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

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Abstract

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}) = 5×10^6 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} = $\sim 10^2$ 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.

Keywords: Influenza A virus; H5N1 subtype; Duck meat; Pathogenicity

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 (Webster et al., 2002; Guan et al., 2002a; Ellis et al., 2004; Li et al., 2004). 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 (Claas et al., 1998; Hien et al., 2004; Peiris et al., 2004; World Health Organization, 2004, 2005). 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 (Mase et al., 2005). 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 (Tumpey et al., 2002). This virus was highly pathogenic in

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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.

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 to 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} (50% egg infectious dose)/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 1).

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 (Table 2), and phylogenetically analyzed. Phylogenetic analysis of representative genes (i.e., the genes for HA, PB2, PA, and nucleoprotein [NP]) are shown in Fig. 1. We found that the genotype of Dk/Yokohama/aq10/03 was unique and distinct from any of the H5N1 virus genotypes previously

Table 1
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	Mase et al. (2005)
A/chicken/Kyoto/3/2004 (H5N1)	800	100	100	400	100	<100	<100	<100	<100	<100	Mase et al. (2005)
A/tern/South Africa/61 (H5N3)	6400	200	800	800	400	400	<100	400	<100	<100	Becker (1966)
A/swan/Shimane/499/83 (H5N3)	1600	200	800	400	<100	200	<100	200	400	400	Ito et al. (2001)
A/duck/Hokkaido/67/96 (H5N4)	1600	200	100	<100	<100	400	<100	1600	<100	200	Takada et al. (1999)
A/Hong Kong/156/97 (H5N1)	1600	200	1600	3200	1600	800	200	3200	<100	800	Gao et al. (1999)
A/Hong Kong/483/97 (H5N1)	800	200	200	400	400	400	<100	800	<100	<100	Gao et al. (1999)
A/Hong Kong/213/2003 (H5N1)	3200	800	<100	800	200	800	<100	6400	100	12800	Guan et al. (2004)
A/turkey/Ontario/7732/66 (H5N9)	400	1600	<100	<100	<100	<100	<100	<100	800	6400	Lang et al. (1968)

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

Table 2
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.

reported, including a recent isolate in Japan (Table 2 and Fig. 1). 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. 1a). 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 2 and Figs. 1b–d). 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 Office International des Epizooties (OIE), the strain was judged highly pathogenic for chickens, killing all chickens exposed to the virus within 2–3 days of intravenous injection (OIE, 2004). Intranasal inoculation of 6-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 (Lipatov et al., 2003). 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 3A).

