Phylogenetic and pathogenic analysis of avian H9N2 and H5N1 influenza A viruses isolated in Japan

（日本で分離されたH9N2およびH5N1亜型鳥インフルエンザウイルスの分子疫学的および病原学的解析）

Masaji MASE

（真瀬昌司）

Contents

Abbreviations

AI: avian influenza

EID_{50} : 50% egg-infectious dose

HA: hemagglutinin

HI: hemagglutination inhibition

HPAI: highly pathogenic avian influenza

LPAI: low pathogenic avian influenza

M: matrix protein

MID_{50}: 50% mouse-infectious dose

MLD_{50}: 50% mouse-lethal dose

MPAI: mildly pathogenic avian influenza

NA: neuraminidase

NI: neuraminidase inhibition

NP: nucleoprotein

NS: nonstructural protein

OIE: Office International Epizooties

PA: acidic polymerase

PB1: basic polymerase 1

PB2: basic polymerase 2

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

SPF: specific-pathogen free

Preface

Influenza viruses, members of the family *Orthomyxoviridae*, cause acute, rapidly spreading catarrhal fevers in humans (28). Influenza viruses are recognized as the cause of significant numbers of natural infections and disease, usually of the upper respiratory tract, in humans, horses, domestic pigs, and various bird species and sporadic cases of naturally occurring disease in mink and a variety of marine mammals (64). Infection of domestic poultry by avian influenza (AI) viruses typically produces syndromes ranging from asymptomatic infection to respiratory disease and decreased egg production to severe, systemic disease with near 100% mortality (60). The latter form of the disease is the result of infection by highly pathogenic (HP) AI viruses.

Avian influenza has been recognized as a highly lethal, systemic disease (i.e., highly pathogenic avian influenza) by the World Organization for Animal Health (Office International des Epizooties; OIE), a world trade organization that codifies sanitary and health standards, which identifies HPAI as a List A disease. OIE List A contains transmissible diseases that have the potential for rapid spread, irrespective of national borders, which are of serious economic or public health consequence and of major importance in the international trade of animals and animal products.

Milder forms of AI were first recognized in various domestic poultry species between 1949 and the mid-1960s and have been termed low pathogenic avian influenza (LPAI) or mildly pathogenic avian influenza (MPAI) (60). Their impact on poultry production and trade has been much less severe than that of HPAI. They have not been listed by OIE as either a List A or B disease. In this thesis, MPAI will be used to refer to influenza illnesses not caused by HPAI.

Phylogenetic analysis of influenza A viruses and the presence of all 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of viruses in aquatic birds suggest that these hosts are natural reservoirs of such viruses (64). Although aquatic birds do not show any

symptoms when infected with the virus, it multiplies in their intestine, particularly in the simple columnar epithelial cells, which form crypts in the colon. Thus, many influenza viruses are shed via feces into the lake water these birds inhabit, where it can spread among waterfowl and to mammals.

Influenza A viruses have also been isolated from psittacines (4,40), although little is known about their role in the ecology and epizootiology of these viruses. Because they are captured in the wild and distributed worldwide for sale as pets, psittacines might serve as biological vectors in the spread of influenza A viruses to animals in other countries. In fact, a Newcastle disease virus originating from imported psittacines caused outbreaks of the disease among domestic poultry in southern California, USA, in the early 1970s (63).

Recently, avian influenza viruses, particularly of subtypes H5N1 and H9N2, have been spreading in Asian countries, where they are now enzootic, causing multiple outbreaks in poultry and even being transmitted to wild birds (5,12,17,31,32,65). Among them, highly pathogenic H5N1 influenza viruses attracted international attention when they were transmitted to 18 humans in Hong Kong in 1997, resulting in six deaths. (8,59). The viruses continued to circulate in Asia and, in 2003, two more people in Hong Kong were infected, leading to one additional fatality (47). These H5N1 viruses were not only highly pathogenic to chickens and quail, but they were also lethal to mice without adaptation. Accordingly, the H5N1 avian influenza viruses continue to be a threat to public health.

The wide distribution of H9N2 avian influenza viruses from East Asia to points in the Middle East, Europe, and Southern Africa highlights their potential to cause a pandemic via direct transmission from poultry to humans (5). In 1999, for the first time, H9N2 avian influenza viruses were isolated from two humans in Hong Kong. More recently, in 2003, H9N2 virus was isolated again from a human in Hong Kong. These incidents raise the possibility that H9N2 virus could give rise to a pandemic. H9N2 viruses isolated in 1999 shared common internal gene components with H5N1 viruses from 1997 that were lethal to humans in Hong Kong (16). Thus, H9N2 viruses along with H5N1 viruses are high on the

list of candidates that could potentially cause the next human influenza pandemic. During routine virologic survey of birds imported to Japan, influenza A viruses were isolated from Indian ring-necked parakeets (*Psittacula Krameri manilleusis*) originating from Pakistan.

Since late 2003, highly pathogenic H5N1 viruses have continued to cause outbreaks in Asian countries, including Vietnam, Korea, Cambodia, Laos, Thailand, Indonesia, and China. Some of these viruses have been transmitted to humans, for example, in Vietnam and Thailand (23,66), resulting in over 50 documented deaths (69). Although most of these countries declared themselves to be free of avian influenza in the spring of 2004 (67), these viruses appear to have returned to at least some of these countries (68). From the end of December 2003 to March 2004, outbreaks of acute, highly transmissible and lethal disease in chickens occurred in Japan, involving three chicken farms and a group of chickens raised as pets.

Furthermore, a highly pathogenic H5N1 influenza virus has been isolated from duck meat imported from China to Korea (62). This virus was highly pathogenic in chickens and replicated efficiently in the lungs of mice without prior adaptation. Thus, the importation of poultry products is a potential source of highly pathogenic H5N1 virus and a risk to public health. In Japan, to prevent such introduction of highly pathogenic H5N1 influenza viruses through imported poultry products, duck meat has been randomly sampled by the Animal Quarantine Service for virus isolation. During this routine surveillance, H5N1 viruses were isolated from duck meat imported from China in May of 2003.

To understand the potential of these avian influenza viruses for pandemic threat, I studied their genetic and biologic properties.

Chapter I

Imported parakeets harbor H9N2 influenza A viruses that are genetically closely related to those transmitted to humans in Hong Kong

Introduction

Phylogenetic analysis of influenza A viruses, together with the presence of all 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of viruses in aquatic birds, suggest that these hosts are natural reservoirs of such viruses (64). Influenza A viruses have also been isolated from psittacines among other avian species (4,40), although little is known about their roles in the ecology, epizootiology, and epidemiology of these viruses. Because they are captured in the wild and distributed worldwide for sale as pets, psittacines might serve as biological vectors in the spread of influenza A viruses to animals in other countries. In fact, a Newcastle disease virus originating from imported psittacines caused outbreaks of the disease among domestic poultry in southern California, USA, in the early 1970s (63).

In recent years, H9N2 viruses have caused influenza outbreaks in poultry worldwide, including Europe, Pakistan, and Asia (4,5,21,39). H9N2 viruses are genetically distinct in Asia, where at least three lineages defined by the nucleotide sequence of the NP gene are circulating (21). Some of these Asian H9N2 viruses have been transmitted to pigs and humans (21) in mainland China, as well as Hong Kong (32,45,46). For example, an H9N2 virus, A/quail/Hong Kong/G1/97(G1), that was genetically related to the H9N2 viruses from humans, was isolated during surveillance for influenza A viruses in Hong Kong birds (16). Moreover, the genes encoding the internal proteins (PA, PB1, PB2, NP, M, and NS) of the H9N2 viruses isolated from two children in Hong Kong were genetically closely related to those of the H5N1 viruses that had been directly transmitted from birds to humans, killing 6 out of the 18 people infected (8,24,33,54,55,58,59).

During routine virologic diagnosis of birds imported to Japan, influenza A viruses were isolated from Indian ring-necked parakeets (Psittacula Krameri manilleusis) imported from Pakistan. Here we describe the properties of these isolates.

Materials and methods

Viruses.

The first virus, A/parakeet/Chiba/1/97, was isolated from the respiratory organ (trachea) of a bird that died at a pet shop within 10 days of importation in March 1997. The second virus, A/parakeet/Narita/92A/98, also isolated from respiratory organs (mixture of trachea and lung), came from a bird that died at the animal quarantine station at the Narita airport in Japan in June 1998. Briefly, the homogenates were then centrifuged at low speed (3,000 x g for 10 min) and the supernatants were filtered through a sterile 0.45μm membrane filter before inoculation into the allantoic cavity of embryonated specific pathogen-free eggs. Both isolates were identified as influenza A viruses of the H9N2 subtype by conventional hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (2,36). The viruses were plaque-purified in primary chicken kidney cells, and stock viruses were prepared by inoculation into the allantoic cavities of 10-day-old chick embryos. To our knowledge, these are the first H9N2 influenza viruses isolated from psittacine birds.

Molecular characterization and phylogenetic analysis.

To determine the genetic relationship of these isolates with other H9N2 viruses, all eight genes of both isolates were sequenced. Viral RNA was extracted with a commercial kit (ISOGEN, Nippongene, Tokyo, Japan) from allantoic fluids containing virus. After reverse transcription with Superscript II (Life Technologies, Gaithersburg, MD) using random 9 mers, cDNAs were amplified by the polymerase chain reaction (PCR). PCR amplification of the coding regions of the viral gene segments was performed with gene-specific primer sets (Table.1). PCR-derived dsDNA was used as a template for sequencing on an Applied Biosystems 373S automated DNA sequencer using cycle sequencing dye terminator chemistry (Perkin-Elmer/Applied Biosystems, Foster City, CA). The nucleotide sequences were analyzed using version 10.0 of the sequence analysis software package GENETYX-MAC (Software Development, Tokyo, Japan). Identical results were obtained when the

isolates were resequenced.

Pathogenicity tests.

Chickens. Six-week-old specific pathogen-free (SPF) chickens were used in this study. Eight chickens were inoculated either intravenously or intranasally with 0.1 ml of virus (10^7 EID_{50}) and observed daily for 14 days.

Mice. The organ tropism of the virus was assessed by intranasally inoculating mice with 50 ul of virus (10^7EID_{50}). Three mice were then sacrificed on day 3 and another three on day 6 postinfection so that viral titers in their lung, brain, spleen, kidney, and liver could be determined by inoculating tissue homogenates into the allantoic cavity of 10-day-old embryonated eggs.

Nucleotide sequence accession numbers.

All sequences used in this study were sent to DDBJ, and the accession numbers are AB049153 to AB049168.

Results

Genetic analysis

Both H9N2 isolates were genetically closely related to each other (>99% identity by nucleotide analysis of all eight RNA segments) (Table 2), indicating that they belong to the same lineage. Since the viruses were identified one year apart, their lineage must have been established in Pakistan for at least a year.

The entire coding regions of the HA and NA genes of the H9N2 parakeet viruses showed >97% identity with those of the H9N2 viruses isolated from humans in 1999 and from quail in 1997 (G1 virus), but there were appreciable differences in the PA, HA, NP, NA, M and NS genes compared with a chicken isolate (A/chicken/Hong Kong/G9/97) and in all genes compared with A/duck/Hong Kong/Y439/97 (Table 3). Potential HA N-glycosylation sites with the N-X-T/S motif (in which X may be any amino acid except proline) are shown in Table 4. Unlike most other H9 HAs, those of the two parakeet viruses had a glycosylation site at Asn-87, consistent with findings in the H9N2 human and G1 quail viruses. However, the potential glycosylation site at Asn-188 in the three Hong Kong isolates is not represented in the parakeet viruses or other H9N2 viruses. A/parakeet/Narita/92A/98 virus lost a glycosylation site at Asn-200 that is conserved in all other H9 HAs. The significance of this variability in glycosylation sites remains unknown. Both of the parakeet viruses possessed an R-S-S-R sequence at the HA cleavage site (Table 5). It is identical to that found in the H9N2 human and G1 quail viruses, but differs from that found in other H9 viruses.

The parakeet viruses also differed from the H9N2 human and G1 quail viruses in length of the NA stalk. In contrast to the latter viruses, whose NA stalks are two amino acid residues shorter than those of other N2 NAs, the parakeet virus NAs had the same number of amino acid residues in this region as other N2 NAs (Fig. 1). Interestingly, the Hong Kong H5N1 viruses also have a shorter NA stalk than do other N1 viruses (58, 73). Although the biological significance of this finding is uncertain, a shorter NA stalk has been associated with high virulence in poultry (11).

Phylogenetic trees for the HA and NA genes constructed by the neighbor-joining method (52)

reinforced viral RNA sequencing results suggesting that the parakeet viruses share an immediate ancestor with the H9N2 human and G1 quail viruses (Fig. 2,3). Phylogenetic analysis of the entire coding regions of the NP, PB2, PB1, PA, M and NS genes showed that the parakeet viruses cluster with the H9N2 human and G1 quail as well as human H5N1 Hong Kong viruses (A phylogenetic tree of the NP gene is shown as an example; Fig. 4).

A mutation at the initiation codon of the NP gene of A/parakeet/Narita/92A/98 virus (CUG instead of AUG) was detected. Sequencing analysis of this region was repeated five times with identical results. The CUG initiation codon has not been reported in the genes of any influenza virus; however, it is the most efficient non-AUG initiation codon (36) and is used in the synthesis of both eukaryotic and viral proteins (10,37,49). Thus, we suggest that this CUG codon is used as an initiation codon for NP translation, although we cannot speculate on its biologic significance in the NP gene of A/parakeet/Narita/92A/98.

Pathogenicity

G1 quail virus replicated in chickens and ducks without producing any disease signs, but spread to the brain in mice (21). We therefore examined the pathogenicity of our H9N2 parakeet viruses in chickens and mice. Viruses were recovered from tracheal and cloacal swabs, but none of the infected chickens showed any disease signs. When Mice were intranasally inoculated with virus, all survived without signs of disease. Of all organs tested, lung was the only site from which virus was recovered at 3 and 6 days postinfection (Table 6). These results indicate that the parakeet viruses differ from G1 quail virus in both tissue tropism and virulence in mice and hence contain amino acid alterations that would account for the discrepant phenotypes.

Discussion

Here, we indicate that H9N2 viruses, genetically closely related to those transmitted to humans (33,45,46), were circulating in psittacines in Pakistan in 1997-1998 and were introduced into Japan during this period. Thus, viruses with the potential for bird-to-human transmission may still be circulating in countries other than China, and may be spreading across geographical boundaries through the importation and sale of wild psittacine birds as pets.

An H9N2 influenza virus caused an influenza outbreak in poultry in Pakistan in 1999 (39). Although the causative virus was never fully characterized, the HA cleavage site sequence was identical to that of our viruses. Efforts to clarify the relationship between the Pakistan virus and ours will depend on more complete molecular characterization of the former.

Avian influenza A viruses are genetically divided into two geographically based lineages: Eurasian and American. The G1 quail strain and the 1997 H5N1 Hong Kong virus belong to the Eurasian lineage. Recent surveillance studies indicate that Eurasian avian viruses can be subdivided further and that the NP genes of the Hong Kong H5N1 and H9N2 genes belong to one of these sublineages (44). Although earlier experimental attempts to infect human volunteers with avian influenza viruses had failed (6), the Hong Kong H5N1 and H9N2 viruses were clearly able to infect humans. Thus, the H5N1 and H9N2 Hong Kong viruses seem unique in their ability to replicate efficiently in humans. Although amino acid differences were found between these Hong Kong and other avian viruses, it is still not clear which differences account for the formers' direct transmissibility to humans or, alternatively, which simply represent unique traits of the different avian lineages in nature. Thus, further genetic studies of Eurasian avian influenza viruses are needed to evaluate the potential of avian viruses to cross host range barriers.

The PB2, PA, NP, and M proteins of the 1997 H5N1 viruses contained human virus-like amino acid residues that might have been responsible for direct transmission of the avian virus to humans (73). Some of these residues are also found in the H9N2 parakeet viruses:#position 661 (Ala-avian; Thr-human) in PB2, the residue located in the region responsible for interaction with other

polymerase components (48); and #position 136 (Leu-avian; Met-human) in the NP, the residue in the RNA binding domain of the protein (1). To unequivocally determine the contributions of these amino acid residues to the viruses' replicative capacity in mammals, one needs to generate viruses with specific mutations, using reverse genetics (25,41).

In conclusion, the international trade of exotic pet birds carrying influenza A viruses may pose a serious health threat to humans. Previously, psittacine birds have not been thought to play a major role in the epizootiology and epidemiology of influenza A viruses. Since the trading of these birds across regional and international boundaries is extensive (over 400,000 non-poultry birds, 50,000 from Pakistan, were imported to Japan each year for the last 5 years), the risk of worldwide dissemination of potentially virulent influenza A virus is considerable. Thus, adequate quarantine and surveillance systems should be established in countries engaging in such trade.

