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

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Abstract

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.
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Keywords: Influenza A virus; H5N1 subtype; Antigenecity; Pathogenecity; Phylogenetic analysis; NA inhibitor

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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. (Claas et al., 1998; Subbarao et al., 1998). The virus continued to circulate in Asia and, in 2003, two more people in Hong Kong were infected, leading to one additional fatality (Peiris et al., 2004). 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 (Guan et al., 2002a; Sims et al., 2003; Webster et al., 2002). 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 (Guan et al., 2002b) 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, e.g., in Vietnam and Thailand (Hien et al., 2004; World Health Organization, 2004a), resulting in over 30 documented deaths (World Health Organization, 2004d). Although most of these countries declared themselves to be free of avian influenza in the spring of 2004 (World Health Organization, 2004b), these viruses appear to have returned to at least some of these countries (World Health Organization, 2004c).

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.

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. 1). 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 conventional hemagglutination inhibition (HI) and neuraminidase inhibition (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 seven of these chickens led to the slaughter of the remaining six 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 fourth outbreak 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 1). 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,

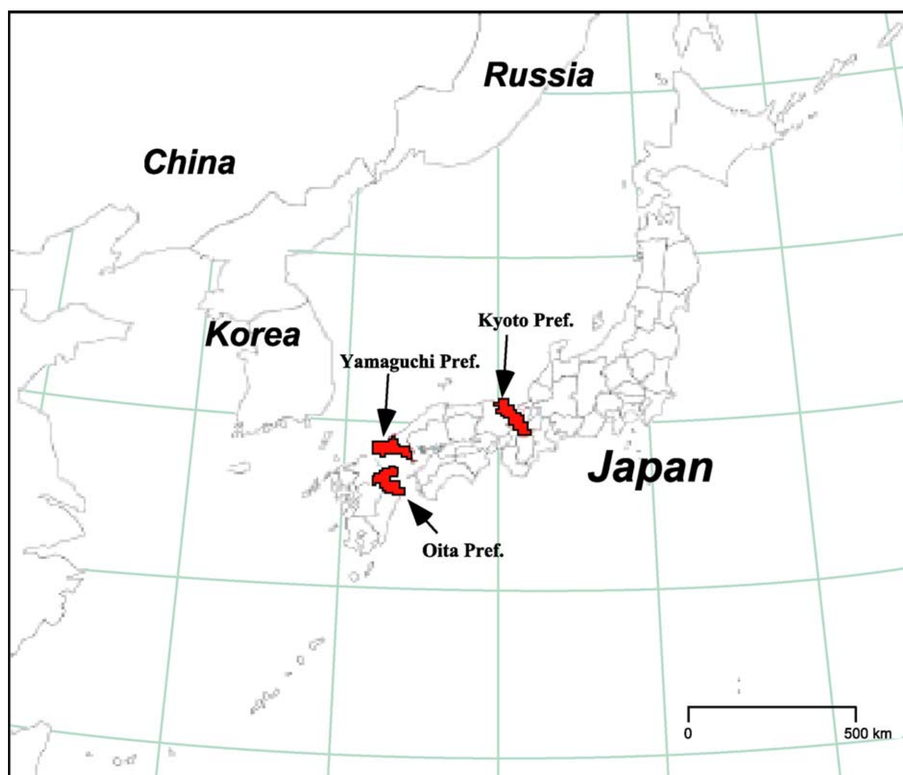

Fig. 1. The location of the Japanese prefectures in which H5N1 highly pathogenic avian influenza outbreaks occurred.

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 2), 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 3; the result 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. 2). These results further suggest that H5N1 viruses of distinct genotypes were circulating in late 2003–early 2004; that is, genotype Z in Indonesia, Thailand, Vietnam, (Li et al., 2004), and genotype V in Japan (this study).

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 4). 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 (Guan et al., 2002b). 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 (Li et al., 2004).