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

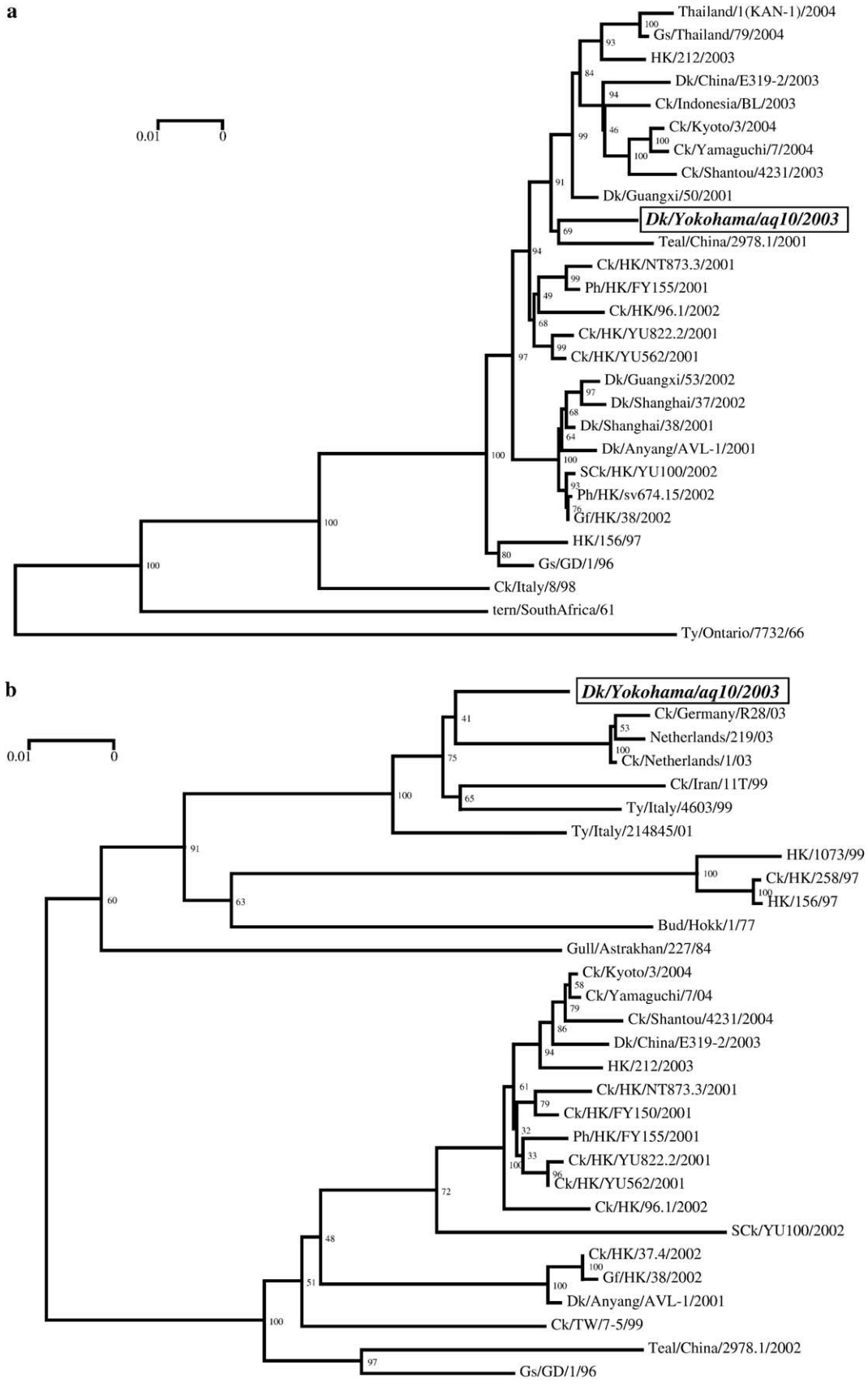
To determine the extent of mutations required for the duck meat isolate to exhibit high virulence in mice, we examined virus recovered from the dead mice infected with Dk/Yokohama/aq10/2003. Two mice died on day 11 postinfection and virus was isolated only from the brain, but not from other organs tested, of these mice (Table 3B). 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 4).

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. 2) 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 (Monto, 2003)) 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