Summary

In 1997 and 1998, H9N2 influenza A viruses were isolated from the respiratory organs of Indian ring-necked parakeets (*Psittacula Krameri manilleusis*) that had been imported from Pakistan to Japan. The two isolates were closely related to each other (>99% by nucleotide analysis of eight RNA segments), indicating that H9N2 viruses of the same lineage were maintained in these birds for at least one year. The hemagglutinin and neuraminidase of both isolates showed >97% nucleotide identity with those of H9N2 viruses isolated from humans in Hong Kong in 1999, while the six genes encoding internal proteins were >99% identical to the corresponding genes of H5N1 viruses recovered during the 1997 outbreak in Hong Kong. These results suggest that the H9N2 parakeet viruses originating in Pakistan share an immediate ancestor with the H9N2 human viruses. Thus, influenza A viruses with the potential to be transmitted directly to humans may be circulating in captive birds worldwide.

Table.1. Nucleotide sequences of the primers used in this chapter.

Gene	Primer	Sequence
PB2	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-57F(PB2-95F)	CCACTGTGGATCATATGGCCA
	FLU-65R(PB2-750R)	CCTCCTGGTGTGTACATCTG
	FLU-66F(PB2-1621R)	TCATCGTCTATGATGTGGGA
	FLU-71F(PB2-1003F)	TCAAAAGAGAGGAAGAAGTGCT
	FLU-74R(PB2-1702R)	TCTCCCAATTCCTAATGATCCA
	FLU-73F(PB2-679F)	CTACCAGTGGCTGGTGGGACAAG
	FLU-72R(PB2-1203R)	TTGCTCGTCCCTCCCGCTGACTAT
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
PB1	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-69F(PB1-398F)	TCGCCAGACCTATGACTGGACATT
	FLU-70R(PB1-1135R)	GTGTCCGTAGCTTCATGCTCTT
	FLU-85F(PB1-933F)	GGAGACAACACCAAATGGAATGA
	FLU-86R(PB1-1619R)	TATCACTGTAACTCCAATGCTCAT
	FLU-87F(PB1-1480F)	GGACAGGAACATTTGAGTTCAC
	FLU-89R(PB1-514R)	CTCATTGGCTGTTAGACCGTTCGA
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
PA	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-79R(PA-633R)	CGCCTCTCTCGGACTGACGAAAGGA
	FLU-90F(PA-365F)	CCGATTCACTGAAATTGGTGTGA
	FLU-67F(PA-740F)	CTGCATTGAGGGCAAGCTTTCTCA
	FLU-68R(PA-1368R)	TTCCTTCTCATGCTTGCAATGTG
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
	FLU-81F(PA-1351R)	AGATCGCTAGCAAGCTGGAT
	FLU-82R(PA-1765R)	CTCATTTCCATCCCCCATTTCAT

HA	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	H9-514R	CCATCTCATACTCCTGTAGAATGAA
	FLU-47(H9-463F)	CAG CGG AAC AAG CAG AGC ATG
	FLU-48(H9-1588R)	CTC AGA TTC CAG CTT TAC CCC TTC
	H9 1472FF	AAATGTGATGACCAGTGCATGGA
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
NP	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	NP 184R	GTTTGAGTTCAGTGCACATCTGTA
	FLU-61F(NP-81F)	AAC TGG TGG AGA ACG CCA GAA TGC
	FLU-56R(NP-1464R)	AGG CAC GAT CGG GTT CGT TG
	FLU-62R(NP-1154R)	TCCACGTTCTCATTTGAAGC
	NP 1087F	ACAAGAGTAATCCCAAGAGGACAA
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
NA	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	N2413R	AAAGTGGTTCCCTGCCCAAGTGC
	FLU-51F(N2-345F)	GGT GAC GAG AGA ACC TTA TG
	FLU-52R(N2-959R)	CCT GAG CAC ACA TAA CTG GA
	N2853F	GTTAGATGTGTTTGCAGAGACAA
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
M	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-04R(M-508R)	TGCTGGGAGTCAGCAATCTG
	FLU-15F(M-489F)	CACGCTCACCGTGCCCAGTG
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
NS	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-20R(NS-656R)	CCCATTCTCATTACTGCTTC
	FLU-21F(NS-108F)	GGT GAT GCC CCA TTC CTT GA
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC

Table 2. Nucleotide and amino acid differences between the two parakeet isolates

Segment	Number of different	
	Nucleotides (% identity)	Amino acids (% identity)
PB2	7 (99.7)	3 (99.6)
PB1	17 (99.3)	4 (99.5)
PA	9 (99.6)	1 (99.9)
HA	14 (99.2)	9 (98.4)
NP	10 (99.3)	3 (99.4)
NA	9 (99.4)	5 (98.9)
M	5 (99.5)	M1 1 (99.6) M2 0 (100)
NS	3 (99.6)	NS1 1(99.6) NS2 0 (100)

Table 3. Sequence comparison of A/parakeet/Chiba/1/97 with H9N2 Hong Kong influenza virus[a]

Segment	No. of nucleotides sequenced	% Homology with A/parakeet/Chiba/1/97							
		A/Hong Kong/1073/99		A/quail/Hong Kong/G1/97		A/chicken/Hong Kong/G9/97		A/duck/Hong Kong/Y439/97	
		Nucleotides	Amino acids	Nucleotides	Amino acids	Nucleotides	Amino acids	Nucleotides	Amino acids
PB2	2280	98.5	99.1	98.5	98.7	98.2	98.3	87.6	96.4
PB1	2277	98.9	99.7	98.9	99.6	98.4	99.1	90.6	97.3
PA	2151	97.6	98.7	98.4	99.2	89.0	94.2	89.9	96.1
HA	1683	97.7	97.5	98.0	97.4	92.5	92.9	85.1	88.1
NP	1497	98.9	99.4	99.3	99.4	90.6	96.3	94.7	97.0
NA	1410	97.9	97.7	98.1	97.4	94.0	93.4	89.7	89.3
M	982	99.1	99.6 (M1)	99.1	99.6 (M1)	96.4	98.0 (M1)	92.9	95.6 (M1)
			96.9 (M2)		97.9 (M2)		96.9 (M2)		93.8 (M2)
NS	838	98.2	96.1 (NS1)	98.6	96.5 (NS1)	93.3	92.5 (NS1)	91.1	89.5 (NS1)
			98.3 (NS2)		99.2 (NS2)		95.7 (NS2)		97.5 (NS2)

[a] Nucleotide and amino acid sequences were compared, and identity was determined by Genbank searches.

Table 4. Potential glycosylation sites on HA proteins of parakeet and closely related H9N2 viruses

	Positions:							
Virus	11	87	123	188	200	280	287	474
A/parakeet/Chiba/1/97	+	+	+	-	+	+	+	+
A/parakeet/Narita/92A/98	+	+	+	-	-	+	+	+
A/quail/Hong Kong/G1/97	+	+	+	+	+	+	+	+
A/Hong Kong/1073/99	+	+	+	+	+	+	+	+
A/Hong Kong/1074/99	+	+	+	+	+	+	+	+
A/chicken/Beijing/1/94	+	-	+	-	+	+	+	+
A/chicken/Hong Kong/739/94	+	-	+	-	+	+	+	+
A/chicken/Hong Kong/G9/97	+	-	+	-	+	+	+	+
A/duck/Hong Kong/Y439/97	+	-	+	-	+	+	+	+
A/quail/Hong Kong/AF157/93	+	-	+	-	+	+	+	+
A/turkey/California/189/66	+	-	+	-	+	+	+	+
A/turkey/Minnesota/1/95	+	-	+	-	+	+	+	+

+: presence of a glycosylation site.

-: absence of a glycosylation site.

Table 5. HA connecting peptide sequences of H9N2 viruses

Viruses	Connecting peptide amino acid sequence						Reference
A/turkey/Wisconcin/66	P	A	V	S	S	R	42
A/quail/Arizona/29209/93	P	A	A	S	N	R	16
A/duck/Hong Kong/168/77	P	A	A	S	G	R	16
A/duck/Hong Kong/784/79	P	A	A	S	D	R	16
A/chicken/Beijing/1/94	P	A	R	S	S	R	16
A/quail/Hong Kong/G1/97	P	A	R	S	S	R	16
A/chicken/Hong Kong/G9/97	P	A	R	S	S	R	16
A/Hong Kong/1073/99	P	A	R	S	S	R	33
A/parakeet/Chiba/1/97	P	A	R	S	S	R	This study
A/parakeet/Narita/92A/98	P	A	R	S	S	R	This study

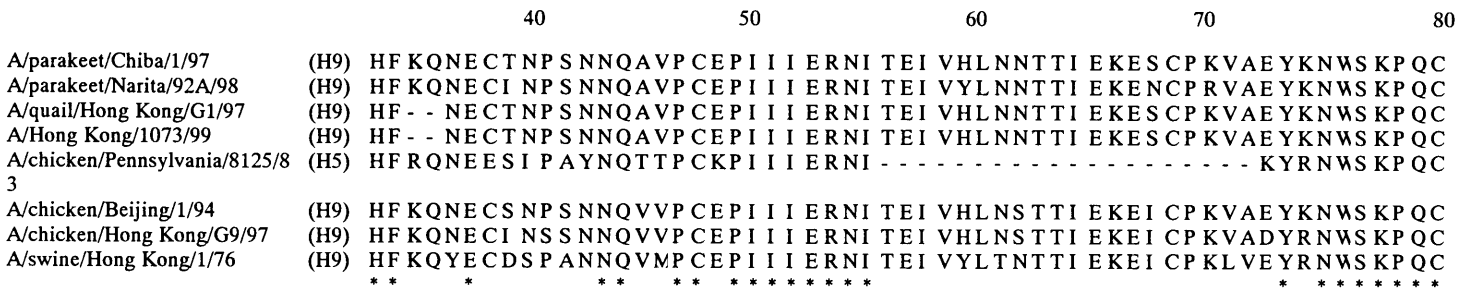

Fig.1.Comparison of NA stalks of representative N2 influenza A viruses. Identical amino acids are shown by asterisk (*)

and the positions of deletions by dashes. The HA subtype of each virus is shown in parenthesis.

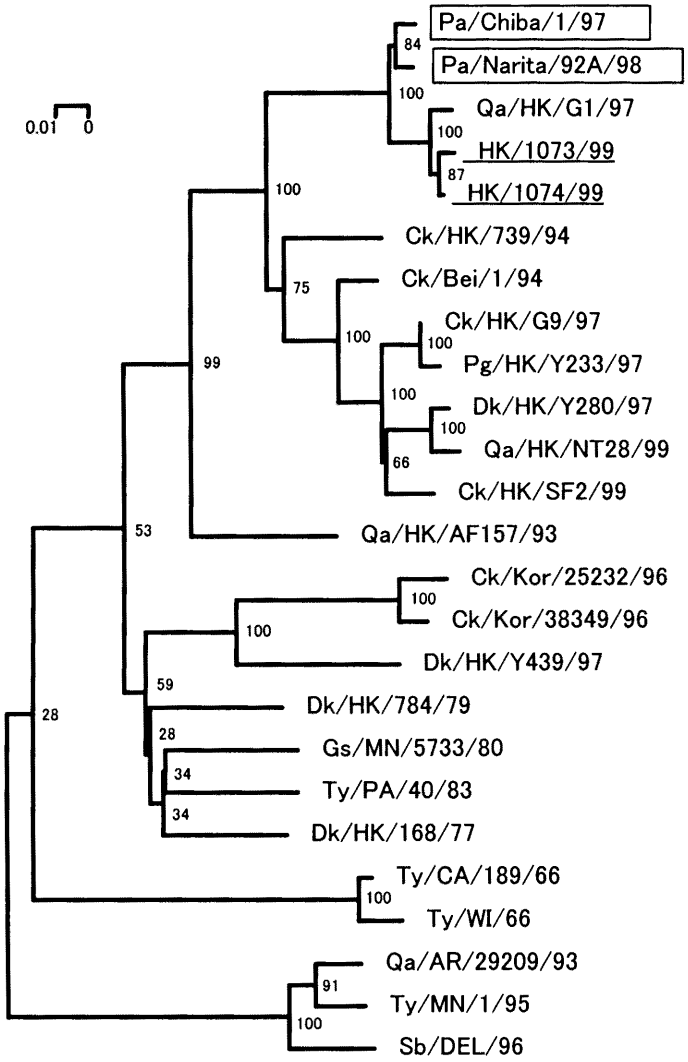

Fig.2. Phylogenetic trees of the H9 HA gene of influenza A viruses. Nucleotides 46-1091 (1046bp) of the H9 HAs was used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated from parakeets or humans are boxed or underlined, respectively. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value. Abbreviations: Pa (parakeet), Qa (quail), HK (Hong Kong), Ck (chicken), Bei (Beijing), Pg (pigeon), Dk (duck), Kor (Korea), Gs (goose), MN (Minnesota), Ty (turkey), PA (Pavia), CA (California), WI (Wisconcin), AR (Arizona), Sb (shorebird), DEL (Delaware), Penn (Pennsylvania), Sw (swine).

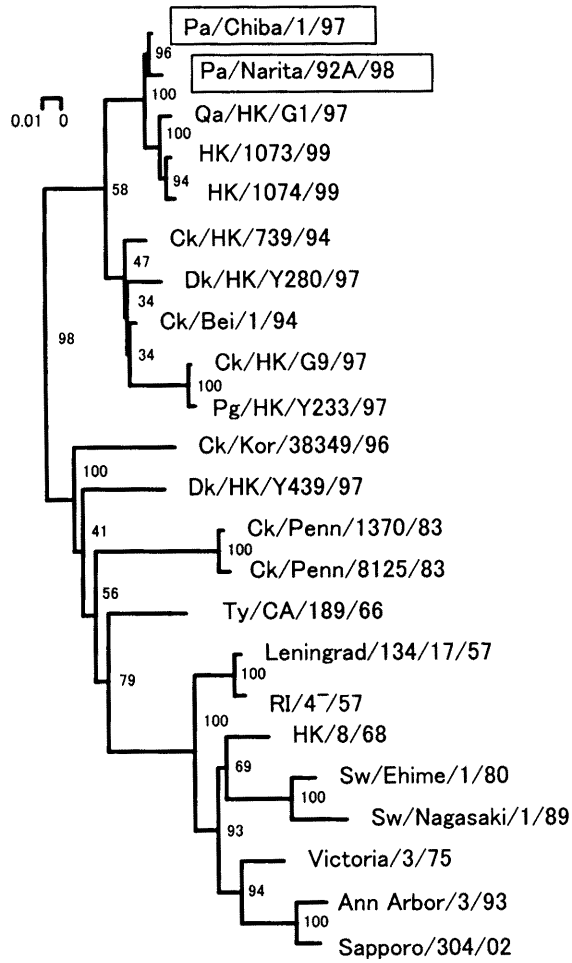

Fig.3. Phylogenetic trees of the N2 gene of influenza A viruses. Nucleotides 1-1386 (1386bp) of the N2 NAs was used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated from parakeets or humans are boxed or underlined, respectively. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value. Abbreviations: Pa (parakeet), Qa (quail), HK (Hong Kong), Ck (chicken), Bei (Beijing), Pg (pigeon), Dk (duck), Kor (Korea), Gs (goose), MN (Minnesota), Ty (turkey), PA (Pavia), CA (California), WI (Wisconcin), AR (Arizona), Sb (shorebird), DEL (Delaware), Penn (Pennsylvania), Sw (swine).