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 (Hatta et al., 2001) 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 (Seo et al., 2002). 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 (Monto, 2003). 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 (Crumpacker, 2001); viruses become resistant to oseltamivir through a single amino acid substitution at position 119, 152, 274, or

Table 1
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/7732/66		
			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	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
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[a])	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, S503N)	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)

[a] 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.

292 in the NA active center (Gubareva et al., 2002). The Japanese isolates do not contain the amino acid residues in these proteins that would confer to them resistance to these antiviral compounds.

Pathogenecity

Pathogenicity testing, according to the guidelines established by the Office International Epizootics (OIE), showed 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 (A/crow/Osaka/102/2004 was not tested). Upon intranasal inoculation of 6-week-old specific pathogen-free chickens (n = 8) with 10^6 50% egg infectious doses (EID_{50}) of the A/chicken/Yamaguchi/7/2004 virus, each chicken died within 3 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 5×10^5 EID_{50}, indicating that this strain is not as virulent as some of the H5N1 viruses isolated from humans (Gao et al., 1999), but is as virulent as most H5N1 viruses isolated from poultry (Lipatov et al., 2003). 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 5).

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 (Ito et al., 2001). 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 south-eastern Asia were thought to have been caused by viruses of genotype Z (Li et al., 2004). This genotype emerged in 2002, and has become a dominant H5N1 virus in southern China (Guan et al., 2004; Li et al., 2004). 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 1 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 2 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. (2003). 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

