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Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia

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Summary. Influenza A viruses of different subtypes were isolated from fecal samples of ducks in their nesting areas in Siberia in summer from 1996 to 1998. Phylogenetic analysis of the NP genes of the isolates in Siberia and those in Hokkaido, Japan on their flyway of migration from Siberia to the south in autumn revealed that they belong to the Eurasian lineage of avian influenza viruses. It is noted that the genes of the isolates in Siberia are closely related to those of H5N1 influenza virus strains isolated from chickens and humans in Hong Kong in 1997 as well as to those of isolates from domestic birds in southern China. The results indicate that influenza viruses perpetuated in ducks nesting in Siberia should have contributed genes in the emergence of the H5N1 virus in Hong Kong. Vaccine prepared from avirulent A/duck/Hokkaido/4/96 (H5N3) influenza virus was potent enough to protect mice from challenge with lethal dose of the pathogenic H5N1 virus [19]. Intensive surveillance study of aquatic birds especially in Siberia is, therefore, stressed to provide information on the future pandemic influenza virus strains and for vaccine preparation.

Introduction

Pandemic influenza viruses arise by genetic reassortment between human and non-human viruses [10, 12, 21]. All of the known subtypes of influenza A virus (H1 to H15 and N1 to N9) are circulating in aquatic birds, especially in migratory ducks [13, 20]. Antigenic and genetic analyses of influenza virus isolates from migratory ducks, domestic ducks, pigs, and humans indicated that the hemagglutinin (HA) gene of A/Kong Kong/68 (H3N2) strain was introduced into the precedent human H2N2 Asian influenza virus by genetic reassortment in pigs through domestic ducks from an H3 influenza virus circulating in migratory ducks in southern China [8, 9, 22]. In experimental infection of pigs, most tested strains of each HA subtype of H1–H13 avian influenza viruses replicate in the upper respiratory tracts of the animals. Co-infection of pigs with a swine virus and with an avian virus enable to replicate in this animal generated reassortant viruses that could be passaged in them [7]. The results indicate that avian viruses of any subtype can contribute genes in the generation of reassortants and so that none of the 15 HA and 9 neuraminidase (NA) subtypes can be ruled out as potential candidates for future pandemics. The direct transmisssion of H5N1 influenza viruses from poultry to humans in Hong Kong in 1997 [1, 17, 18] further emphasized the need to have information on influenza viruses circulating in avian species in the world.

Influenza A viruses of different subtypes were isolated from fecal samples of ducks and water of the lakes where they nest in summer on Yukon Flat in central Alaska [6]. It was the first isolation of influenza viruses from fecal samples of ducks in their nesting areas in their breeding season in Alaska. Influenza viruses were also isolated from the water samples of lakes where ducks breed even in autumn when they had left to the south for migration. We, thus, proposed the mechanism of year-by-year perpetuation of the viruses in the frozen lake water while ducks are absent. Phylogenetic analysis of the NP genes of the isolates showed that they belonged to the North American lineage of avian influenza viruses, suggesting that the host ducks nesting in the lakes on Yukon Flat and reservoiring influenza viruses migrate to North America and not to southern China, an influenza epicenter [6, 16].

In the present study, to provide information on the precursor genes of future pandemic influenza viruses, virological surveillance of avian influenza was carried out in Siberia where ducks nest in summer and in Hokkaido, Japan where ducks congregate on their flyway of migration from Siberia from 1995 to 1998.

Materials and methods

Virus isolation and identification

A total of 3,078 fecal samples were collected from 2,875 ducks, including mallards (*Anas platyrhynchos*), teals (*Anas crecca*), and pintails (*Anas acuta*), 131 geese (*Anser sp.*), 4 swans (*Cygnus sp.*), 44 mew gulls (*Larus canus*), and 24 shorebirds at 11 different points in Siberia of Russia in August 1995–1998 and in September 1996. In addition, fecal samples of 474 ducks and 345 swans were collected in October 1996–1998 at Lake Ohnuma in