Fig. 1. Phylogenetic trees of the H5 HA (a), PB2 (b), PA (c), and NP (d) genes of influenza A viruses. Dk/Yokohama/aq10/03 is shown boxed. Nucleotides 13–1607 (1595 bases) of the H5 HA, 995–2108 (1114 bases) of the PB2, 1411–2148 (738 bases) of the PA, and 1–972 (972 bases) of the NP genes were 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).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Gs/Thailand/79/2004
HK/212/2003
Dk/China/E319-2/2003
Ck/Indonesia/BL/2003
Ck/Kyoto/3/2004
Ck/Yamaguchi/7/2004
Ck/Shantou/4231/2003
Dk/Guangxi/50/2001
Dk/Yokohama/aq10/2003
Teal/China/2978.1/2001
Ck/HK/NT873.3/2001
Ph/HK/FY155/2001
Ck/HK/96.1/2002
Ck/HK/YU822.2/2001
Ck/HK/YU562/2001
Dk/Guangxi/53/2002
Dk/Shanghai/37/2002
Dk/Shanghai/38/2001
Dk/Anyang/AVL-1/2001
SCk/HK/YU100/2002
Ph/HK/sv674.15/2002
Gf/HK/38/2002
HK/156/97
Gs/GD/1/96
Ck/Italy/8/98
tern/SouthAfrica/61
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Dk/Yokohama/aq10/2003
Ck/Germany/R28/03
Netherlands/219/03
Ck/Netherlands/1/03
Ck/Iran/11T/99
Ty/Italy/4603/99
Ty/Italy/214845/01
HK/1073/99
Ck/HK/258/97
HK/156/97
Bud/Hokk/1/77
Gull/Astrakhan/227/84
Ck/Kyoto/3/2004
Ck/Yamaguchi/7/04
Ck/Shantou/4231/2004
Dk/China/E319-2/2003
HK/212/2003
Ck/HK/NT873.3/2001
Ck/HK/FY150/2001
Ph/HK/FY155/2001
Ck/HK/YU822.2/2001
Ck/HK/YU562/2001
Ck/HK/96.1/2002
SCk/YU100/2002
Ck/HK/37.4/2002
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Dk/Anyang/AVL-1/2001
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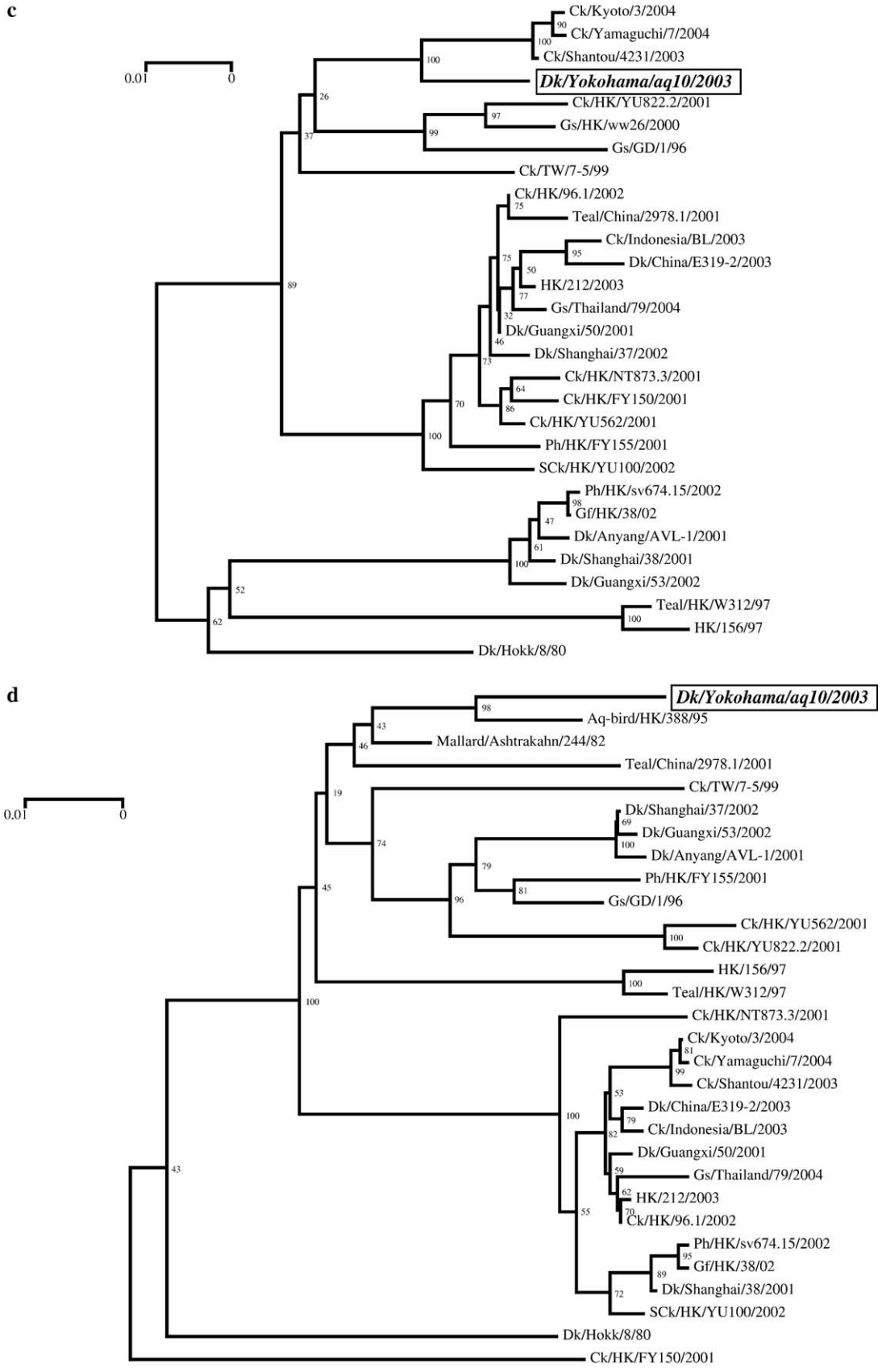