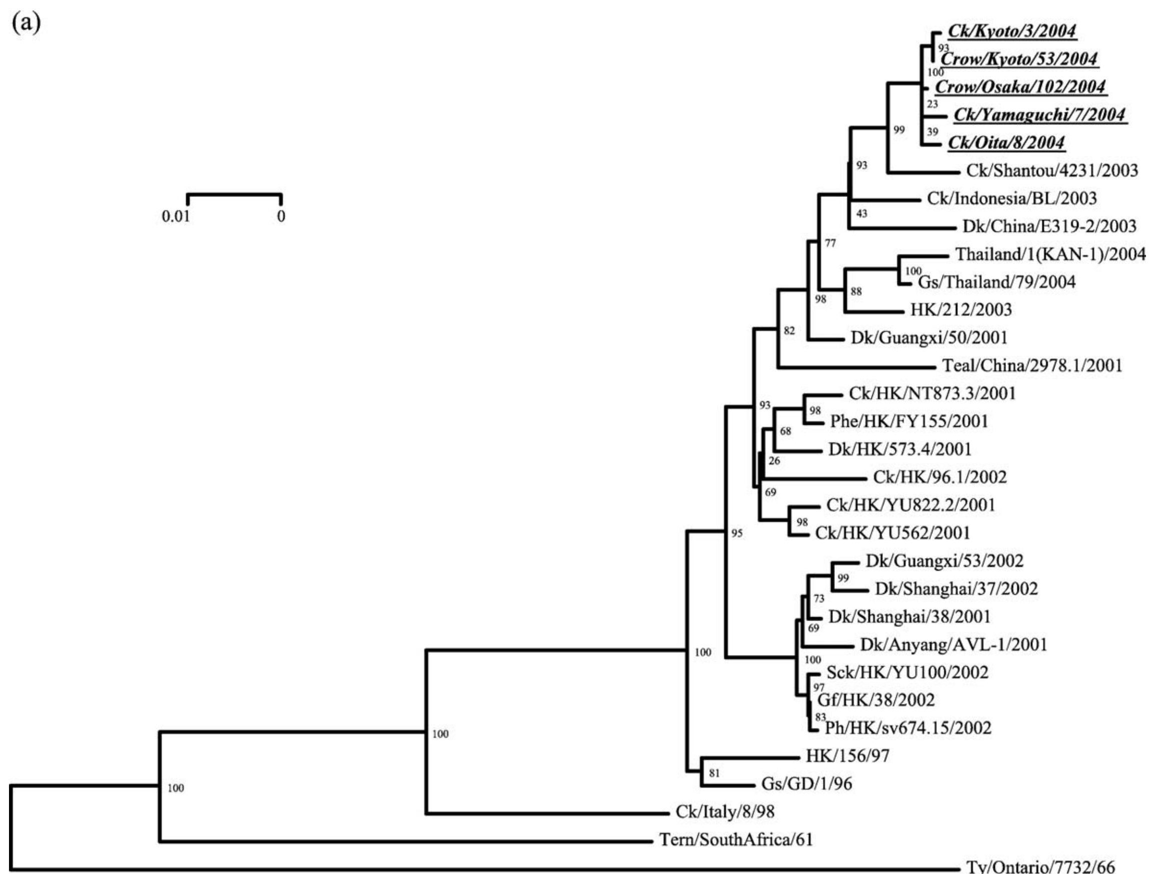

Fig. 2. Phylogenetic trees of the H5 HA (a), N1 NA (b), PA (c) genes of influenza A viruses. Nucleotides 62–1564 (1503 bases) of the H5 HAs, 510–1061 (552 bases) of the N1 NAs, and 1435–2172 (738 bases) of the PA genes were used for phylogenetic analysis. Horizontal distances are proportional to the 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 1,000 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 (silkey chicken), TW (Taiwan), Ty (turkey).

