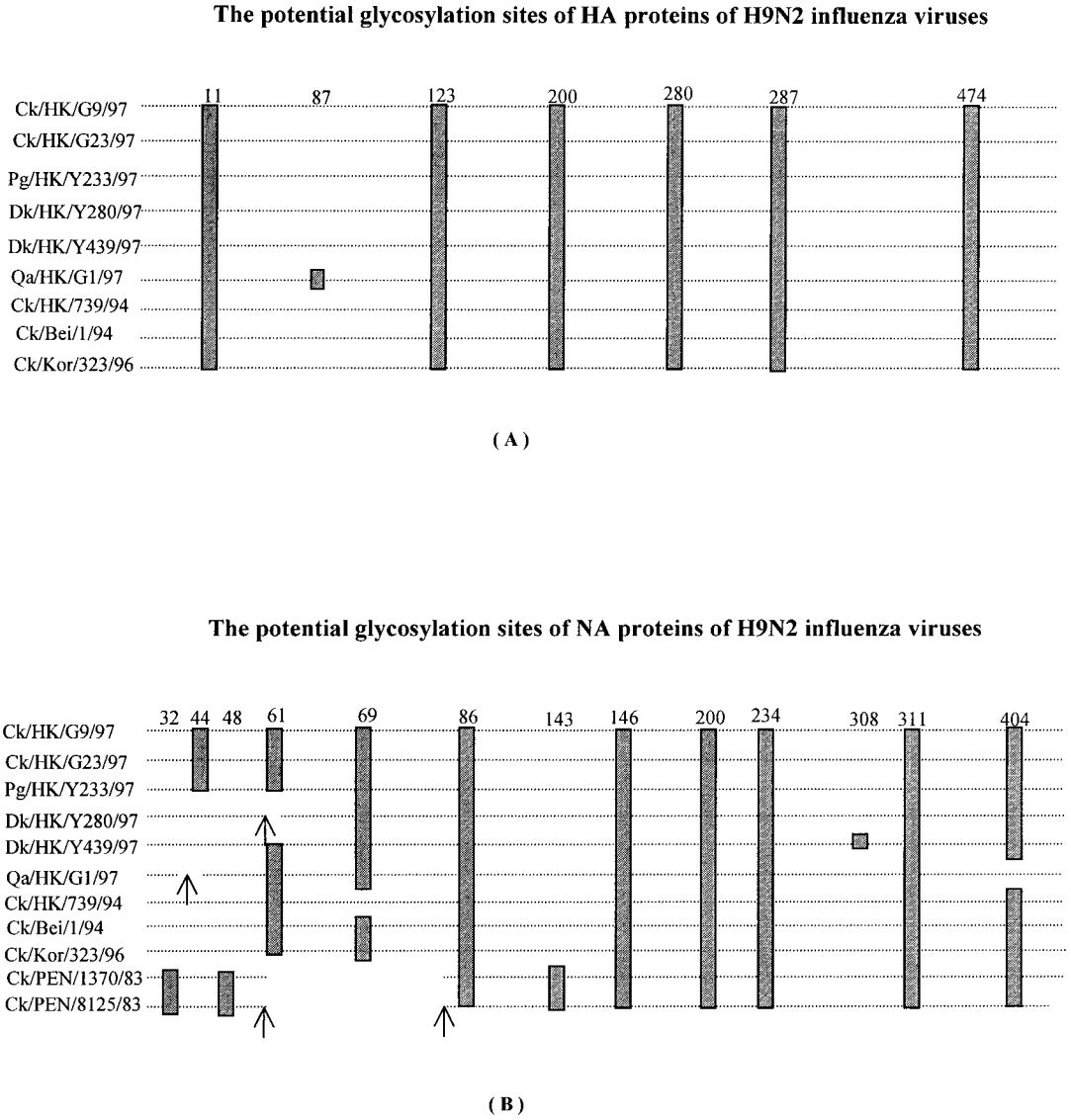

FIG. 2. The locations of potential glycosylation sites on HA (A) and NA (B) proteins of H9N2 influenza viruses. The dotted lines represent protein sequences of the HA and NA molecules. The numbered positions show the potential glycosylation sites. The arrows indicate the positions of deletions.