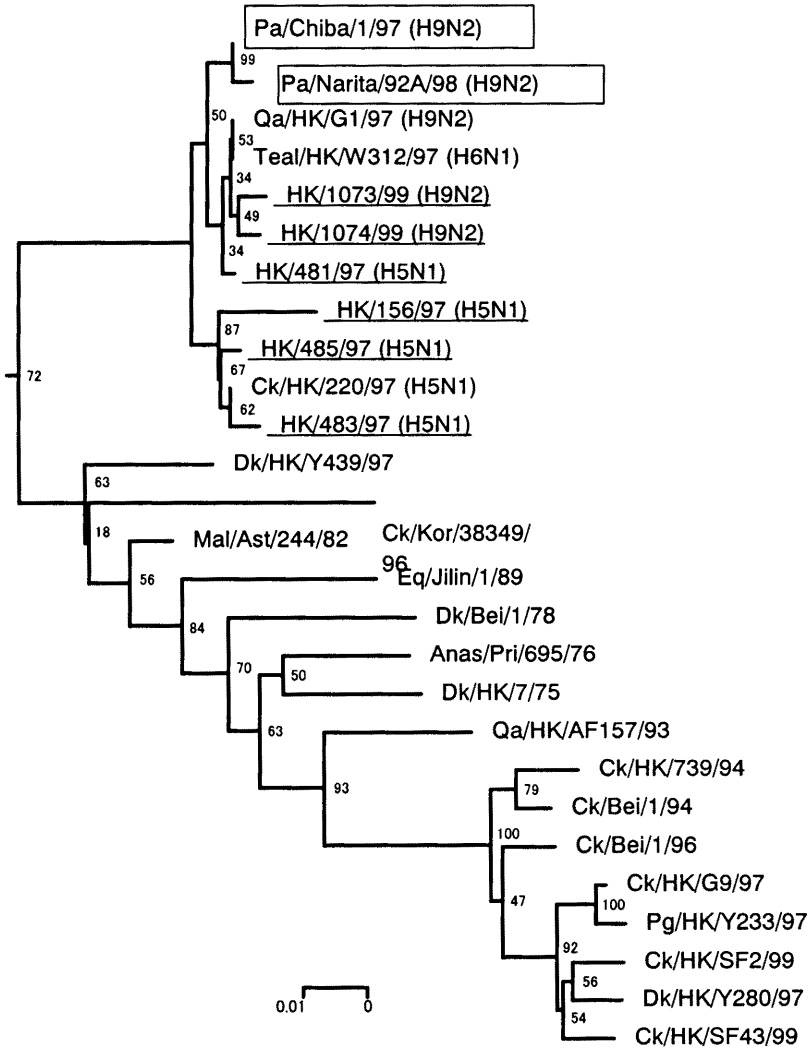

Fig.4. Evolutionary relationship of influenza A virus NP genes. Nucleotides 741-1398 (658bp) of the NP genes were used for phylogenetic analysis. All viruses underlined are isolated from human. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated from parakeets or humans are boxed or underlined, respectively. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value. Abbreviations: Pa (parakeet), Qa (quail), HK (Hong Kong), Ck (chicken), Dk (duck), Kor (Korea), Mal (mallard), Ast (Astrakhan), Eq (equine), Bei (Beijing), Anas (Anas acuta), Pri (Primorje), Pg (pigeon).

Table 6. Virus isolation from infected mice

Virus	Organs	Virus isolation at days postinoculation	
		3	6
Pa/Chiba/1/97	Brain	-	-
	Liver	-	-
	Spleen	-	-
	Kidney	-	-
	Lung	3/3 (4.8*)	1/3 (3.8)
Pa/Narita/92A/98	Brain	-	-
	Liver	-	-
	Spleen	-	-
	Kidney	-	-
	Lung	3/3 (5.2)	3/3 (4.7)

- : 0/3 (Not Isolated) *: average Log_{10} EID_{50}/g

Chapter II

Characterization of H5N1 influenza A viruses isolated during the 2003-2004 influenza outbreaks in Japan

Introduction

Highly pathogenic H5N1 influenza viruses attracted international attention when they were transmitted to 18 humans in Hong Kong in 1997, resulting in six deaths. (8,59). The virus continued to circulate in Asia and, in 2003, two more people in Hong Kong were infected, leading to one additional fatality (47). The viruses responsible for these human cases originate from the H5N1 viruses circulating among birds in southern China. In fact, the HAs of these H5N1 viruses can be traced back to a virus isolated from a goose in southern China in 1996 (A/goose/Guandong/1/96 (Go/GD; H5N1)), and H5N1 viruses of multiple genotypes have been circulating in this part of the world since that time (17,56,65). The NA of the virus responsible for the 1997 Hong Kong outbreak was derived from an N1 virus distinct from the Go/GD-like virus. Viruses of this genotype (i.e., those isolated from humans in Hong Kong in 1997) have since disappeared. Although H5N1 viruses emerged in land-based poultry in the markets of Hong Kong after 1997, immense depopulation of poultry in that country (18) quelled the outbreaks before the virus became widely disseminated. These H5N1 viruses were not only highly pathogenic to chickens and quail, but they were also lethal to mice without adaptation (although a high virus inoculum is required). Accordingly, the H5N1 avian influenza viruses continue to be a threat to public health.

Since late 2003, highly pathogenic H5N1 viruses have continued to cause outbreaks in Asian countries, including Vietnam, Korea, Cambodia, Laos, Thailand, Indonesia, and China. Some of these viruses have been transmitted to humans, for example, in Vietnam and Thailand (23,66), resulting in over 30 documented deaths (69). Although most of these countries declared themselves to be free of avian influenza in the spring of 2004 (67), these viruses appear to have returned to at least some of these countries (68).

From the end of December 2003 to March 2004, outbreaks of acute, highly transmissible and lethal disease in chickens occurred in Japan, involving three chicken farms and a group of chickens raised as pets. Here, we characterized the highly pathogenic H5N1 influenza viruses isolated from these outbreaks.

Materials and methods

Virus isolation and identification.

Tissue homogenates (i.e., trachea, lung, spleen, kidney, and rectum) from infected birds were inoculated into the allantoic cavity of 10-day-old embryonated eggs. Inoculated eggs were incubated at 37 °C for 1-2 days. The subtypes of the isolates were determined by HI and NI assays described in chapter I.

Virus stocks.

To prepare stocks for this study, viruses were propagated in the allantoic cavity of embryonated eggs once at 37 °C for 1-2 days and then stored at -80 °C until use.

Genetic and phylogenetic analyses.

To determine the genetic relationship between these isolates and other viruses, all eight genes of the isolates were sequenced. Viral RNA was extracted from virus-containing allantoic fluid by using a commercial kit (ISOGEN-LS, Nippongene, Tokyo, Japan). After reverse transcription with Superscript II (Life Technologies, Gaithersburg, MD) using random 9-mers, cDNAs were amplified by the polymerase chain reaction (PCR). PCR amplification of the coding regions of the viral gene segments was performed with gene-specific primer sets (Table.7). PCR-derived dsDNA was used as a template for sequencing on an Applied Biosystems 310 automated DNA sequencer using cycle sequencing dye terminator chemistry (Perkin-Elmer/Applied Biosystems, Foster City, CA). The nucleotide sequences were analyzed using version 12.0 of the sequence analysis software package GENETYX-MAC (Software Development, Tokyo, Japan). The phylogenetic trees were constructed, as described in chapter I. The nucleotide sequences obtained from this study are available from GenBank under accession numbers AB166859 to AB166866, AB188813 to AB188824, and AB189046 to AB189065.

Antigenic analysis.

The antigenic relationships among H5N1 influenza viruses were determined in HI tests by using polyclonal chicken antiserum and a panel of monoclonal antibodies. (26).

Pathogenicity tests.

Chickens. Six-week-old specific pathogen-free chickens were used in this study. Eight chickens were inoculated either intravenously or intranasally with 0.1 ml of virus (10^6 EID_{50}) and observed daily for 14 days.

Mice. To determine the mouse lethal $dose_{50}$ (MLD_{50},) six-week-old female BALB/c mice (n=32; SLC Japan) were anesthetized by pentobarbital inhalation. Mice were intranasally infected in groups of four with 50 ul of allantoic fluid in PBS (10-fold serial dilutions from 10^{-1} to 10^{-8} virus) and observed for 20 days. The organ tropism of the virus was assessed by intranasally inoculating mice with 50 ul of virus (10^6EID_{50}). Three mice were then sacrificed on day 3 and another three on day 6 postinfection so that viral titers in their lung, brain, spleen, kidney, and liver could be determined by inoculating tissue homogenates into the allantoic cavity of 10-day-old embryonated eggs.

Oseltamivir carboxylate sensitivity

The sensitivity of the viral NA to oseltamivir carboxylate was evaluated with an NA enzyme inhibition assay based on the method of Gubareva et al (20). Methylumbelliferyl-N-acetylneuraminic acid (MUNANA, Sigma), at a final concentration of 0.1 mM, was used as a fluorescent substrate. Virus dilutions containing between 800 and 1200 fluorescence units were used in the NA inhibition assay. Briefly, diluted virus and drug (0.01 nM - 1 mM) in 33 mM 2-[N-morpholino]ethanesulfonic acid (pH 6.0) containing 4 mM $CaCl_2$ were mixed and incubated for 30 min at 37°C, at which point, the substrate was added. After 1 hr at 37°C, the reaction was stopped by adding 0.1M NaOH in 80% ethanol (pH 10.0). Fluorescence was quantified at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The relationship between the concentration of inhibitor and the percentage of fluorescence inhibition was determined and IC_{50} values were obtained by extrapolating those findings.

Results

Outbreak information.

In late December 2003, layer chickens began to die in one of the seven chicken houses on a farm in the Yamaguchi prefecture located in the western part of Japan (Fig.5). Infection spread throughout the other chicken houses, resulting in the loss of a total of 34,000 chickens to either infection or to slaughtering in an effort to control the outbreak. Homogenates of organs (such as trachea, lung, spleen, kidney, and rectum) from the infected birds were inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs. Hemagglutinating agents were isolated from all the organs tested. We further evaluated the hemagglutinating agent isolated from the spleen, and it was identified to be an influenza A virus of H5N1 subtype (A/chicken/Yamaguchi/7/2004) by HI and NI assays. The second outbreak occurred in February 2004 in a flock of 13 chickens raised as pets in the Oita prefecture, which is located 150 Km from the site of the first outbreak. The sudden death of 7 of these chickens led to the slaughter of the remaining 6 chickens as well as one duck. H5N1 viruses were isolated from the lungs, tracheas, and spleens of these chickens and one of the isolates from spleen, A/chicken/Oita/8/2004, was used in this study. The third outbreak also occurred in February 2004, but 350-450 km away from the Yamaguchi and Oita prefectures in a commercial layer chicken farm in the Kyoto prefecture. This outbreak resulted in the loss of 225,000 chickens; the trachea from one of which was the source of another H5N1 virus isolate (A/chicken/Kyoto/3/2004). The fourthoutbreak also occurred in March 2004 in a commercial broiler chicken farm in the Kyoto prefecture, and involved the loss of 15,000 chickens. Following the third outbreak, H5N1 viruses were also isolated from a total of nine crows found dead within a 30 Km radius of the farm in Kyoto. A/crow/Kyoto/53/2004 and A/crow/Osaka/102/2004 were isolated from trachea samples and chosen as representative isolates for subsequent studies.

Antigenic characterization

Some antigenic differences were detected among the H5N1 viruses isolated in Japan; two isolates

from the third outbreak (A/chicken/Kyoto/3/2004 and A/crow/Kyoto/53/2004) failed to react with monoclonal antibody 77B1, whereas the other isolates were reactive (Table 8). These Japanese H5N1 isolates were also antigenically different from other previously characterized H5N1 viruses, including those isolated in Hong Kong in 1997 and 2003 (see reactivity with monoclonal antibodies 94F1 and 17C5), leaving the origin of these Japanese H5N1 isolates unclear.

Phylogenetic analysis

To determine whether the H5N1 strains isolated in Japan are related to those circulating in Asia since 1996, all eight gene segments of the five Japanese isolates were sequenced. All of the Japanese H5N1 isolates were genetically closely related to one another (>99% identity at the nucleotide level in all eight RNA segments; see Table 9), suggesting that these isolates share an immediate ancestor. All of the genes of these Japanese H5N1 isolates showed more than 98% nucleotide identity with that of the H5N1 virus isolated from a chicken in China in 2003 (A/chicken/Shantou/4231/2003, genotype V) (see Table 10; the results with the index A/chicken/Yamaguchi/7/2004 strain only is shown).

The phylogenetic analysis for these genes reinforced the above mentioned findings, leading us to conclude that the Japanese H5N1 isolates belong to the same genetic cluster as A/chicken/Shantou/4231/2003 (Fig.6-8). These results further suggest that H5N1 viruses of distinct genotypes were circulating in late 2003-early 2004; i.e., genotype Z in Indonesia, Thailand, Vietnam, China (Li et al, 2004), and genotype V in Japan (this study) and China (32).

Molecular markers

As is typical of all H5N1 viruses isolated since 1997, all of the Japanese isolates contained multiple basic amino acids at the HA cleavage site (Table 11). However, the precise amino acid sequences at this site were not identical among these isolates; the Japanese isolates had a single amino acid deletion at this site, as is the case for A/duck/China/E319-2/2003 and A/duck/Hong Kong/573.4/2001 (18). Of interest, there was divergence even among the Japanese isolates; the Oita isolate differed from other

viruses at this site. The biologic significance of this difference remains unknown.

When the NAs of the Japanese isolates were aligned with that of other H5 viruses, the Japanese isolates had a 20 amino acid deletion (positions at 49-68) identical to that found in isolates of the genotypes V and Z (32).

The presence of specific amino acids in two other gene products are known to affect the virulence of these viruses: glutamic acid-to-lysine mutation at position 627 in PB2 is associated with high virulence in mice in the 1997 Hong Kong viruses (22) and glutamic acid at position 92 in NS1 is associated with high virulence in pigs upon reassortment of the NS gene of H5N1 with A/Puerto Rico/8/34 (53). All of the Japanese isolates had glutamic acid at position 627 in PB2 and glutamic acid at position 92 in NS1.

There are two types of antiviral compounds for influenza A viruses: M2 ion channel blockers, such as amantadine and rimantadine, and NA inhibitors, such as oseltamivir and zanamivir (38). Resistance to these compounds is associated with particular mutations. Viruses become resistant to amantadine through a single amino acid substitution at position 26, 27, 30, 31, or 34 in the transmembrane region of the M2 protein (9); viruses become resistant to oseltamivir through a single amino acid substitution at position 119, 152, 274 or 292 in the NA active center (20). The Japanese isolates do not contain the amino acid residues in these proteins that would confer to them resistance to these antiviral compounds.

Pathogenicity

Pathogenicity testing, according to the guidelines established by the OIE, showed all five Japanese H5N1 isolates to be highly pathogenic for chickens, given that all chickens died within a day of being intravenously inoculated with one of these viruses. Upon intranasal inoculation of six-week-old specific pathogen-free chickens (n=8) with 10^6 EID_{50} of the A/chicken/Yamaguchi/7/2004 virus, each chicken died within three days.

To test the virulence of A/chicken/Yamaguchi/7/2004 in mammalian species, we determined the virus dose that would be lethal to 50% of mice exposed. This dose was 5 x 10^5 EID_{50}, indicating that this strain is not as virulent as some of the H5N1 viruses isolated from humans (14), but is as virulent as

most H5N1 viruses isolated from poultry (34). Although a high dose of virus was required to kill mice, this strain clearly has the ability to replicate well in mouse lung without prior adaptation and to spread to the brain. Its ability to spread to internal organs, however, was limited (Table 12).

Sensitivity to an NA inhibitor

NA inhibitors are thought to play an important role in controlling annual influenza epidemics, as well as pandemics caused by new influenza strains. One such inhibitor, oseltamivir, was used to limit infection among people involved in the effort to contain the avian influenza outbreaks in Japan. For this reason, we tested the sensitivity of one of the Japanese isolates to oseltamivir. The amount of virus required to inhibit 50% of the activity (IC_{50}) of oseltamivir carboxylate was 3.4 nM for A/chicken/Yamaguchi/7/2004, which is within the IC_{50} range for influenza viruses deemed sensitive to this drug (i.e., 1-10 nM; Gubareva et al., 2002). This finding thus validates the prophylactic use of oseltamivir during the eradication and containment effort.

Discussion

Here, we have shown that H5N1 influenza A viruses isolated in Japan between late 2003 and 2004 were closely related to each other. Pathogenic avian influenza viruses can emerge from repeated passages of non- or low-pathogenic viruses in poultry (27). However, we are unaware of any recent influenza cases caused by such viruses in poultry in Japan. Therefore, the emergence of these Japanese H5N1 viruses likely stems from either a single source that subsequently spread within the country or from multiple sources where viruses highly related to the Japanese isolates were circulating.

Recent outbreaks of H5N1 avian influenza in southeastern Asia were thought to have been caused by viruses of genotype Z (32). This genotype emerged in 2002, and has become a dominant H5N1 virus in southern China (19,32). Our genetic analysis showed that the Japanese H5N1 isolates clustered with a single genotype V virus isolated in Shantou, in the Guandong province of mainland China. However, because there is limited information regarding the viruses currently circulating in Asia, we were unable to pinpoint the exact origin of the Japanese isolates, which would be valuable for the prevention of future influenza outbreaks in Japanese poultry.

Of interest, the Japanese H5N1 viruses were isolated from dead crows. A large number of crows were observed near the chicken houses on the farms in Kyoto, suggesting that these crows were infected with the virus by direct contact with virus-contaminated material. Since crow deaths were not observed prior to chicken deaths and chickens did not have direct contact with crows, it is unlikely that chickens were infected from crows. Although many other species of dead birds, including dove, bulbul, thrush, and sparrow (as identified in a study by the Kyoto prefecture), were tested for virus during this period, none harbored the virus. The possibility exists, however, that there are avian species that harbor the virus without showing disease symptoms. Thus, crows may be exceptionally sensitive to these H5N1 viruses, although morbidity and mortality rates remain unknown. Given that the last virus-positive crow was found one month after the clean up of the farm in Kyoto, it is possible that the virus may have spread among crows. However, because no new viruses have been isolated for more than two months since the last virus isolation from a crow, it is likely that this virus is no longer

circulating in birds in Japan.