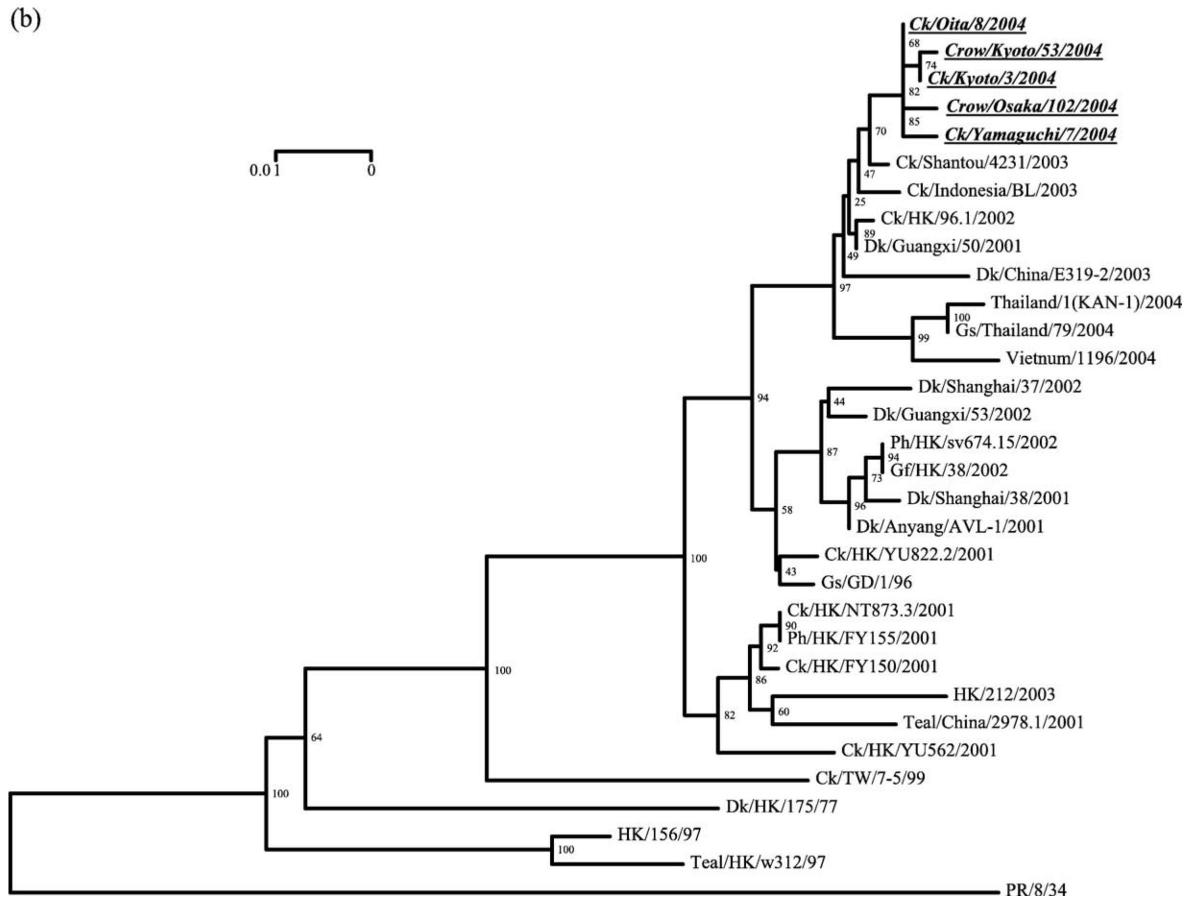

Fig. 2 (*continued*).

recently demonstrated in Thailand and Vietnam (Hien et al., 2004; World Health Organization, 2004b). 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 (Neumann et al., 1999; World Health Organization, 2004e).

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 conventional hemagglutination inhibition (HI) and neuramidase inhibition (NI) assays.

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 (the sequences of the primers are available upon request). PCR-derived dsDNA was used as a