Fig. 1 (*continued*).

Table 3A
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

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

(Crumpacker, 2001), 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 (Gubareva et al., 2002; Kiso et al., 2004). 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 nucleotides, and concomitantly, three amino acid substitutions, although the amino acid substitutions found in these variants were unique to each other (Table 5). 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 (Hatta et al., 2001). 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 (Tumpey et al., 2002). 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 (Guan et al., 2002b). Dk/Yokohama/aq10/03, however, became lethal upon acquiring three amino acid substitutions. Interestingly, in a similar study by Lipatov et al. (2003), of four mouse brain variants, two of the variants had 12 and 17 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 (Neumann et al., 1999).

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. (2003). This PB2 mutation is responsible for the high virulence of Hong Kong H5N1 virus in mice (Hatta et al., 2001). It was also detected in an H7N7 virus isolated from a lethal human case during an outbreak in the Netherlands in 2003 (Fouchier et al., 2004) and in H5N1 viruses isolated from patients who died from their infection in 2004 (Govorkova et al., 2005; Li et al., 2004; Puthavathana et al., 2005). Notably, the PB2 lineage to which Dk/Yokohama/aq10/03 and the Netherlands H7N7 virus belong is different from that of the 1997 Hong Kong H5N1 viruses (see Fig. 1b), 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 (Sturm-Ramirez et al., 2004). This discovery changed our belief that ducks are resistant to influenza viruses that are highly pathogenic to chickens (Alexander et al., 1986). 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

Table 3B
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 4
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	9.3×10^1	9.3×10^1	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

Virus titers in the organs shown were tested immediately after the animals' deaths (on day 9 or 10 postinfection).
[a] BALB/c mice ($n=3$) were intranasally infected with $10^{2.5}$ EID_{50} of virus.
[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.

pathogenic viruses. It appears that H5N1 viruses are now enzootic in Asia (Li et al., 2004), 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.

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 (500–3000 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, 1 g of meat, which was pooled and made into a 10% homogenate in PBS. The homogenates were then centrifuged at low speed (3000 rpm for 10 min) and the supernatants were filtered through a sterile 0.45 μm 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 conventional hemagglutination inhibition and neuraminidase inhibition assays. Virus was propagated for 24–36 h 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 previously (Mase et al., 2001). RNA segments were PCR-amplified and sequenced using the primers below.