How were these viruses introduced into Japan? Several possibilities exist: feral birds, virus-contaminated material, and illegally imported infected birds are all possible sources. The exact source of the Japanese influenza outbreaks is unlikely to be found given the low likelihood of isolating virus-positive samples from any of these potential sources.

The H5N1 Japanese isolates were highly pathogenic for chickens, killing every exposed chicken within a day of intravenously injection. Many of the virus-inoculated chickens died suddenly without clinical signs and presented no apparent gross regions on postmortem examination. Indeed, this was also the case in the field. It is therefore important for veterinarians to recognize that these highly pathogenic avian influenza viruses may not cause classic signs and symptoms in their host, such as extensive hemorrhage.

The index Japanese H5N1 virus was lethal to mice without prior adaptation, replicating in the brain, lung, and spleen. The pathogenicity of this virus was thus similar to that reported by Lipatov et al. (34). Fortunately, there were no human cases reported during the outbreaks in Japan; however, these viruses clearly have the potential to infect mammals and cause severe illness, as has been recently demonstrated in Thailand and Vietnam (23,67). One possible explanation for the lack of human infection in Japan may be the prophylactic use of oseltamivir by the personnel involved in the effort to contain and eradicate the H5N1 virus, which we have shown to be sensitive to this drug. Alternatively, infectivity to humans might differ between the viruses of genotype Z that were responsible for human deaths in Thailand and Vietnam and those of genotype V isolated in Japan. Considering that H5N1 viruses have been circulating in Asia since 1996, they will no doubt remain in this part of the world and will likely be transmitted to other countries. It is, therefore, essential that we continue to be vigilant and prepare for future outbreaks by, for example, stockpiling anti-influenza drugs and vaccines recently produced with new technology, such as reverse genetics (41,70).

Summary

In Japan, between the end of December, 2003 and March, 2004, four outbreaks of acute, highly transmissible and lethal disease occurred in birds in three prefectures separated by 150-450 km, involving three chicken farms and a group of chickens raised as pets. The cause of each outbreak was an H5N1 influenza A virus - the first highly pathogenic virus to be isolated from the outbreaks in Japan since 1925. The H5N1 virus was also isolated from dead crows, apparently infected by contact with virus-contaminated material. These H5N1 viruses were antigenically similar to each other, but could be differentiated from other H5 viruses, including those isolated from Hong Kong in 1997 and 2003, by use of a panel of monoclonal antibodies in hemagglutination inhibition assays. Genetically, the H5N1 viruses in Japan were closely related to each other in all genes and were genetically closely related to a single isolate of genotype V that was isolated in 2003 in the Guandong Province of mainland China (A/chicken/Shantou/4231/2003). The virulence of the index isolate (A/chicken/Yamaguchi/7/2004) was studied in chickens and mice. Chickens intravenously or intranasally inoculated with the isolate died within 1 or 3 days of inoculation, respectively. In mice, although this virus replicated well in the lung without prior adaptation and spread to the brain, the dose lethal to 50% of the mice was 5×10^5 50% egg infectious doses (EID_{50}), which is less pathogenic than the Hong Kong 1997 H5N1 viruses isolated from humans. Our findings indicate that the H5N1 viruses associated with the influenza outbreaks in chickens in Japan were genotypically closely related to an H5N1 virus isolated from chicken in China in 2003 (genotype V), but were different from those prevalent in southeastern Asia in 2003-2004 (i.e., genotype Z) and that these highly pathogenic viruses can be transmitted to crows, which are highly susceptible to these viruses

Table.7. Nucleotide sequences of the primers used in this chapter.

Gene	Primer	Sequence
PB2	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-57F(PB2-95F)	CCACTGTGGATCATATGGCCA
	FLU-65R(PB2-750R)	CCTCCTGGTGTGTACATCTG
	FLU-66F(PB2-1621R)	TCATCGTCTATGATGTGGGA
	FLU-71F(PB2-1003F)	TCAAAAGAGAGGAAGAAGTGCT
	FLU-74R(PB2-1702R)	TCTCCCAATTCCTAATGATCCA
	FLU-73F(PB2-679F)	CTACCAGTGGCTGGTGGGACAAG
	FLU-72R(PB2-1203R)	TTGCTCGTCCCTCCCGCTGACTAT
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
PB1	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-69F(PB1-398F)	TCGCCAGACCTATGACTGGACATT
	FLU-70R(PB1-1135R)	GTGTCCGTAGCTTCATGCTCTT
	FLU-85F(PB1-933F)	GGAGACAACACCAAATGGAATGA
	FLU-86R(PB1-1619R)	TATCACTGTAACTCCAATGCTCAT
	FLU-87F(PB1-1480F)	GGACAGGAACATTTGAGTTCAC
	FLU-89R(PB1-514R)	CTCATTGGCTGTTAGACCGTTCGA
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
PA	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-79R(PA-633R)	CGCCTCTCTCGGACTGACGAAAGGA
	FLU-90F(PA-365F)	CCGATTCACTGAAATTGGTGTGA
	FLU-67F(PA-740F)	CTGCATTGAGGGCAAGCTTTCTCA
	FLU-68R(PA-1368R)	TTCCTTCTCATGCTTGCAATGTG
	FLU-81F(PA-1351R)	AGATCGCTAGCAAGCTGGAT
	FLU-82R(PA-1765R)	CTCATTTCCATCCCCCATTTCAT
	M13-20-Rconsensus'	GTAAAACGACGGCCAGT AGTAGAAAC
HA	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	H5-198R	ATC GCA GAG CTT CCC GTT GTG
	H5-155F	ACA CAT GCY CAR GAC ATA CT

HA	H5-699R	CTY TGR TTY AGT GTT GAT GT
	FLU-37Fm (H5-427F)	TAATGCCTCRTCAGGRGTGA
	H5-1066R	TTT ATA GAG GGA GGA TGG CAG
	FLU-26R (H5-1105R)	CCATACCAWCCRTCTACCATTCC
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
NP	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	NP 184R	GTTTGAGTTCAGTGCACATCTGTA
	FLU-61F(NP-81F)	AAC TGG TGG AGA ACG CCA GAA TGC
	FLU-56R(NP-1464R)	AGG CAC GAT CGG GTT CGT TG
	FLU-62R(NP-1154R)	TCCACGTTCTCATTTGAAGC
	NP 1087F	ACAAGAGTAATCCCAAGAGGACAA
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
NA	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	N1N-R(N1-593R)	CAA CCA ACT AGT GCC ATC ATG
	FLU-11Fm(N1-534F)	TTGCTTGGTCAGCAAGTGC
	FLU-12R(N1-1149R)	TCTGTCCATCCATTAGGATCC
	N1-1076F	ATA CGG CAA TGG TGT TTG GAT
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
M	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-04R(M-508R)	TGCTGGGAGTCAGCAATCTG
	FLU-15F(M-489F)	CACGCTCACCGTGCCCAGTG
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC
NS	M13-20-Fconsensus	GTAAAACGACGGCCAGT AGCAAAAGCAGG
	FLU-20R(NS-656R)	CCCATTCTCATTACTGCTTC
	FLU-21F(NS-108F)	GGT GAT GCC CCA TTC CTT GA
	M13-20-Rconsensus	GTAAAACGACGGCCAGT AGTAGAAAC

Table 8. Antigenic analysis of H5N1 influenza viruses isolated in Japan in 2004

viruses	HI titers with the following:									Reference
	Polyclonal antiserum to Tern/South Africa/61 (Hyperimmune)	Polyclonal antiserum to Ty/Ontario/ 7732/66 (Hyperimmune)	Monoclonal antibodies to HK/156/97				Monoclonal antibodies to HK/486/97		Monoclonal antibodies to Ty/Ontario/77 32/66		
			31G1	61B2	62H7	94F1	14F8	17C5	24B9	77B1	
A/chicken/Yamaguchi/7/2004 (H5N1)	800	100	800	3200	800	100	200	<100	<100	1600	This study
A/chicken/Oita/8/2004 (H5N1)	800	100	100	400	200	100	<100	<100	<100	400	This study
A/chicken/Kyoto/3/2004 (H5N1)	800	100	100	400	100	<100	<100	<100	<100	<100	This study
A/crow/Kyoto/53/2004 (H5N1)	800	100	400	200	100	<100	<100	<100	<100	<100	This study
A/crow/Osaka/102/2004 (H5N1)	400	100	200	200	100	<100	<100	<100	<100	400	This study
A/tern/South Africa/61 (H5N3)	6400	200	800	800	400	400	<100	400	<100	<100	7
A/swan/Shimane/499/83 (H5N3)	1600	200	800	400	<100	200	<100	200	400	400	27
A/duck/Hokkaido/67/96 (H5N4)	1600	200	100	<100	<100	400	<100	1600	<100	200	61
A/Hong Kong/156/97 (H5N1)	1600	200	1600	3200	1600	800	200	3200	<100	800	14
A/Hong Kong/483/97 (H5N1)	800	200	200	400	400	400	<100	800	<100	<100	14
A/Hong Kong/213/2003 (H5N1)	3200	800	<100	800	200	800	<100	6400	100	12800	19
A/turkey/Ontario/7732/66 (H5N9)	400	1600	<100	<100	<100	<100	<100	<100	800	6400	30

Note: Polyclonal and monoclonal antibodies were used at a starting dilution of 1: 100.

Table 9. Nucleotide and amino acid differences among Japanese H5N1 influenza viruses

Segment	No. of nucleotides compared	No. of nucleotide (amino acid) differences between the index A/chicken/Yamaguchi/7/2004 strain and:			
		A/chicken/Oita/ 8/2004	A/chicken/Kyoto/ 3/2004	A/crow/Kyoto/ 53/2004	A/crow/Osaka/ 102/2004
PB2	2280	5 (0)	5 (1; *I463V)	6 (1; I463V)	8 (2; T339M, G698S)
PB1	2274	7 (2; C80S,F94Y)	1 (1; C80S)	1 (1; C80S)	1 (1; C80S)
PA	2151	4 (2; G66D, V596I)	7 (1; V596I)	7 (1; V596I)	7 (2; E352D, V596I)
HA	1704	9 (4; A83V, I219V, R325K, S513N)	7 (2; K152T, I219V)	6 (2; K152T, I219V)	5 (2; S123P, I219V)
NP	1497	1 (0)	1 (0)	1 (0)	2 (0)
NA	1350	7 (1; Q45K)	7 (1; N289D)	8 (2; P252S, N289D)	10 (2; V129A, I294M)
M	982	0 (M1, 0) (M2, 0)	1 (M1, 0) (M2, 0)	1 (M1, 0) (M2, 0)	1 (M1, 0) (M2, 0)
NS	823	3 (NS1 1; L3P) (NS2 2; L3P, A90T)	3 (NS1 2; L3P, S73P) (NS2 2; L3P, A90T)	4 (NS1 2; L3P, S73P) (NS2 2; L3P, A90T)	2 (NS1 1; L3P) (NS2 2; L3P, A90T)

*The amino acid differences found between A/chicken/Yamaguchi/7/2004 and others are shown as follows:
amino acid residue found in A/chicken/Yamaguchi/7/2004, amino acid position,
and amino acid residue found in the virus in question.

Table 10. Sequence comparison between A/chicken/Yamaguchi/7/2004 and other recent H5N1 influenza viruses

Segment	% nucleotide (amino acid) homology with A/chicken/Yamaguchi/7/2004		
	A/chicken/Shantou/4231/2003 (genotype V)	A/goose/Thailand/79/2004 (genotype Z)	A/HongKong/212/2003 (genotype Z+)
PB2	99.2 (98.9)	98.9 (99.6)	98.9 (99.3)
PB1	99.3 (99.6)	98.5 (99.3)	99.1 (99.6)
PA	99.6 (99.2)	93.6 (97.9)	93.8 (97.8)
HA	98.5 (98.1)	97.2 (97.0)	97.3 (97.3)
NP	99.5 (99.1)	98.7 (100)	99.1 (99.8)
NA	98.8 (98.4)	97.3 (96.4)	89.8 (92.1)
M	99.4 (M1 99.6) (M2 100)	98.8 (M1 99.6) (M2 96.9)	98.9 (M1 100) (M2 96.9)
NS	99.3 (NS1 97.8) (NS2 96.7)	98.2 (NS1 97.3) (NS2 98.3)	98.5 (NS1 98.2) (NS2 97.5)

Table 11. HA connecting peptide sequences of recent Asian H5N1 viruses

Viruses	Connecting peptide amino acid sequence								Reference
A/chicken/Yamaguchi/7/2004	R	E	-	R	R	K	K	R	This study
A/chicken/Oita/8/2004	R	E	-	K	R	K	K	R	This study
A/chicken/Kyoto/3/2004	R	E	-	R	R	K	K	R	This study
A/crow/Kyoto/53/2004	R	E	-	R	R	K	K	R	This study
A/crow/Osaka/102/2004	R	E	-	R	R	K	K	R	This study
A/HongKong/156/97	R	E	R	R	R	K	K	R	59
A/chicken/Hong Kong/YU822.2/2001	R	E	R	R	R	K	K	R	18
A/chicken/Hong Kong/YU562/2001	R	E	R	R	R	K	K	R	18
A/pheasant/Hong Kong/FY155/2001	R	E	R	R	R	K	K	R	18
A/chicken/Hong Kong/FY150/2001	R	E	R	R	R	K	K	R	18
A/chicken/Hong Kong/NT873.3/2001	R	E	I	R	R	K	K	R	18
A/duck/Hong Kong/573.4/2001	R	E	-	R	R	K	K	R	17
A/duck/AVL-1/Anyang/2001	R	E	R	R	R	K	K	R	62
A/teal/China/2978.1/2001	R	E	I	R	R	K	K	R	32
A/pheasant/Hong Kong/sv674.15/2002	R	E	R	R	R	K	K	R	19
A/chicken/Hong Kong/96.1/2002	R	E	R	R	R	K	K	R	19
A/Hong Kong/213/2003	R	E	R	R	R	K	K	R	19
A/duck/China/E319-2/2003	R	E	-	R	R	R	K	R	32
A/chicken/Shantou/4231/2003	R	E	R	R	R	K	K	R	32
A/chicken/Indonesia/BL/2003	R	E	R	R	R	K	K	R	32
A/Vietnam/1196/2004	R	E	R	R	R	K	K	R	32
A/goose/Thailand/79/2004	R	E	R	R	R	K	K	R	32

Table 12. Growth of A/chicken/Yamaguchi/7/2004 in mice

Organs	days after infection (log_{10} EID_{50}/g)*	
	3	6
Brain	0/3	2/3 (4.0, 3.3)
Lung	3/3 (5.4±1.0)	3/3 (5.2±1.9)
Spleen	3/3 (4.3±1.0)	1/3 (3.3)
Liver	0/3	0/3
Kidney	0/3	0/3

* Virus titers in the organs shown were determined. Mean±SD for the number of virus-positive mice that were sacrificed.

Fig.5. The location of the Japanese prefectures in which H5N1 highly pathogenic avian influenza outbreaks occurred.