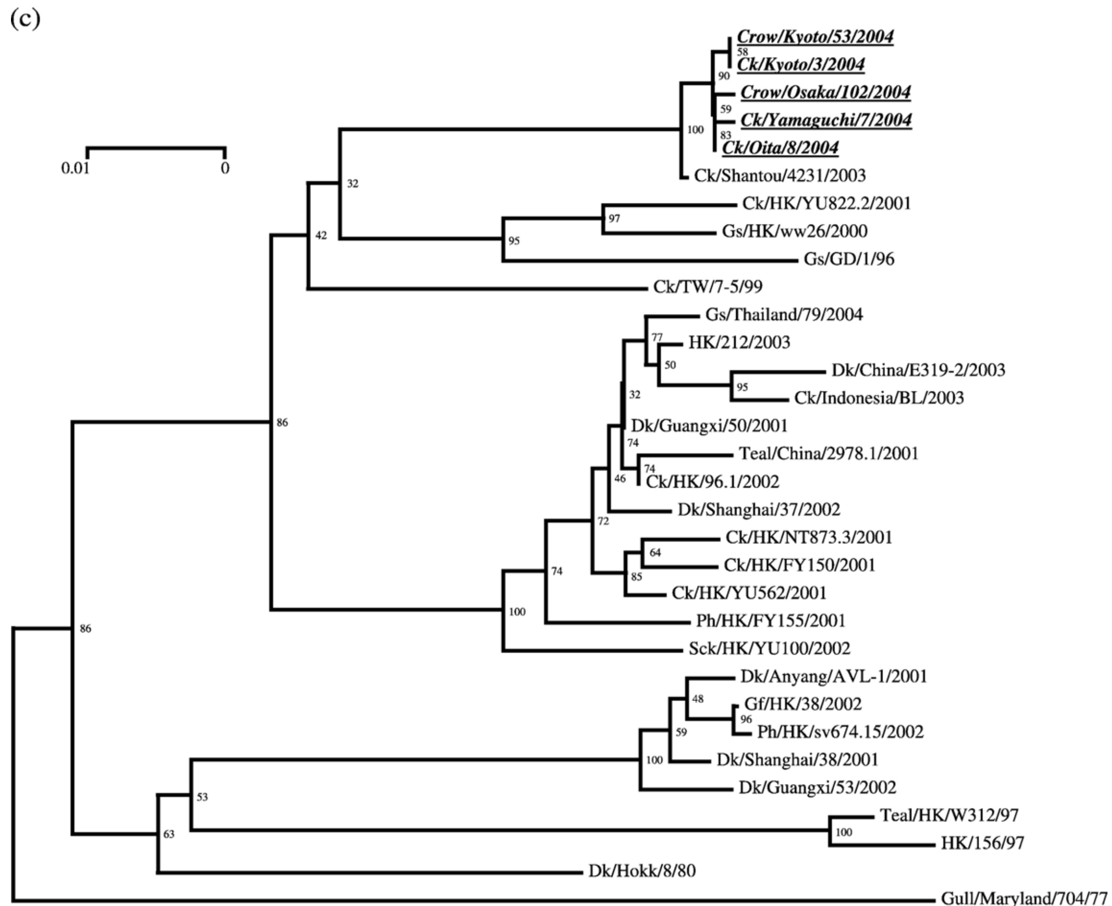

Fig. 2 (*continued*).

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 previously. (Mase et al., 2001).

The nucleotide sequences obtained from this study are available from GenBank under accession numbers AB166859–AB166866, AB188813–AB188824, and AB189046–AB189065.

Antigenic analysis

The antigenic relationships among H5N1 influenza viruses were determined in hemagglutination inhibition (HI) tests by using polyclonal chicken antiserum and a panel of monoclonal antibodies (Horimoto et al., 2004).

Pathogenecity tests

Chickens

Six-week-old specific pathogen-free chickens were used in this study. Eight chickens were inoculated either intra-

Table 3
Sequence comparison between A/chicken/Yamaguchi/7/2004 and other recent H5N1 influenza viruses

Segment	Percent 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 4
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	Subbarao et al. (1998)
A/chicken/Hong Kong/YU822.2/2001	R	E	R	R	R	K	K	R	Guan et al. (2002b)
A/chicken/Hong Kong/YU562/2001	R	E	R	R	R	K	K	R	Guan et al. (2002b)
A/pheasant/Hong Kong/FY155/2001	R	E	R	R	R	K	K	R	Guan et al. (2002b)
A/chicken/Hong Kong/FY150/2001	R	E	R	R	R	K	K	R	Guan et al. (2002b)
A/chicken/Hong Kong/NT873.3/2001	R	E	I	R	R	K	K	R	Guan et al. (2002b)
A/duck/Hong Kong/573.4/2001	R	E	–	R	R	K	K	R	Guan et al. (2002b)
A/duck/AVL-1/Anyang/2001	R	E	R	R	R	K	K	R	Tumpey et al. (2002)
A/teal/China/2978.1/2001	R	E	I	R	R	K	K	R	Li et al. (2004)
A/pheasant/Hong Kong/sv674.15/2002	R	E	R	R	R	K	K	R	Guan et al. (2004)
A/chicken/Hong Kong/96.1/2002	R	E	R	R	R	K	K	R	Guan et al. (2004)
A/Hong Kong/213/2003	R	E	R	R	R	K	K	R	Guan et al. (2004)
A/duck/China/E319-2/2003	R	E	–	R	R	R	K	R	unpublished
A/chicken/Shantou/4231/2003	R	E	R	R	R	K	K	R	Li et al. (2004)
A/chicken/Indonesia/BL/2003	R	E	R	R	R	K	K	R	Li et al. (2004)
A/Vietnam/1196/2004	R	E	R	R	R	K	K	R	Li et al. (2004)
A/goose/Thailand/79/2004	R	E	R	R	R	K	K	R	Li et al. (2004)

venously 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},) 6-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 µl 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 µl of virus (10^6 EID_{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. (2002). 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 h at 37 °C, the reaction was stopped by adding 0.1 M 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.

Table 5
Growth of A/chicken/Yamaguchi/7/2004 in mice

Organs	Days after infection ($\log_{10}$ EID_{50}/g)[a]	
	3	6
Brain	0/3	2/3 (3.7 ± 0.5)
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

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

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