and Pg/HK/Y233/97) possessed an additional potential glycosylation site at Asn44 in the stalk caused by a Phe → Ser substitution at residue 45. Similarly, Dk/HK/Y439/97 possessed an additional potential carbohydrate at residue 308 (Asn). Two of the H9N2 NAs are of special interest because they have deletions in the stalk of the NA: Qa/HK/G1/97 has a deletion of six nucleotides (amino acid residues 45–46), whereas Dk/HK/Y280/97 has a deletion of nine nucleotides (amino acid residues 62–64). The deletion in Dk/HK/Y280/97 results in the loss of one potential glycosylation site (Asn61). Deletions in the NA of the H9N2 viruses have not been correlated with pathogenicity in chickens, but their possible role in host range transmission has not been fully elucidated, and these deletions may serve as useful markers.

DISCUSSION

In Asia, H9N2 influenza viruses were isolated only from ducks before 1992 (Shortridge, 1992); however, subsequent surveillance studies showed H9N2 viruses to be prevalent in domestic poultry (mainly chickens) in the region (Mo *et al.,* 1997; Guan, 1997). Genetic and phylogenetic analyses suggest that a stable lineage has become established in poultry in southern China. The current study revealed that one of the H9N2 influenza viruses isolated from chickens in southern China is pathogenic in that species. Although this virus does not satisfy the criteria for highly pathogenic avian influenza virus, our genetic findings suggest that recent H9N2 isolates from southern China may have the potential to

acquire the basic amino acids in the HA connecting peptide needed to become highly pathogenic strains. The current study also showed that some H9N2 avian influenza viruses possessing the internal genes similar to H5N1 from Hong Kong in 1997 could directly infect mice without adaptation, raising the possibility of interspecies zoonotic transfer.

Our studies showed that replication patterns differ among H9N2 influenza viruses. The North American H9N2 isolates from turkeys (Ty/WI/66, Ty/MN/95) and from shore birds (Sb/DE/9/96) showed limited ability to replicate in chickens: approximately half of the inoculated birds supported limited virus replication, mainly in the intestinal tract. In contrast, viruses from the three lineages of H9N2 influenza viruses isolated from chickens in Asia replicated in most of the chickens inoculated. Thus influenza viruses isolated from different avian species differ in host range restriction. Similar differences were observed when ducks were inoculated with viruses from the three Asian H9N2 lineages. Replication of Dk/HK/Y439/97 in the trachea was very limited, but higher titers of virus were shed from the cloaca. By contrast, Ck/HK/G9/97 and Qa/HK/G1/97 replicated mainly in the trachea, supporting the notion of emergence of multiple H9N2 lineages in Asia and adaptation to multiple avian hosts.

Ck/Bei/1/94 virus inoculation of chickens caused 80% mortality rates in 12-week-old birds, despite its failure to meet the criteria for a highly pathogenic influenza virus (i.e., it does not possess enough basic amino acids at the HA cleavage site and did not kill six of eight 3-week-old chickens after intravenous injection). The disease signs caused by the Ck/Bei/1/94 virus were confined to the lungs, with no evidence of systemic spread. In contrast, the two H9N2 isolates from chickens in Korea and the Ck/HK/G9/97 virus failed to cause any signs of disease in inoculated chickens, despite having caused substantial morbidity and limited deaths on chicken farms. Examination of the connecting peptides of the HAs revealed that the H9N2 Korean isolates contain the typical nonpathogenic sequence A-S-Y-R at the cleavage site of HA1/HA2 (Klenk and Garten, 1994; Horimoto and Kawaoka, 1994). By contrast, the Ck/Bei/1/94 and Ck/HK/G9/97 viruses contain an additional basic amino acid at the −4 position (-R-S-S-R). However, the failure of Ck/HK/G9/97 virus to cause disease signs suggests that this difference does not account for the increased pathogenicity of the Ck/Bei/1/94 virus. Because pathogenicity is a polygenic trait (Rott *et al.,* 1979), other genes of Ck/Bei/1/94 are likely to contribute to its increased pathogenicity. Another possibility is the exacerbation of pathogenicity by bacterial infection. This explanation is supported by the absence of death associated with Ck/HK/739/94 (H9N2) virus infections in chickens treated with tetracycline in Hong Kong in 1994 (Guan, 1997). This virus strain had an additional basic amino acid at the HA cleavage site (-R-S-S-R), allowing possible bacterial protease cleavage of HA, which has been demonstrated in swine influenza viruses (Callan *et al.,* 1997). At the level of nucleotide sequence, a single additional substitution in the region encoding the connecting peptide could potentially convert the virus to a highly pathogenic strain. These findings suggest that some of the H9N2 influenza viruses circulating in poultry in southern China have the potential to become highly pathogenic.