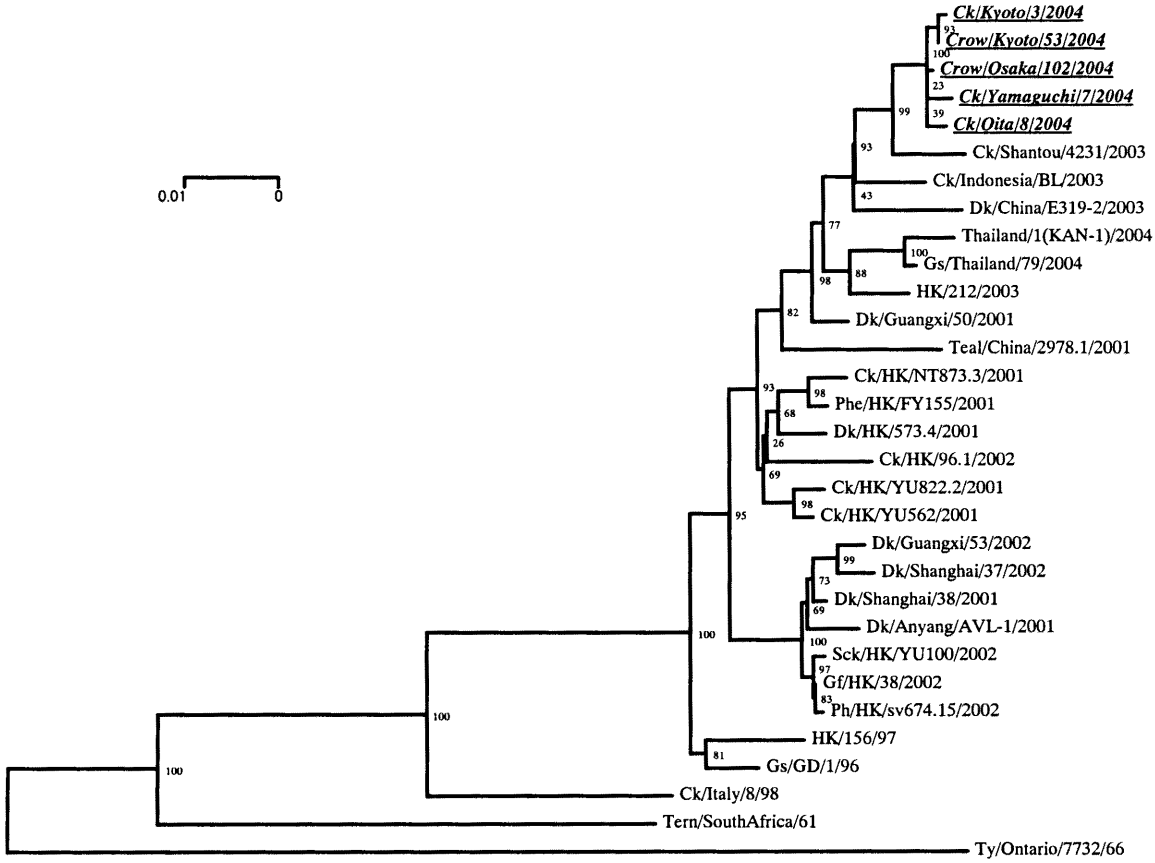

Fig.6. Phylogenetic trees of the H5 gene of influenza A viruses. Nucleotides 42-1544 (1503 bases) of the H5 HAs was used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated in Japan are underlined in italic. Numbers at the nodes indicate confidence levels of bootstrap analysis with 100 replications as a percentage value. Abbreviations: Ck (chicken), Dk (duck), GD (Guandong), Gf (guinea fowl), Gs (goose), HK (Hong Kong), Hokk (Hokkaido), Ph(pheasant), PR (Puerto Rico), Sck (silky chicken), TW (Taiwan), Ty (turkey).

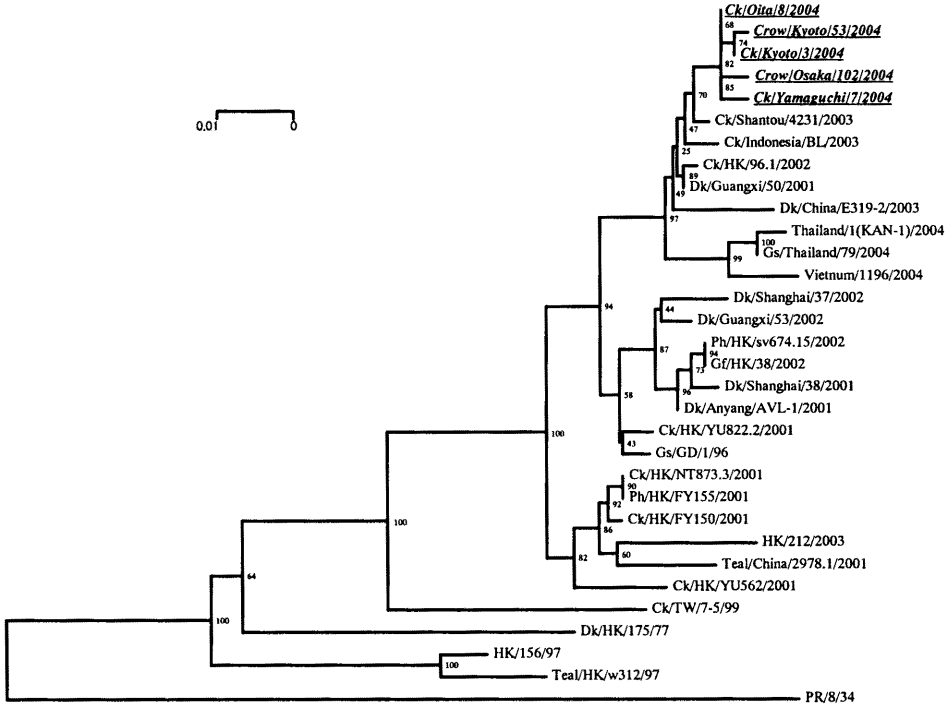

Fig.7. Phylogenetic trees of theN1 NAgene of influenza A viruses. Nucleotides 510-1061 (552 bases) of the N1 Nas gene was used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated in Japan are underlined in italic. Numbers at the nodes indicate confidence levels of bootstrap analysis with 100 replications as a percentage value. Abbreviations: Ck (chicken), Dk (duck), GD (Guandong), Gf (guinea fowl), Gs (goose), HK (Hong Kong), Hokk (Hokkaido), Ph(pheasant), PR (Puerto Rico), Sck (silky chicken), TW (Taiwan), Ty (turkey).

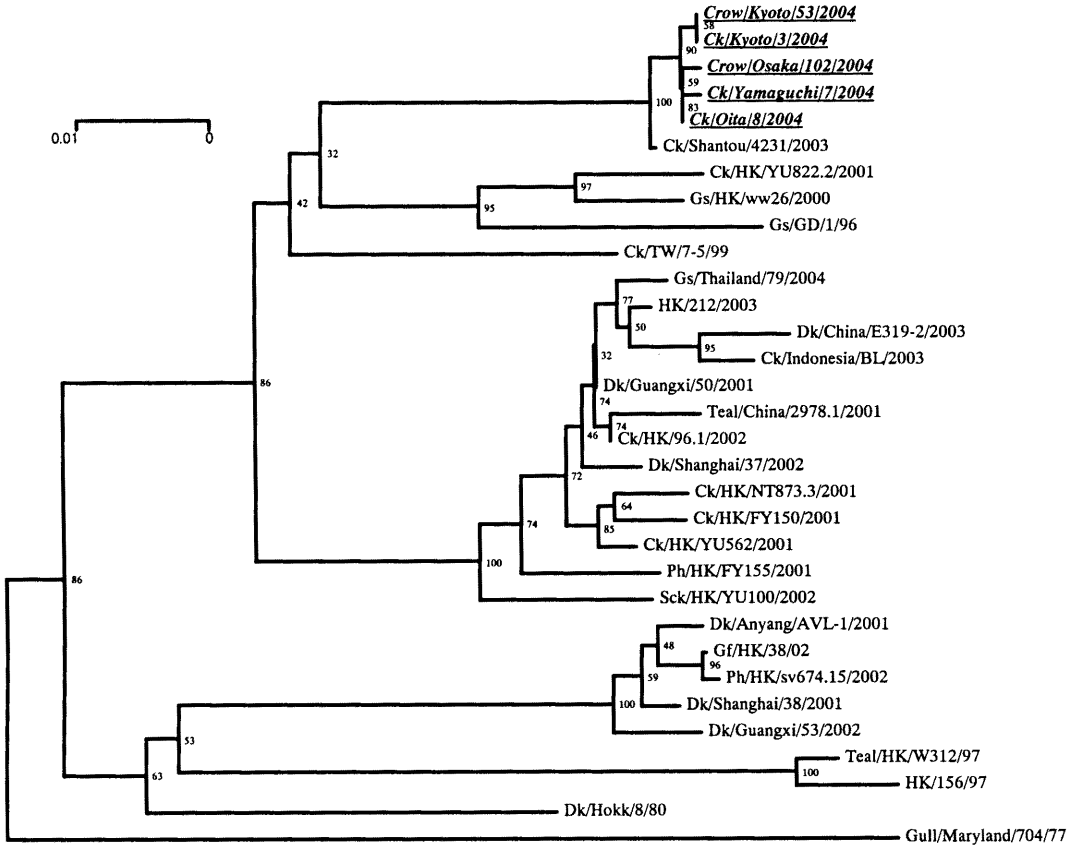

Fig.8. Phylogenetic trees of the PA gene of influenza A viruses. Nucleotides 1431-2168 (738 bases) of the PA gene was used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated in Japan are underlined in italic. Numbers at the nodes indicate confidence levels of bootstrap analysis with 100 replications as a percentage value. Abbreviations: Ck (chicken), Dk (duck), GD (Guandong), Gf (guinea fowl), Gs (goose), HK (Hong Kong), Hokk (Hokkaido), Ph(pheasant), PR (Puerto Rico), Sck (silky chicken), TW (Taiwan), Ty (turkey).

Chapter III

Isolation of a genotypically unique H5N1 influenza virus from duck meat imported into Japan from China

Introduction

Since its first detection in southern China (i.e., A/goose/Guandong/1/96), H5N1 influenza A virus has spread in Asian countries, where it is now enzootic, causing multiple outbreaks in poultry and even transmission to wild birds (12,17,32,65). H5N1 influenza A viruses pose a serious threat to public health, having been directly transmitted from birds to humans multiple times since 1997, resulting in over 40 confirmed deaths (8,23,47,71,72). In Japan, between the end of December 2003 and February 2004, influenza outbreaks caused by an H5N1 virus occurred in birds located at three distinct chicken farms and among a group of chickens raised as pet birds described in chapter II. However, the route of introduction and dissemination of the virus remain unknown.

A highly pathogenic H5N1 influenza virus has been isolated from duck meat imported from China to Korea (62). This virus was highly pathogenic in chickens and replicated efficiently in the lungs of mice without prior adaptation. Thus, the importation of poultry products is a potential source of highly pathogenic H5N1 virus and a risk to public health.

In Japan, to prevent such introduction of highly pathogenic H5N1 influenza viruses through imported poultry products, duck meat has been randomly sampled by the Animal Quarantine Service for virus isolation. During this routine surveillance in May of 2003, H5N1 viruses were isolated from duck meat imported from China. Here we describe the properties of one of these isolates.

Materials and Methods

Virus isolation and identification.

Virus isolation from duck breast meat was performed at the Animal Quarantine Service (AQS). Briefly, applications were submitted to AQS to import lots of duck breast meat for human consumption (500-3,000 cartons/lot under a single import application). One carton from each imported lot was randomly selected and tested for the presence of influenza virus by sampling from each of 10 packages per carton, one gram of meat, which was pooled and made into a 10% homogenate in PBS. The homogenates were then centrifuged at low speed (3,000 x g for 10 min) and the supernatants were filtered through a sterile 0.45μm membrane filter before inoculation into the allantoic cavity of embryonated specific pathogen-free eggs. The viruses were identified as influenza A virus of the H5N1 subtype by HI and NI assays described in chapter I. Virus was propagated for 24-36 hours in the allantoic cavity of eggs at 37°C, after which time the allantoic fluid was harvested, aliquoted, and stored at -80 °C until use.

Genetic and phylogenetic analysis.

RNA extraction, RT-PCR, and sequencing of the PCR products were carried out as described in chapter I. The primers are listed in Table. 8. in chapter II. The nucleotide sequences were analyzed using version 12.0 of the sequence analysis software package GENETYX-MAC (Software Development, Tokyo, Japan). Phylogenetic trees were constructed as described in chapter I.

The nucleotide sequences of the genes of A/duck/Yokohama/aq10/2003 are available from GenBank under accession numbers AB212277 to AB212284.

Antigenic analysis.

Antigenic relationships of the viruses were determined by HI tests using a panel of monoclonal antibodies as previously described in chapter II.

Pathogenicity tests.

Chickens. Six-week-old specific pathogen-free chickens were inoculated either intravenously (n=8) (43) or intranasally (n=10) with 0.1 ml of virus (10^6 EID_{50}), and clinical signs were observed daily.

Mice. Six-week-old female BALB/c mice (SLC Japan, Tokyo) were used in all experiments. Mice were anesthetized by pentobarbital inhalation, before they were inoculated intranasally (i.n.) with 50 ul of infectious virus diluted in PBS. MID_{50} and MLD_{50} titers were determined by inoculating groups of eight mice i.n. with serial 10-fold dilutions of virus, as described by Lu (35). Four days later, four mice from each group were euthanized, and their lungs removed and homogenized. Solid debris was pelleted by centrifugation, and tissues were titrated for virus infectivity in eggs. The four remaining mice in each group were checked daily for signs of disease and death for 14 days postinfection (p.i.). MID_{50} and MLD_{50} titers were calculated by the method of Reed and Muench (51). To determine the organ tropism of the virus, mice (n=24) were intranasally inoculated with 50 ul of virus (10^6 EID_{50}). Three mice were sacrificed on days 3 and 6 p.i. and viral titers in brain, lung, liver, spleen, and kidney were determined by inoculating tissue homogenates into the allantoic cavity of 10-day old embryonated eggs. The remaining mice (n=18) were observed for clinical signs of disease and mortality. Virus titers in representative organs from the dead mice were also determined using embryonated eggs.

Recovery of variants from mouse brain.

Brain homogenates from two mice that died following inoculation with the original duck meat isolate were inoculated into the allantoic cavity of 10-day old embryonated eggs. The viruses isolated from these samples were designated as brain variants 1 and 2 (MBV-1 and MBV-2). Complete nucleotide sequences of both MBVs were determined as described above.

Results

Virus isolation and identification.

Since the isolation of highly pathogenic H5N1 influenza A viruses from duck meat imported from China to South Korea, the Animal Quarantine Service in Japan has monitored poultry meat imported from China for influenza viruses. From March-May 2003, 14 lots of duck breast meat were processed and shipped from a food factory in China. Of these 14 lots, three received from Shandong province tested positive for H5N1 influenza viruses. Virus titers in samples from the meat were approximately $10^{0.5}$ - $10^{4.5}$ EID_{50} /g. Partial nucleotide sequence analysis revealed that these isolates were more than 99% genetically similar in all eight segments, indicating that they all originated from the same source. One, A/duck/Yokohama/aq10/2003 (Dk/Yokohama/aq10/03), was chosen as a representative isolate for subsequent characterization.

Antigenic and phylogenetic analyses.

Using a panel of monoclonal antibodies to H5 hemagglutinin (HA), we found the antigenicity of Dk/Yokohama/aq10/03 to be different from that of other H5N1 viruses, including those strains isolated from humans in Hong Kong in 1997 and 2003 (see reactivity with monoclonal antibodies, 62H7 and 77B1, Table 1). Dk/Yokohama/aq10/03 was also antigenically different from the H5N1 viruses A/chicken/Yamaguchi/7/04 and A/chicken/Kyoto/3/2004, recently isolated in Japan (see reactivity with 17C5, Table 13).

To assess the genetic relationship of Dk/Yokohama/aq10/03 to other H5N1 viruses circulating in Asia since 1996, the sequences of all eight of its gene segments were determined, compared with those in GenBank (Tables 14), and phylogenetically analyzed. Phylogenetic analysis of representative genes (i.e., the genes for HA, PB2, PA, and nucleoprotein [NP]) are shown in Fig. 9-12. We found that the genotype of Dk/Yokohama/aq10/03 was unique and distinct from any of the H5N1 virus genotypes previously reported, including a recent isolate in Japan (Tables 14 and Fig. 9-12). Six genes (PB1, PA, HA, NA, M,

and NS) of Dk/Yokohama/aq10/03 showed > 97 % nucleotide identity with those of the Asian H5N1 viruses isolated from 2000 to 2004. Phylogenetic analysis of the HA gene confirmed this similarity (Fig.9). However, the other genes of Dk/Yokohama/aq10/03 were distinct from those of known H5N1 viruses. For example, the PB2 gene was closely related to a Dutch H7N7 human isolate (A/Netherlands/1/2003), which was transmitted directly from a chicken. Similarly, the PA gene was most closely related to a A/chicken/Shantou/4231/2004 strain and to a recent H5N1 isolate from Japan, and the NP gene most closely related to an H3N8 isolate from an aquatic bird (A/aquatic bird/Hong Kong/399/99) (Table 14 and Fig. 10 -12). These results indicate that an H5N1 virus with a novel, previously unreported genotype was circulating in China.

Pathogenicity of the original Dk/Yokohama/aq10/2003 strain.

Upon testing the pathogenicity of Dk/Yokohama/aq10/2003 using a procedure recommended by the OIE, the strain was judged highly pathogenic for chickens, killing all chickens exposed to the virus within 2-3 days of intravenous injection (43). Intranasal inoculation of six-week-old specific pathogen-free chickens (n=10) with 10^6 EID_{50} of virus killed all 10 chickens within 2-6 days (average 4.6 days).

To test the virulence of the Dk/Yokohama/aq10/2003 strain to mammalian species, we determined the 50% mouse infectious dose (MID_{50}) and 50% mouse lethal dose (MLD_{50}). The MID_{50} and MLD_{50} were 1.6×10^6 and 5×10^6 EID_{50}, respectively, indicating that the virulence of this strain is similar to that of the majority of H5N1 strains isolated from poultry in Hong Kong in 2001 (34). Although a high viral load was required to kill the mice in this experiment, it is worth noting that this strain had the ability to replicate in mouse lung without prior adaptation (Table 15).

Pathogenicity of variants isolated from the brain of mice infected with Dk/Yokohama/aq10/2003.