PB2 gene:

M13 + consensus F (5′-GTAAAACGACGGCCAGTAGCAAAAGCAGG-3′)
PB2-150R (5′-CTTCTCCTGTCTTCCTGATGTGTA-3′)
PB2-95F (5′-CCACTGTGGATCATATGGCCA-3′)
PB2-770R (5′-CCTCCTGGTGTGTACATCTG-3′)
PB2-679F (5′-CTACCAGTGGCTGGTGGGACAAG-3′)
PB2-1203R (5′-TTGCTCGTCCCTCCCGCTGACTAT-3′)
PB2-1040F (5′-TCAAAAGAGAGGAAGAAGTGCT-3′)
PB2-1702R (5′-TCTCCCAATTCCTAATGATCCA-3′)
PB2-1621F (5′-TCATCGTCTATGATGTGGGA-3′)
M13 + consensus R (5′-GTAAAACGACGGCCAGTAGTAGAAAC-3′)

NP gene:

M13 + consensus F (5′-GTAAAACGACGGCCAGTAGCAAAAGCAGG-3′)
NP-190R (5′-GTTTGAGTTCAGTGCACATCTGTA-3′)
NP-87F (5′-AACTGGTGGAGAACGCCAGAATGC-3′)
NP-720R (5′-GATGTTGCACATTCTCTCATATGCAAT-3′)

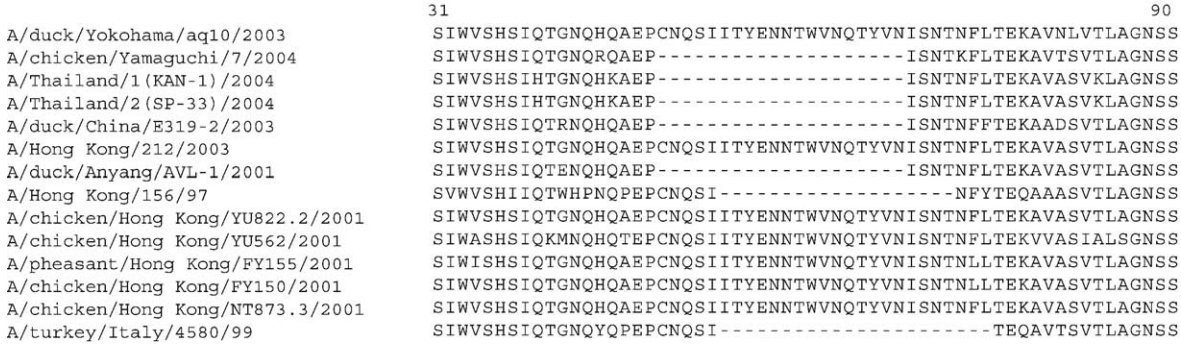

Fig. 2. Comparison of NA stalks among representative N1 influenza A viruses. The sites of amino acid deletions are shown by dashes.

Table 5
Nucleotide and amino acid substitutions found in mouse brain variants derived from A/duck/Yokohama/aq10/2003

Gene	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.

NP-616F (5′-ATGGAACTAATTCGGATGATAAAGC-3′)
NP-1160R (5′-TCCACGTTCTCATTTGAAGC-3′)
NP-1093F (5′-ACAAGAGTAATCCCAAGAGGACAA-3′)
M13 + consensus R (5′-GTAAAACGACGGCCAG-TAGTAGAAAC-3′)

The sequences of the primers for the other segments will be provided upon request. 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 previously (Mase et al., 2005). The nucleotide sequences of the genes of A/duck/Yokohama/aq10/2003 are available from GenBank under accession numbers AB212277-AB212284.

Antigenic analysis

Antigenic relationships of the viruses were determined by hemagglutination inhibition (HI) tests using a panel of monoclonal antibodies as previously described (Horimoto et al., 2004).

Pathogenicity tests

Chickens

Six-week-old specific pathogen-free chickens were inoculated either intravenously (n = 8) (OIE, 2004) 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 μl of infectious virus diluted in phosphate buffered saline (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 et al. (1999). 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 (1938). To determine the organ tropism of the virus, mice (n = 24) were intranasally inoculated with 50 μl 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.

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