After the inoculation of BALB/c mice, the Qa/HK/G1/97 virus grew to high titers in the lungs, spread to the brain, and killed on initial infection, as do most H5N1 strains (Gubareva *et al.,* 1998; Lu *et al.,* 1999; Gao *et al.,* 1999). The Ck/HK/G9/97 strain also killed mice but did not spread to the brain. The almost identical sequences of the 1997 Hong Kong H5N1 and Qa/HK/G1/97 influenza viruses' internal genes and their differences from those of Ck/HK/G9/97-like influenza viruses (Guan *et al.,* 1999) may offer clues to the gene segments that contribute to pathogenicity in mice and to transmission to the brain. The PB1 and PB2 gene segments of Ck/HK/G9/97 and Qa/HK/G1/97 are almost identical, whereas their other four internal genes (PA, NP, M, NS) differ greatly. Although further studies are needed to identify the genes involved, it is reasonable to speculate that more than one of the six internal genes (PB2, PB1, PA, NP, M, NS) of Qa/HK/G1/97 contribute to its lethality in mice and that PB2, PB1, or both, plus one or more of the other genes, are involved in transmission to the brain.

An essential question is whether an avian H9 virus is able to infect and become established in mammals, thereby acquiring the opportunity to reassort or to be directly transmitted to humans. In this study, Ck/HK/G9/97 virus replicated efficiently in the tracheas of pigs. The isolation of H9N2 influenza viruses from both humans and pigs in southern China (Guo *et al.,* 1999; Markwell *et al.,* unpublished information) suggests that such infections may occur naturally. This information, together with the replication of avian H9 influenza viruses in mice, supports the possible ability of some avian H9N2 influenza viruses in southern China to spread to mammalian hosts and to cause disease.

Surveillance studies and phylogenetic analyses have established that Ck/HK/793/94-like or Ck/HK/G9/97-like influenza viruses continue to circulate in southern China. Thus these H9N2 influenza viruses have been prevalent in that region for at least 5 years, since the earliest viruses (Ck/Bei/1/94 or Ck/HK/739/94) were isolated in 1994. This finding suggests that the H9N2 influenza viruses in Asia have established a stable lineage.

Our studies showed that the mode of transmission of the H9N2 influenza viruses (Qa/HK/G1/97 and Ck/HK/G9/97) differs from that of H5N1 viruses from Hong Kong: the H5N1 viruses are transmitted mainly in feces (Shortridge *et al.,* 1998), whereas the H9N2 viruses are transmitted mainly by aerosol (and, to a lesser extent, in

feces). This finding may help to explain the mechanism of the interspecies transmission of H9N2 influenza viruses. It may also explain the wider host range of the avian H9N2 strains isolated from chickens, quail, pigeons, and ducks in Hong Kong poultry markets in 1997. We found that the quail isolate Qa/HK/G1/97 replicates less efficiently in chickens and ducks than do the chicken isolates (Ck/HK/G9/97), suggesting that low species barriers also exist between different types of birds.