To determine the extent of mutations required for the duck meat isolate to exhibit high virulence in mice, we examined virus recovered from the dead mice infected with Dk/Yokohama/aq10/2003. Two mice died on day 11 postinfection and virus was isolated only from the brain, but not from other organs tested, of these mice (Table 16). The virus from the brain of each of these dead mice, designated mouse brain

variant-1 and 2 (MBV-1 and MBV-2), was markedly more virulent than the original virus, although their replication was largely limited to lung and brain tissue. Approximately $10^{4.7}$ fold less of the brain variant virus was required to exhibit similar infectivity and lethality to that achieved by the original virus (Table 17).

Comparison of the original Dk/Yokohama/aq10/03 and its mouse brain variants.

Like other H5N1 viruses, Dk/Yokohama/aq10/03 contains multiple basic amino acids (PQRERRRKKR/G) at its HA cleavage site. However, the NA stalk, which contains a deletion in some H5N1 viruses, especially those from land-based birds and from most humans, was retained intact in Dk/Yokohama/aq10/03 (Fig.13) and in both of the mouse brain variants.

Resistance to the two types of influenza antiviral compounds (M2 ion channel blockers, e.g., amantadine and rimantadine, and NA inihibitors, e.g., oseltamivir and zanamivir (37)) is associated with particular mutations. Viruses become resistant to amantadine through a single amino acid substitution at position 26, 27, 30, 31, or 34 in the transmembrane region of the M2 protein (9), and they can become resistant to oseltamivir through a single amino acid substitution at position 119, 152, 274, 292 or 294 in the NA active center (20,29). None of these amino acid substitutions were found in either the original virus or the mouse brain variants.

Compared to the original virus, both of our variants had three nucleotide, and concomitantly, three amino acid substitutions, although the amino acid substitutions found in these variants were unique to each other (Table 18). One of the PB2 mutations found in MBV-1 (E-to-K at position 627) is noteworthy because this mutation was responsible for the high virulence of the 1997 Hong Kong H5N1 viruses in mice (22). These results show that only a limited number of substitutions are necessary to convert the viruses isolated from duck meat to ones exhibiting high virulence in mice.

Discussion

Here, we have shown that a highly pathogenic H5N1 virus was present in imported duck breast meat and that only a few substitutions are required for this virus to exhibit high virulence in mice.

An H5N1 virus was also isolated from duck meat in Korea in 2001 (62). The origin of this Korean isolate was a duck farm near Shanghai and thus different from that of the Japanese H5N1 isolate characterized here, which came from duck meat originating in Shandong province. Phylogenetic analysis demonstrated that Dk/Yokohama/aq10/03 is genetically distinct from the Korean isolate and from any other H5N1 isolate reported to date. These results indicate that H5N1 viruses, of multiple genotypes, with potential virulence to poultry and humans may still be circulating in ducks in China and that these viruses could be a source for the introduction of highly pathogenic viruses into other countries.

The pathogenicity of Dk/Yokohama/aq10/03 to chickens and mice was similar to that observed with the majority of A/goose/Guandong/1/96-like H5N1 viruses from chickens or pheasants in Hong Kong (18). Dk/Yokohama/aq10/03, however, became lethal upon acquiring three amino acid substitutions. Interestingly, in a similar study by Lipatov et al. (33), of four mouse brain variants, two of the variants had 12 and 18 amino acid substitutions, respectively, in a single passage in mice. In the Lipatov study, all four mouse brain variants had mutations in their PA gene (although the amino acid substitutions found in these viruses were unique to each strain), whereas we found no PA substitutions in our variants. Rather, our variants possessed mutations in PB2, NP and NS2. Thus, multiple different amino acid mutations seem to be associated with virulence to mice. To unequivocally determine the specific contribution of these various amino acid residues to virulence in mice, viruses containing these particular mutations should be generated by reverse genetics (41).

Although only one of our two mouse brain variants possessed the E-to-K mutation at position 627 in PB2, this was the only common amino acid substitution detected in the variants studied by Lipatov et al (34). This PB2 mutation is responsible for the high virulence of Hong Kong H5N1 virus in mice (22). It was also detected in an H7N7 virus isolated from a lethal human case during an outbreak in the Netherlands in 2003 (13) and in H5N1 viruses isolated from patients who died from their infection in 2004

(15,32,50). Notably, the PB2 lineage to which Dk/Yokohama/aq10/03 and the Netherlands H7N7 virus belong is different from that of the1997 Hong Kong H5N1 viruses (see Fig. 1a), which may reflect the dominant nature of this mutation. Thus, while highly pathogenic avian viruses become lethal to mammalian species as a result of multiple different mutations, the E-to-K mutation in PB2 appears to be an important event in this process.

Waterfowl are the natural reservoir of all influenza A viruses, which are usually nonpathogenic in wild aquatic birds. However, an H5N1 virus isolated in 2002 in Hong Kong replicated to high titers in ducks, causing systemic infection and pathology in multiple organs, particularly the brain (57). This discovery changed our belief that ducks are resistant to influenza viruses that are highly pathogenic to chickens (3). The pathogenicity of Dk/Yokohama/aq10/03 to waterfowl, including ducks, is unknown. However, the isolation of H5N1 viruses from duck meat reveals a previously unrecognized source for human exposure to potential highly pathogenic viruses. It appears that H5N1 viruses are now enzootic in Asia (32), and with the use of inactivated poultry vaccines that do not prevent infection per se, these viruses will likely remain in the region for some time. Clearly, in countries where highly pathogenic avian influenza viruses are not yet enzootic, extensive monitoring of poultry products originating from the countries where the H5N1 viruses are enzootic should be continued to eliminate the risk of human infection and the possibility of outbreaks in poultry.

Summary

An H5N1 influenza A virus was isolated from duck meat processed for human consumption, imported to Japan from Shandong Province, China in 2003. This virus was antigenically different from other H5 viruses, including the Hong Kong H5N1 viruses isolated from humans in 1997 and 2003. Sequence analysis revealed that six genes (PB1, PA, HA, NA, M, and NS) of this virus showed >97 % nucleotide identity with their counterparts from recent H5N1 viruses, but that the remaining two genes (PB2 and NP) were derived from other unknown viruses. This duck meat isolate was highly pathogenic to chickens upon intravenous or intranasal inoculation, replicated well in the lungs of mice and spread to the brain, but was not as pathogenic in mice as H5N1 human isolates (with a dose lethal to 50% of mice (MLD_{50}) = 5 x 10^6 50% egg infectious doses [EID_{50}]). However, viruses isolated from the brain of mice previously infected with the virus were substantially more pathogenic (MLD_{50}. = ~ 10^2 EID_{50}) and possessed some amino acid substitutions relative to the original virus. These results show that poultry products contaminated with influenza viruses of high pathogenic potential to mammals are a threat to public health even in countries where the virus is not enzootic and represent a possible source of influenza outbreaks in poultry.

Table 13. Antigenic analysis of H5N1 influenza viruses isolated from duck meat imported from China in 2003

Viruses	hemagglutination inhibition (HI) titers with :										Reference
	Polyclonal antiserum to Tern/South Africa/61 (Hyperimmune)	Polyclonal antiserum to Ty/Ontario/ 7732/66 (Hyperimmune)	Monoclonal antibodies to HK/156/97				Monoclonal antibodies to HK/486/97		Monoclonal antibodies to Ty/Ontario/ 7732/66		
			31G1	61B2	62H7	94F1	14F8	17C5	24B9	77B1	
A/duck/Yokohama/aq10/2003 (H5N1)	800	100	100	400	<100	1600	<100	1600	<100	<100	This study
A/chicken/Yamaguchi/7/2004 (H5N1)	800	100	800	3200	800	100	200	<100	<100	1600	This study
A/chicken/Kyoto/3/2004 (H5N1)	800	100	100	400	100	<100	<100	<100	<100	<100	This study
A/tern/South Africa/61 (H5N3)	6400	200	800	800	400	400	<100	400	<100	<100	7
A/swan/Shimane/499/83 (H5N3)	1600	200	800	400	<100	200	<100	200	400	400	27
A/duck/Hokkaido/67/96 (H5N4)	1600	200	100	<100	<100	400	<100	1600	<100	200	61
A/Hong Kong/156/97 (H5N1)	1600	200	1600	3200	1600	800	200	3200	<100	800	14
A/Hong Kong/483/97 (H5N1)	800	200	200	400	400	400	<100	800	<100	<100	14
A/Hong Kong/213/2003 (H5N1)	3200	800	<100	800	200	800	<100	6400	100	12800	19
A/turkey/Ontario/7732/66 (H5N9)	400	1600	<100	<100	<100	<100	<100	<100	800	6400	30

Note: Polyclonal and monoclonal antibodies were used at a starting dilution of 1: 100.

Table 14. Genetic homology of A/duck/Yokohama/aq10/2003 to other influenza A viruses

Segment	Number of nucleotides examined	Viruses with the highest homology [a]	% homology
PB2	2280	A/chicken/Netherlands/1/2003 (H7N7)	96.9
PB1	2274	A/duck/Zhejiang/52/2000 (H5N1)	98.8
PA	2151	A/chicken/Shantou/4231/2004 (H5N1)	97.5
HA	1707	A/chicken/Jilin/9/2004 (H5N1)	97.0
NP	1497	A/aquatic bird/Hong Kong/399/99 (H3N8)	97.2
NA	1410	A/egret/Hong Kong/757.2/2003 (H5N1)	98.9
M	982	A/goose/Hong Kong/3014.8/2000 (H5N1)	99.1
NS	823	A/duck/Hong Kong/380.5/2001 (H5N1)	98.9

[a] Nucleotide sequences of A/duck/Yokohama/aq10/2003 were compared to those in Genbank

Table 15. Growth of A/duck/Yokohama/aq10/2003 in mice[a]

Organ	No. of animals with virus/No. tested (log_{10} EID_{50}/g)	
	Day 3	Day 6
Brain	0/3	0/3
Lung	3/3 (2.7±0)	1/3 (4.5)
Spleen	0/3	0/3
Liver	0/3	0/3
Kidney	0/3	0/3

[a]BALB/c mice (n=3 per time point) were intranasally infected with 10^6 EID_{50} of virus. Virus titers in the organs shown were tested on days 3 and 6 postinfection.

Table 16. Viral titers in organs of dead mice infected with A/duck/Yokohama/aq10/2003

No.dead/ no. infected	No. of animals with virus/No. died (virus titers in $\log_{10}$ EID_{50}/g)				
	Brain	Lung	Spleen	Liver	Kidney
2/18	2/2 (6.0, 3.3)	0/2	0/2	0/2	0/2

Note: BALB/c mice (n=18) were intranasally infected with 10^6 EID_{50} of virus and were observed for 14 days.
Virus titers in the organs shown were tested immediately after the animals' deaths (on day 11 postinfection).

Table 17. Replication of mouse brain variants of A/duck/Yokohama/aq10/2003 in mice[a]

	MID_{50}[b] (EID_{50})	MLD_{50}[c] (EID_{50})	Brain	Lung	Liver	Spleen	Kidney
MBV-1	$9.3X10^{1}$	$9.3X10^{1}$	2/3 (4.7, 4.5)	3/3 (5.4±1.1)	0/3	1/3 (2.3)	0/3
MBV-2	10^{2}	10^{2}	3/3 (3.2±1.1)	2/3 (2.0, 5.3)	0/3	0/3	0/3

[a]BALB/c mice (n=3) were intranasally infected with $10^{2.5}$ EID_{50} of virus.

Virus titers in the organs shown were tested immediately after the animals' deaths (on day 9 or 10 postinfection).

[b]MID_{50} values are the numbers of EID_{50}s resulting in 50% infection in mice.

[c]MLD_{50} values are the numbers of EID_{50}s resulting in 50% mortality in mice.

Table 18. Nucleotide and amino acid substitutions found in mouse brain variants derived from A/duck/Yokohama/aq10/2003.

Segment	No of substitutions (position)			
	MBV-1		MBV-2	
	nucleotide	amino acid	nucleotide	amino acid
PB2	2 (T554A[a], G1879A)	2 (I185K, E627K)	1(A1891C)	1(M631L)
PB1	0	0	0	0
PA	0	0	0	0
HA	0	0	0	0
NP	0	0	1(A1342G)	1(M448V)
NA	0	0	0	0
M1	0	0	0	0
M2	0	0	0	0
NS1	0	0	0	0
NS2	1(G223A)	1 (E75K)	1(C269T)	1 (T90I)

[a]Two variants (MBV-1 and MBV-2) isolated from the brains of mice infected with A/duck/Yokohama/aq10/2003 were sequenced and their nucleotide and amino acid sequences were compared with those of the parent virus.

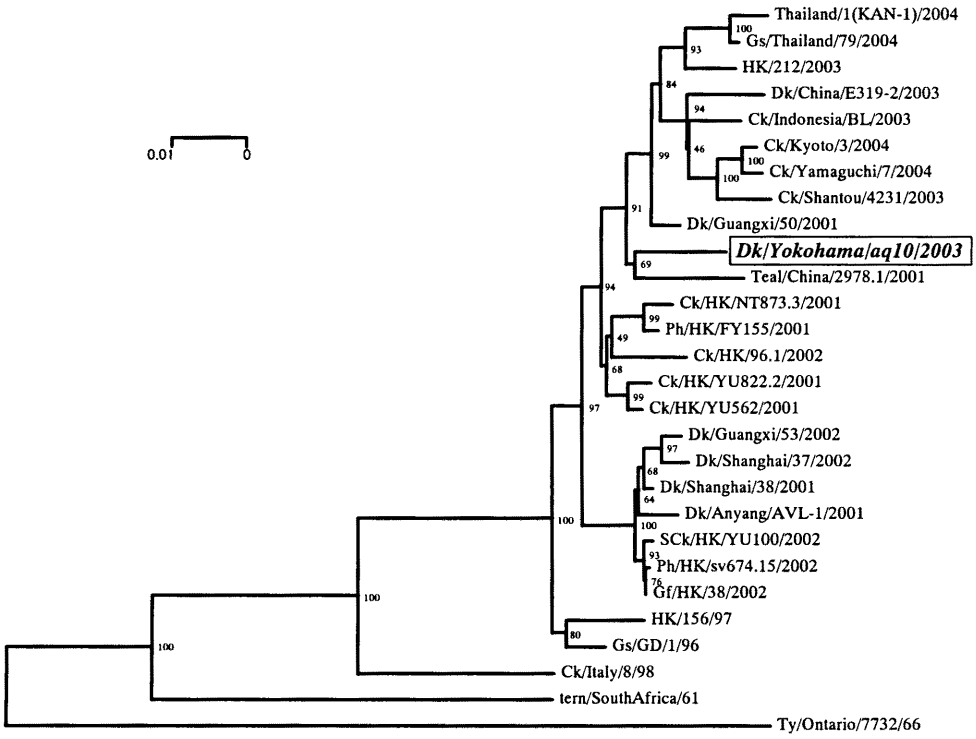

Fig.9. Phylogenetic trees of the H5 HA gene of influenza A viruses. Dk/Yokohama/aq10/03 is shown boxed. Nucleotides 13-1607 (1595 bases) of the H5 HA was subjected to phylogenetic analysis. The nucleotide numbers were derived by counting from the start codon. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate confidence levels of bootstrap analysis with 100 replications as a percentage value. Abbreviations: Bud (budgerigar). Ck (chicken), Dk (duck), GD (Guandong), Gf (guinea fowl), Gs (goose), HK (Hong Kong), Hokk (Hokkaido), Ph (pheasant), SCk (silky chicken), TW (Taiwan), Ty (turkey).

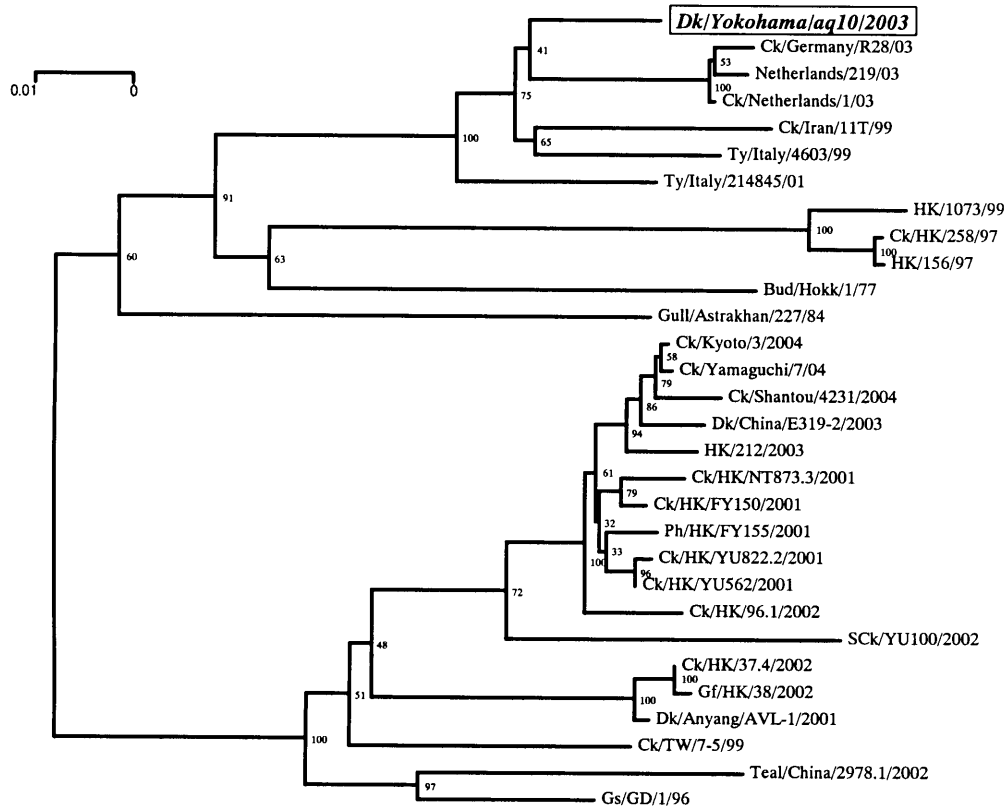

Fig.10. Phylogenetic trees of the PB2 gene of influenza A viruses. Dk/Yokohama/aq10/03 is shown boxed. Nucleotides 995-2108 (1114 bases) of the PB2 gene was subjected to phylogenetic analysis.

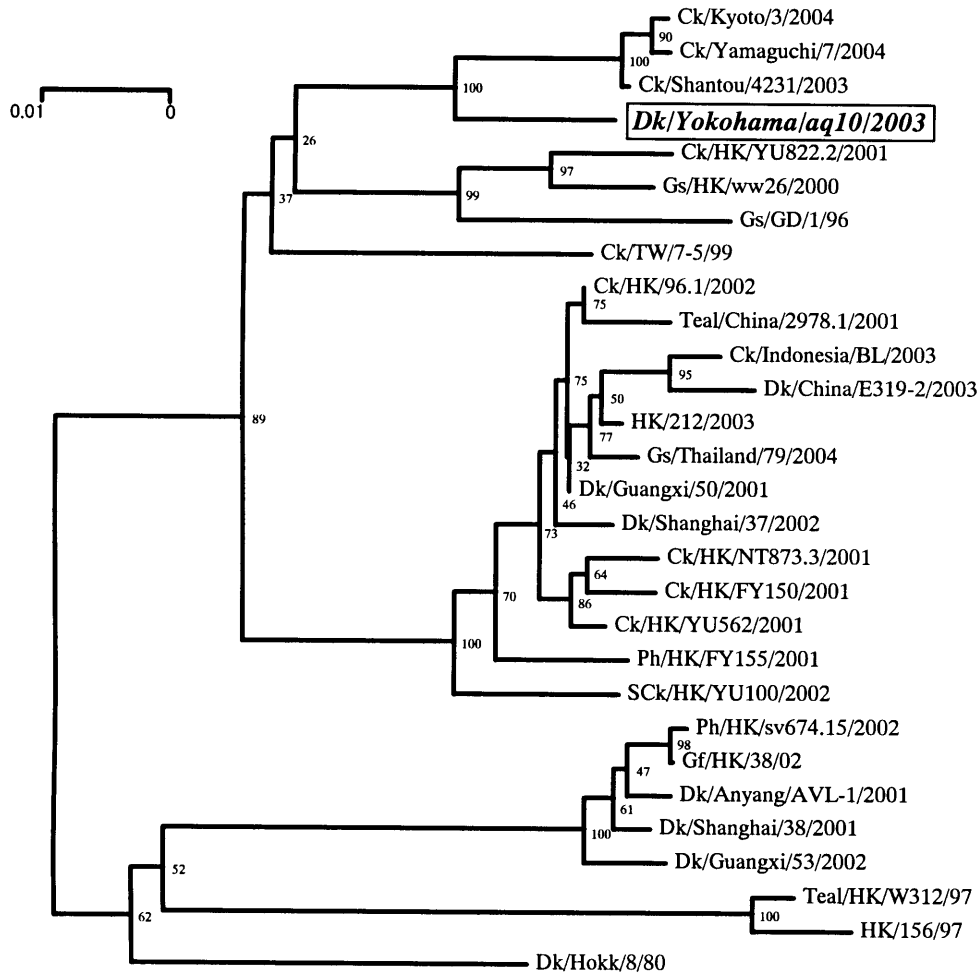

Fig.11. Phylogenetic trees of the PA gene of influenza A viruses. Dk/Yokohama/aq10/03 is shown boxed. Nucleotides 1411-2148 (738 bases) of the PA gene was subjected to phylogenetic analysis.

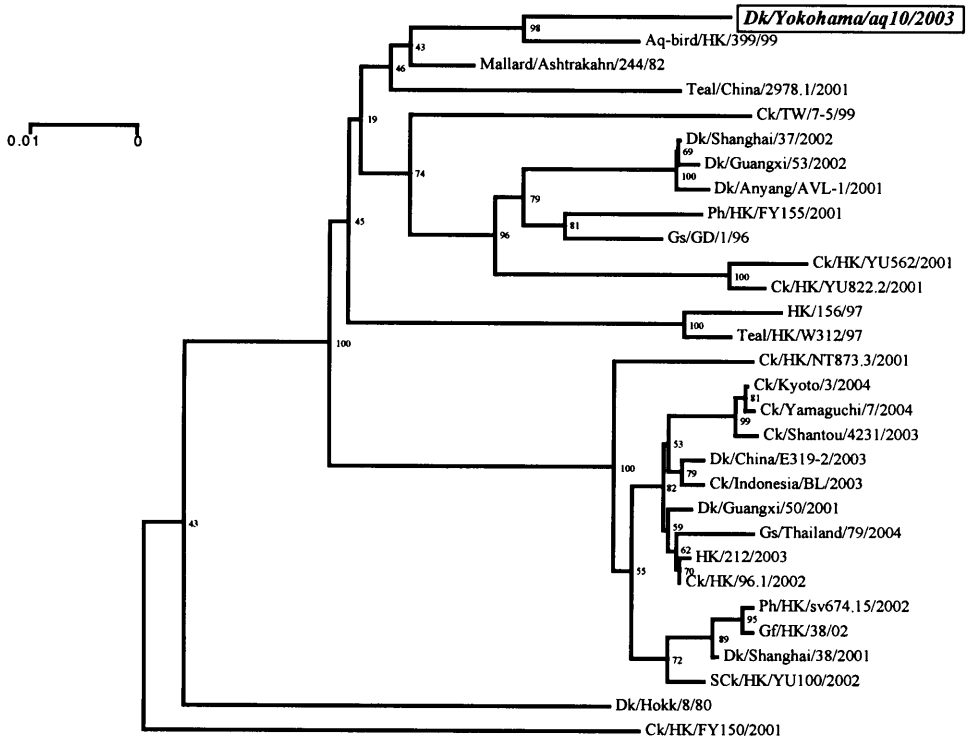

Fig.12. Phylogenetic trees of the NP gene of influenza A viruses. Dk/Yokohama/aq10/03 is shown boxed. Nucleotides 1-972 (972 bases) of the NP genes was subjected to phylogenetic analysis.

```
                                  31                                                                90
A/duck/Yokohama/aq10/2003         SIWVSHSIQTGNQHQAEPCNQSIITYENNTWVNQTYVNISNTNFLTEKAVNLVTLAGNSS
A/chicken/Yamaguchi/7/2004        SIWVSHSIQTGNQRQAEP-------------------ISNTKFLTEKAVTSVTLAGNSS
A/Thailand/1(KAN-1)/2004          SIWVSHSIHTGNQHKAEP-------------------ISNTNFLTEKAVASVKLAGNSS
A/Thailand/2(SP-33)/2004          SIWVSHSIHTGNQHKAEP-------------------ISNTNFLTEKAVASVKLAGNSS
A/duck/China/E319-2/2003          SIWVSHSIQTRNQHQAEP-------------------ISNTNFFTEKAADSVTLAGNSS
A/Hong Kong/212/2003              SIWVSHSIQTGNQHQAEPCNQSIITYENNTWVNQTYVNISNTNFLTEKAVASVTLAGNSS
A/duck/Anyang/AVL-1/2001          SIWVSHSIQTENQHQAEP-------------------ISNTNFLTEKAVASVTLAGNSS
A/Hong Kong/156/97                SVWVSHIIQTWHPNQPEPCNQSI-------------------NFYTEQAAASVTLAGNSS
A/chicken/Hong Kong/YU822.2/2001  SIWVSHSIQTGNQHQAEPCNQSIITYENNTWVNQTYVNISNTNFLTEKAVASVTLAGNSS
A/chicken/Hong Kong/YU562/2001    SIWASHSIQKMNQHQTEPCNQSIITYENNTWVNQTYVNISNTNFLTEKVVASIALSGNSS
A/pheasant/Hong Kong/FY155/2001   SIWISHSIQTGNQHQAEPCNQSIITYENNTWVNQTYVNISNTNLLTEKAVASVTLAGNSS
A/chicken/Hong Kong/FY150/2001    SIWVSHSIQTGNQHQAEPCNQSIITYENNTWVNQTYVNISNTNLLTEKAVASVTLAGNSS
A/chicken/Hong Kong/NT873.3/2001  SIWVSHSIQTGNQHQAEPCNQSIITYENNTWVNQTYVNISNTNFLTEKAVASVTLAGNSS
A/turkey/Italy/4580/99            SIWVSHSIQTGNQYQPEPCNQSI----------------------TEQAVTSVTLAGNSS
```

Fig.13. Comparison of NA stalks among representative N1 influenza A viruses.

The sites of amino acid deletions are shown by dashes.

Conclusion

We isolated two H9N2 influenza A viruses from the respiratory organs of Indian ring-necked parakeets (*Psittacula Krameri manilleusis*) that had been imported from Pakistan to Japan in 1997 and 1998. The hemagglutinin and neuraminidase of both isolates showed >97% nucleotide identity with those of H9N2 viruses isolated from humans in Hong Kong in 1999, while the six genes encoding internal proteins were >99% identical to the corresponding genes of H5N1 viruses recovered during the 1997 outbreak in Hong Kong. These results suggest that the H9N2 parakeet viruses originating in Pakistan share an immediate ancestor with the H9N2 human viruses. Thus, influenza A viruses with the potential to be transmitted directly to humans may be circulating in captive birds worldwide.

We showed that the H5N1 viruses associated with outbreaks of HPAI in chickens in Japan were genotypically closely related to an H5N1 virus isolated from a chicken in China in 2003 (genotype V), but were different from those prevalent in southeastern Asia in 2003-2004 (i.e., genotype Z). Furthermore, we demonstrated that these highly pathogenic viruses can be transmitted to crows, which are highly susceptible to these viruses.

H5N1 viruses were isolated from duck meat imported from China during this routine surveillance in May of 2003. We characterized these H5N1 isolates and found that poultry products contaminated with influenza viruses of high pathogenic potential to mammals are a threat to public health even in countries where the virus is not enzootic and represent a possible source of influenza outbreaks in poultry.

Currently, H5N1 influenza viruses remain a serious public health concern in Asia and now in Europe. This study demonstrates the importance of surveillance for avian influenza virus, particularly in imported wild birds and poultry products.

Moreover, our phylogenetic analysis showed that H5N1 viruses of multiple genotypes are circulating in Asia and we detected that the existence of novel amino acid substitutions potentially associated with high virulence in mammals. Thus, the findings obtained in this study will be useful for the epizoology of the avian influenza virus and the establishment of prevention and control methods for

avian influenza.

Acknowledgments

The author would like to acknowledge Prof. Y. Kawaoka, Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, for his valuable advice and detailed review of this thesis. The author is grateful to Dr. S.Yamaguchi, Department of Infectious Diseases, National Institute of Animal Health, for his valuable advice, suggestions, and encouragement throughout this study. The author greatly appreciates the staff of the pathogenic virus section, National Institute of Animal Health, for valuable suggestions and encouragement. Finally, the author thanks Dr. M.Eto, Animal Quarantine Service in Japan, the staff of the Regional Livestock Hygiene Service Centers in Japan, and Drs. Y. Sanada and N. Sanada, Birds Hospital-BIRD HOUSE, for providing clinical materials.

References

1. Albo, C.,Valencia, A., Portela, A. 1995. Identification of an RNA binding region within the N-terminal third of the influenza A virus nucleoprotein. J. Virol. 69, 3799-3806.

2. Alexander, D. J., Allan,W.H., Parsons,G. 1979. Characterisation of influenza viruses isolated from turkeys in Great Britain during 1963-1977. Res. Vet. Sci. 26,17-20.

3. Alexander, D.J., Parsons, G., Manvell, R.J. 1986. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks, and quail. Avian Pathol. 15, 647-662.

4. Alexander, D. J. 2000. A review of avian influenza in different bird species. Vet. Microbiol. 74, 3-13.

5. Banks, J., Speidel,E.C., Harris,P.A., Alexander, D.J. 2000. Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian Pathology 29, 353-359.

6. Beare, A. S., Webster, R.G. 1991. Replication of avian influenza viruses in humans. Arch. Virol. 119, 37-42.

7. Becker, W.B., 1966. The isolation and classification of tern virus: influenza virus A/tern/South Africa/61. J.Hygiene.64, 309-320.

8. Claas, E. C., de Jong, J. C., van Beek, R., Rimmelzwaan, G. F., Osterhaus, A. D. 1998. Human influenza virus A/HongKong/156/97 (H5N1) infection. Vaccine 16, 977-978.

9. Crumpacker, C., 2001. Antiviral therapy. In: Knipe DM, Howley PM, eds. Fields virology. 4th ed.

Philadelphia: Lippincott Williams & Wilkins, 393-s433.

10. Dasso, M. C., Jackson, R. J. 1989. Efficient initiation of mammalian mRNA translation at a CUG codon. Nucleic Acids Res. 17, 6485-6497.

11. Deshpande, K. L., Naeve, C. W., Webster,R.G. 1985. The neuraminidases of the virulent and avirulent A/Chicken/Pennsylvania/83 (H5N2) influenza A viruses: sequence and antigenic analyses. Virology 147, 49-60.

12. Ellis, T.M., Bousfield, R.B., Bissett, L.A., Dyrting, K.C., Luk, G.S., Tsim, S.T, Sturm-Ramirez, K., Webster, R.G., Guan, Y., Peiris, J.S.M., 2004. Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Pathol. 33, 492-505.

13. Fouchier, R.A., Schneeberger, P.M., Rozendaal, F.W., Broekman, J.M., Kemink, S.A., Munster, V., Kuiken, T., Rimmelzwaan, G.F., Schutten, M., Van Doornum, G.J., Koch, G., Bosman, A., Koopmans, M., Osterhaus, A.D., 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A. 101, 1356-1361.

14. Gao, P., Watanabe, S, Ito, T., Goto, H., Wells, K., McGregor, M., Cooley, A.J., Kawaoka, Y., 1999. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol.73, 3184-9.

15. Govorkova, E.A., Rehg, J.E., Krauss, S., Yen, H.L., Guan, Y., Peiris, M., Nguyen, T.D., Hanh, T.H., Puthavathana, P., Long, H.T., Buranathai, C., Lim, W., Webster, R.G., Hoffmann, E., 2005. Lethality to Ferrets of H5N1 Influenza Viruses Isolated from Humans and Poultry in 2004. J Virol. 79, 2191-2198.

16. Guan, Y., Shortridge, K. F., Krauss,S., Webster, R.G. 1999. Molecular characterization of H9N2

influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc. Natl. Acad. Sci. USA 96, 9363-9367.

17. Guan, Y., Peiris, M., Kong, K.F., Dyrting, K.C., Ellis, T.M., Sit, T., Zhang, L.J., Shortridge, K.F., 2002. H5N1 influenza viruses isolated from geese in Southeastern China: evidence for genetic reassortment and interspecies transmission to ducks. Virology 292, 16-23.

18. Guan, Y., Peiris, J.S., Lipatov, A.S., Ellis, T.M., Dyrting, K.C., Krauss, S., Zhang, L.J., Webster, R.G., Shortridge, K.F., 2002. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proc Natl Acad Sci U S A. 99, 8950-5.

19. Guan, Y., Poon, L.L., Cheung, C.Y., Ellis, T.M., Lim, W., Lipatov, A.S., Chan, K.H., Sturm-Ramirez, K.M., Cheung, C.L., Leung, Y.H., Yuen, K.Y., Webster, R.G., Peiris, J.S., 2004. H5N1 influenza: a protean pandemic threat. Proc Natl Acad Sci U S A.101, 8156-61.

20. Gubareva, L.V., Webster, R.G., Hayden, F.G., 2002. Detection of influenza virus resistance to neuraminidase inhibitors by an enzyme inhibition assay. Antiviral Res. 53, 47-61.