MATERIALS AND METHODS

Viruses

The H9N2 influenza A viruses used in this study were propagated in 11-day-old chicken embryos and are listed in Table 5 and Fig. 2.

Polymerase chain reaction and sequencing

Viral RNA was extracted from infected allantoic fluid using RNEasy Mini Kit (Qiagen, Valencia, CA). After reverse transcription, cDNA was amplified by polymerase chain reaction (PCR), as described previously (Shu *et al.,* 1994). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen).

PCR products were sequenced by using synthetic oligonucleotides produced by the Center for Biotechnology at St. Jude Children's Research Hospital. Reactions were performed with Rhodamine Dye-Terminator Cycle Sequencing Ready Reaction Kits used with AmpliTaq DNA Polymerase FS (Perkin–Elmer/Applied Biosystems, Norwalk, CT). Samples were electrophoresed and analyzed on Perkin–Elmer model 377 DNA sequencers (Perkin–Elmer/Applied Biosystems).

Sequence analysis and phylogenetic analysis

All sequence data were edited and translated by using the Wisconsin Sequence Analysis Package, Version 10.0 (GCG, Madison, WI). Nucleotide and deduced amino acid sequences were aligned by the Feng-Doolittle progressive alignment method and manipulated with GeneDoc, version 2.3 (K. B. Nicholas at ketchup@cris.com). Phylogenetic analysis was performed by using PAUP (Phylogenetic Analysis Using Parsimony) software, Version 4.0 (David Swofford, Illinois Natural History Survey, Champaign, IL). Phylogenetic trees with the shortest lengths were identified by implementing a heuristic search. The nucleotide sequences obtained from this study are available from GenBank under accession numbers AF186266 to AF186272.

Animal tests

Chickens. Specific-pathogen-free (SPF) white leghorn chickens (3 or 4 weeks old) were inoculated intravenously with 0.2 ml of a 1:10 dilution of virus containing bacteria-free allantoic fluid [approximately 10^7 50% egg-infectious doses (EID_{50})]. Infected chickens were observed for 2 weeks. To examine virus replication, we also inoculated SPF chickens of different ages (3, 6, and 12 weeks) intranasally, intratracheally, and orally with $\sim 10^7$ EID_{50} of virus. Tracheal and cloacal swabs were collected after inoculation, suspended in phosphate-buffered saline with antibiotics, and injected into 10-day-old embryonated chicken eggs for virus isolation. Virus replication in organs was identified by sacrificing infected chickens 3 days after inoculation. Organ homogenates were prepared and used for virus isolation in eggs.

Ducks. Five-week-old Peking white ducks were inoculated with biologically cloned H9N2 influenza viruses; approximately 10^6 EID_{50} was inoculated into the nares, trachea, eye, and mouth. The birds were observed daily; infectious virus in tracheal and cloacal swab specimens collected on days 3 and 5 was titrated in embryonated eggs.

Mice. Five-month-old BALB/cByJ mice were administered metofane for anesthesia and were injected intranasally with 0.1 ml of 1:10 dilution of biologically cloned H9N2 viruses in phosphate-buffered saline (approximately 10^6 EID_{50}). Mice were sacrificed at specific intervals after injection, and infectious virus in blood, brain, and lung was titrated in embryonated chicken eggs (Shortridge *et al.,* 1998).

Pigs. Two 1-month-old Hanford white minipigs were inoculated intranasally and orally with approximately 10^6 EID_{50} of biologically cloned H9N2 influenza viruses. One uninoculated pig was kept in contact with the infected pigs (in the same cage). Daily nasal swab specimens were collected for virus titration in embryonated chicken eggs.

Statistical analysis

Fisher's Exact method (Agresti, 1990) was used for the analysis of the infection and mortality rates in chickens and ducks. A combination of longitudinal model (Little *et al.,* 1996) and logistical mode (Agresti, 1990) was used for the analysis of the longitudinal change of infection rates in chickens and the weight change in mice.

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