21. Guo, Y. Krauss, J., S., Senne, D. A., Mo, I. P., Lo, K. S., Xiong, X. P., Norwood, M., Shortridge,K.F., Webster, R. G., Guan. Y. 2000. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virology 267, 279-288.

22. Hatta, M., Gao, P., Halfmann, P., Kawaoka, Y., 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293, 1840-2.

23. Hien, T.T., Liem, N.T., Dung, N.T., San, L.T., Mai, P.P., Chau, Nv. N., Suu, P.T., Dong, V.C., Mai, L.T., Thi, N.T., Khoa, D.B., Phat, L.P., Truong, N.T., Long, H.T., Tung, C.V., Giang, L.T., Tho, N.D., Nga, L.H.,

Tien, N.T., San, L.H., Tuan, L.V., Dolecek, C., Thanh, T.T., De Jong, M., Schultsz, C., Cheng, P., Lim, W., Horby, P., Farrar J., 2004. Avian Influenza A (H5N1) in 10 Patients in Vietnam.N Engl J Med , 350, 1179-88.

24. Hiromoto, Y., Yamazaki, Y., Fukushima, T., Saito, T., Lindstrom, S. E., Omoe, K., Nerome, R., Lim,W., Sugita, S., Nerome, K. 2000. Evolutionary characterization of the six internal genes of H5N1 human influenza A virus. J. Gen. Virol. 81, 1293-1303.

25. Horimoto, T., Kawaoka, Y. 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J. Virol. 68, 3120-3128.

26. Horimoto, T., Fukuda. N., Iwatsuki-Horimoto, K., Guan, Y., Lim, W., Peiris, M.,Sugii, S., Odagiri, T., Tashiro, M., Kawaoka, Y., 2004. Antigenic differences between H5N1 human influenza viruses isolated in 1997 and 2003. J.Vet.Med.Sci. 66, 303-305.

27. Ito, T., Goto, H., Yamamoto, E., Tanaka, H., Takeuchi, M., Kuwayama, M,, Kawaoka, Y., Otsuki, K., 2001. Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol. 75, 4439-4443.

28.Kilbourne, E.D. 1987. Influenza. Plenum: NewYork, 1-359.

29. Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F.G., Sugaya, N., Kawaoka, Y., 2004. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet. 364, 9759-9765.

30. Lang, G., Narayan, O., Rouse, B.T., Ferguson, A.E., Connell, M.C.,1968. A new influenza A virus infection in turkeys II. A highly pathogenic variant, A/turkey/Ontario/7732/66. Can Vet J. 9, 151-60.

31. Li, K. S., Xu, K. M., Peiris, J. S., Poon, L. L., Yu. K.Z., Yuen, K. Y., Shortridge, K. F., Webster, R. G. & Guan, Y. (2003). Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans? J.Virol. 77, 6988-94.

32. Li,K.S., Guan, Y., Wang, J., Smith, G.J., Xu, K.M., Duan, L., Rahardjo, A.P., Puthavathana, P., Buranathai, C., Nguyen, T.D., Estoepangestie, A.T., Chaisingh, A., Auewarakul, P., Long, H.T., Hanh, N.T., Webby, R.J., Poon, L.L., Chen, H., Shortridge, K.F., Yuen, K.Y., Webster, R.G., Peiris, J.S., 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430, 209-213.

33. Lin, Y. P., Shaw, M., Gregory, V., Cameron, K., Lim, W., Klimov, A., Subbarao, K., Guan, Y., Krauss, S., Shortridge, K., Webster, R., Cox, N., Hay. A. 2000. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc. Natl. Acad. Sci. USA 97, 9654-9658.

34. Lipatov, A.S., Krauss, S., Guan, Y., Peiris, M., Rehg, J.E., Perez, D.R., Webster, R.G., 2003. Neurovirulence in mice of H5N1 influenza virus genotypes isolated from Hong Kong poultry in 2001. J Virol. 77, 3816-23.

35. Lu, X., Tumpey, T.M., Morken, T., Zaki, S.R., Cox, N.J., Katz, J.M., 1999. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol. 73, 5903-5911.

36. Madeley, C. R., Allan, W. H., Kendal, A. P.. 1971. Studies with avian influenza A viruses: serological relations of the haemagglutinin and neuraminidase antigens of ten virus isolates. J. Gen. Virol. 12, 69-78.

37. Mehdi, H., Ono, E., Gupta, K. C. 1990. Initiation of translation at CUG, GUG, and ACG codons in

mammalian cells. Gene 91, 173-178.

38. Monto, A.S., 2003. The role of antivirals in the control of influenza. Vaccine 21, 1796-1800.

39. Naeem, K., Ullah, A., Manvell, R. J., Alexander, D. J. 1999. Avian influenza A subtype H9N2 in poultry in Pakistan. Vet. Rec. 145, 560.

40. Nestorowicz, A., Kawaoka, Y., Bean, W. J., Webster, R.G. 1987. Molecular analysis of the hemagglutinin genes of Australian H7N7 influenza viruses: role of passerine birds in maintenance or transmission? Virology 160, 411-418.

41. Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann,E., Hobom, G., Kawaoka, Y. 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. USA 96, 9345-9350.

42. Nobusawa, E., Aoyama, T., Kato, H., Suzuki, Y., Tateno, Y., Nakajima, K. 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 182, 475-485.

43. Office International des Epizooties. 2004. Highly pathogenic avian influenza. In: Manual of diagnostic tests and vaccines for terrestrial animals, 5 th edition. Office International des Epizooties, Paris, France. Chapter 2.1.14. http://www.oie.int.

44. Okazaki, K., Takada, A., Ito, T., Imai, M., Takakuwa, H., Hatta, M., Ozaki, H., Tanizaki, T., Nagano, T., Ninomiya, A., Demenev, V. A., Tyaptirganov, M. M., Karatayeva, T. D., Yamnikova, S. S., Lvov, D. K., Kida, H. 2000. Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. Arch. Virol. 145, 885-893.

45. Peiris, M., Yuen, K. Y., Leung, C. W., Chan, K. H., Ip, P. L., Lai, R. W., Orr, W. K., Shortridge, K. F. 1999. Human infection with influenza H9N2. Lancet 354, 916-917

46. Peiris, M., Yam, W. C., Chan, K. H., Ghose, P., Shortridge, K. F. 1999. Influenza A H9N2: aspects of laboratory diagnosis. J. Clin. Microbiol. 37, 3426-3427.

47. Peiris, J.S., Yu, W.C., Leung, C.W., Cheung, C.Y., Ng, W.F., Nicholls, J.M., Ng, T.K., Chan, K.H., Lai, S.T., Lim, W.L., Yuen, K.Y., Guan, Y. 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 363, 617-619.

48. Perales, B., de la Luna, S., Palacios, I., Ortin, J. 1996. Mutational analysis identifies functional domains in the influenza A virus PB2 polymerase subunit. J. Virol. 70, 1678-1686.

49. Prats, A. C., De Billy, G., Wang, P., Darlix, J. L. 1989. CUG initiation codon used for the synthesis of a cell surface antigen coded by the murine leukemia virus. J. Mol. Biol. 205, 363-372.

50. Puthavathana, P., Auewarakul, P., Charoenying, P.C., Sangsiriwut, K., Pooruk, P., Boonnak, K., Khanyok, R., Thawachsupa, P., Kijphati, R., Sawanpanyalert, P., 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J Gen Virol. 86, 423-433.

51. Reed, L., Muench. H., 1938. A simple method of estimating fifty percent endopoints. Am. J. Hyg. 27, 493-497.

52. Saitou, N., Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

53. Seo, S.H., Hoffmann, E., Webster, R.G., 2002. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nature Medicine 8, 950-4

54. Shortridge, K. F., Zhou, N. N., Guan, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K. G., Norwood, M., Senne, D., Sims, L., Takada, A., Webster, R. G. 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252, 331-342.

55. Shortridge, K. F., Gao, P., Guan, Y., Ito, T., Kawaoka, Y., Markwell, D., Takada, A., Webster, R. G. 2000. Interspecies transmission of influenza viruses: H5N1 virus and a Hong Kong SAR perspective. Vet. Microbiol. 74, 141-147.

56. Sims, L.D., Guan, Y., Ellis, T.M., Liu, K.K., Dyrting, K., Wong, H., Kung, N.Y., Shortridge, K.F., Peiris, M., 2003. An update on avian influenza in Hong Kong 2002. Avian Dis. 47, 1083-6.

57. Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., Poon, L., Guan, Y., Peiris, M., Webster, R.G., 2004. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J Virol. 78, 4892-4901.

58. Suarez, D. L., Perdue, M. L., Cox, N., Rowe, T., Bender, C., Huang, J., Swayne, D. E. 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J. Virol. 72, 6678-6688.

59. Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K., Cox, N. 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279, 393-396.

60. Swayne, D.E. Halvorson, D.A. Influenza. In Saif, (eds.). Disease of poultry, 11th ed. Iowa State University Press: Ames, IA, 583-605.

61. Takada, A., Kuboki, N., Okazaki, K., Ninomiya, A., Tanaka, H., Ozaki, H., Itamura, S., Nishimura, H., Enami, M., Tashiro, M., Shortridge, K.F., Kida, H., 1999. Avirulent Avian influenza virus as a vaccine strain against a potential human pandemic. J Virol.73, 8303-7.

62. Tumpey, T.M., Suarez, D.L., Perkins, L.E., Senne, D.A., Lee, J.G., Lee, Y.J., Mo, I.P., Sung, H.W., Swayne, D.E., 2002. Characterization of a highly pathogenic H5N1 avian influenza A virus isolated from duck meat. J Virol. 76, 6344-55.

63. Utterback, W. W., Schwartz, J. H. 1973. Epizootiology of velogenic viscerotropic Newcastle disease in southern California, 1971-1973. J. Am. Vet. Med. Assoc. 163, 1080-1088.

64. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56, 152-179.

65. Webster, R.G., Guan, Y., Peiris, M., Walker, D., Krauss, S., Zhou, N.N., Govorkova, E.A., Ellis, T.M., Dyrting, K.C., Sit, T., Perez, D.R., Shortridge, K.F., 2002. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. J Virol. 76, 118-126.

66. World Health Organization., Avian influenza A (H5N1). 2004. Weekly epidemiol.Rec. 79, 65-76.

67.World Health Organization., Avian influenza A (H5N1). 2004. Weekly epidemiol.Rec. 79, 201-204.

68.World Health Organization., Avian influenza A (H5N1). 2004. Weekly epidemiol.Rec. 79, 265-272.

69.World Health Organization., Avian influenza A (H5N1). 2004. Weekly epidemiol.Rec. 79, 369-376.

70.World Health Organization., Development of a vaccine against avian influenza H5N1 infection in humans: update 4. 2004.

71. World Health Organization., 2004. Weekly epidemiol. Rec. 79, 385-392

72. World Health Organization., 2005. Weekly epidemiol. Rec. 80, 1-8.

73. Zhou, N. N., Shortridge, K. F., Claas, E. C. J., Krauss, S. L., Webster, R. G. 1999. Rapid evolution of H5N1 influenza viruses in chickens in Hong Kong. J. Virol. 73, 3366-3374.

Summary in Japanese

論文の内容の要旨

論文題目　Phylogenetic and pathogenic analysis of avian H9N2 and H5N1 influenza A viruses isolated in Japan

（日本で分離された H9N2 および H5N1 亜型鳥インフルエンザウイルスの分子疫学的および病原学的解析）

氏　名　真瀬昌司

はじめに

近年アジアを中心に鳥インフルエンザが流行している。1997年には、H5N1亜型のウイルスが18名のヒトに直接感染し、そのうち6名が死亡した。本ウイルスは、2003年以降アジア諸国で猛威を奮い、2004年にはわが国でも発生が認められた。また輸入アヒル肉からもH5N1ウイルスが分離された。

一方、H9N2亜型ウイルスもアジアを中心に広く流行しており、ヒトの感染例も報告されている。わが国では流行が認められていないが、流行国から輸入された鳥類から本ウイルスが分離された。

本研究では、輸入インコから分離されたH9N2ウイルス株、わが国で発生した高病原性鳥インフルエンザから分離されたH5N1ウイルス,および輸入アヒル肉から分離されたH5N1ウイルスの遺伝学的、病原学的性状を解析した。

1. 輸入愛玩鳥から分離されたH9N2鳥インフルエンザウイルスの性状

1997年及び1998年に、パキスタンから日本へ輸入されたワカケホンセイインコ*(Psittacula Krameri manillensis)*の呼吸器からH9N2亜型のA型インフルエンザウイルスが2株分離された。両分離株は遺伝学的に極めて近縁であり、このことは同一系統のウイルスが少なくとも1年間これらの鳥の集団で維持されていたことを示す。両株の赤血球凝集素遺伝子およびノイラミニダーゼ遺伝子の塩基配列は、1999年に香港でヒトから分離されたH9N2亜型ウイルス株との間で97%以上の相同性を示し、また6種類の内部蛋白質遺伝子の塩基配列は香港で1997年に分離されたH5N1亜型ウイルス株との間で99%以上の相同性を示した。以上の結果は、パキスタン由来のインコから分離されたH9N2亜型ウイルスとヒトから分離されたH9N2亜型ウイルスは共通の祖先から派生したことを示唆している。以上の結果は、ヒトに直接感染する可能性をもつA型インフルエンザウイルスが愛玩鳥の輸出入を介して世界的に伝播している可能性を示唆した。

2. 2003-2004年にかけ日本で発生したH5N1亜型高病原性鳥インフルエンザの流行で分離されたウイルスの性状.

わが国で2003年12月末から2004年3月にかけて、致死的な鳥の伝染性疾病が150-450km離れた3県下で4件（養鶏場3件と愛玩鶏1件）発生した。原因はH5N1亜型高病原性鳥インフルエンザウイルスで、わが国では初めての発生例であった。本ウイルスは死亡したカラスからも分離された。わが国で分離されたH5N1ウイルス株は、モノクローナル抗体を用いた赤血球凝集反応抑制試験の結果において、その抗原的類似性が示されたが、1997年および2003年に香港でヒトから分離されたウイルス株とは識別された。遺伝学的にも、わが国のウイルス株は相互に近縁であったが、これらのウイルスは中国広東省で分離された遺伝子型Vのウイルス(A/chicken/Shantou/4231/2003株)と最も高い相同性を示し、東南アジアの主流株である遺伝子型Zではなかった。わが国の代表株としてA/chicken/Yamaguchi/7/2004株の鶏およびマウスに対する病原性を調べたところ、鶏では静脈内接種で1日以内に、経鼻接種では3日以内に全羽死亡した。一方、マウスではその50%致死量が5×10^{5} EID_{50} (50%egg-infectious dose)であり、1997年に香港においてヒトから分離された株よりもその病原性は低かったが、馴化を必要とせずとも肺でよく増殖しさらに脳へも拡散した。

3. 2003 年中国から日本に輸入されたアヒル肉から分離された H5N1 亜型高病原性鳥インフルエンザウイルスの性状.

2003 年中国山東省から輸入されたアヒル肉から H5N1 亜型鳥インフルエンザウイルスが分離された。本ウイルス株は、モノクローナル抗体を用いた赤血球凝集反応抑制試験の結果から、他の 1997 年および 2003 年に香港でヒトから分離されたウイルス株などとはその抗原性が異なっていることが示された。遺伝学的には、本ウイルス株の 6 遺伝子(PBA, PA, HA, NA,M および NS)は最近分離された H5N1 ウイルス株に近縁であったが、残り 2 遺伝子(PB2, NP)は他のウイルス株由来と考えられた。本株の鶏およびマウスに対する病原性を調べたところ、鶏に対して高病原性であり、またマウスではその 50%致死量が 5×10^{6} EID_{50} であった。しかし、ウイルスを感染させたマウスの脳から回収されたウイルスには数カ所のアミノ酸変異が認められ、またマウスに対する病原性が著しく上昇していた。以上の結果はインフルエンザウイルス清浄国であってもこのような家禽製品を介して高病原性鳥インフルエンザウイルスが持ち込まれる可能性があり公衆衛生学的な脅威となること、また養鶏産業上、感染源と成りうる可能性を示す。

4. おわりに

2005 年現在でも H5N1 鳥インフルエンザは公衆衛生学的に問題となっている。本研究の結果、鳥インフルエンザの疫学を考える上で、サーベイランス、特に輸入生鳥や輸入家禽物のサーベイランスが重要であることが示された。また、わが国で発生した高病原性鳥インフルエンザから分離された H5N1 亜型の鳥インフルエンザウイルスも含め、アジアには複数の異なる遺伝子型の H5N1 亜型鳥インフルエンザが存在し流行していることが明らかになった。またほ乳類であるマウスに対し容易に強毒化しうること、その変異には複数のアミノ酸置換が関与している可能性が示唆された。本研究成果は鳥インフルエンザの流行疫学の解明、および鳥インフルエンザの予防、防除方法の確立に有用である。