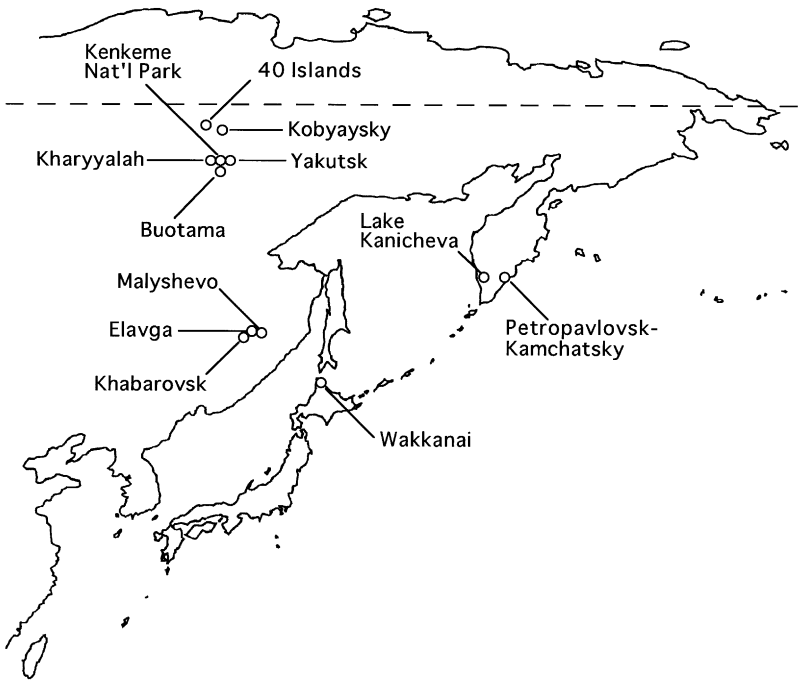

Fig. 1. Location of 12 different points where fecal materials were collected in Siberia and Hokkaido. The broken line indicates the Arctic Circle

Wakkanai, Hokkaido, Japan. Figure 1 illustrates locations of points where the samples were collected. The samples were kept in the cool, transported to Hokkaido University, and stored at −80 °C until assayed. Each sample was inoculated into the allantoic cavities of chicken embroys. After 2 days incubation at 35 °C the allantoic fluids were tested for hemagglutinating activity. All hemagglutinating agents were identified by hemagglutination-inhibition and neuraminidase-inhibition tests using specific antisera to the reference strains of influenza viruses [11].

Nucleotide sequencing

Nucleotide sequences of the NP genes of the isolates were determined as essentially described elsewhere [6]. In brief, viral RNA was extracted from the allantoic fluid of chicken embryos infected with virus and reverse-transcribed using the NP gene-specific oligonucleotide 5′-GCTTCAAATGAGAACATGGA-3′ as a primer. DNA fragments of 380 bp were generated by PCR using the above oligonucleotide and 5′-TTGTCTCCGAAGAAATAAGA-3′ as primers, and sequenced by using an ABI PRISM DNA sequencing kit with the ABI 373A sequencing system (Applied Biosystems Inc., Foster City, CA). Primer for sequencing the NP gene was 5′-ATGTCAAAGGAAGGCACGAT-3′.

Phylogenetic analysis

Phylogenetic analysis was based on partial nucleotide sequences of the NP genes (positions 1,216–1,430) of influenza viruses. Sequence data of the genes together with those from Genebank and our previous report [6] were analyzed by neighbor-joining method [14].

Results

Isolation of influenza A viruses from fecal samples of waterfowls

In total, 3,897 fresh fecal samples of birds were collected at 11 different points in Siberia in summer and at Lake Ohnuma in Wakkanai, Hokkaido where ducks congregate on the way of migration from 1995 to 1998. From these samples, 67 hemagglutinating agents were isolated. Of these, 48 agents were serologically identified as influenza viruses and the others as paramyxoviruses. Thirty-eight isolates, consisting of 5 H3N8, 20 H4N6, 1 H4N9, 1 H11N1, 2 H11N6, 8 H11N9, and 1 H13N6 viruses, were isolated from the samples of ducks collected only in the limited areas in Siberia (Table 1). H4N6, H4N9, H11N1, H11N6, and H11N9 viruses cocirculated in Kobyaysky area in 1996 and an H4N6 virus was isolated in 1997. H3 and H13 viruses were isolated from the samples collected in Yakutsk in 1997 and 1998, respectively. No influenza virus was isolated from the samples

Table 1. Isolation of influenza viruses from fecal samples of waterfowls in Siberia and Hokkaido in 1995–1998

		No. of samples with virus/total no. of samples tested			
Location	Birds	1995	1996	1997	1998
Petropavlovsk-	Duck	0/26			
Kamchatsky	Gull	0/32			
Lake Kanycheva	Duck	0/95			
Khabarovsk	Duck	0/18			
	Gull	0/2			
Malyshevo	Duck	0/83	0/7		
Elavga	Duck		0/66		
Koybyaysky	Duck		31[a]/819	1[b]/124	
40 Islands	Duck			0/1,185	
	Goose			0/126	
	Gull			0/10	
	Shorebirds		0/24		
Yakutsk	Duck			5[c]/62	1[d]/170
Kemkeme	Duck				0/23
National Park	Swan				0/4
	Goose				0/5
Kharyyalah	Duck				0/146
Buotama	Duck				0/51
Wakkanai	Duck		6[e]/143	2[f]/170	2[g]/161
	Swan		0/111	0/156	0/78

No. of each antigenic subtypes of the isolates were as follows:
[a]19 H4N6, 1 H4N9, 1 H11N1, 2 H11N6, and 8 H11N9, [b]1 H4N6, [c]5 H3N8, [d]1 H13N6, [e]1 H1N1, 1 H5N3, 1 H5N4, 1 H6N7, 1 H8N1, and 1 H8N3, [f]1 H9N2 and 1 H11N9, and [g]1 H6N2 and 1 H9N2

collected in the the South of 55° North latitude before migrating season. In late October, 10 influenza virus strains were isolated in Hokkaido, Japan. The numbers of subtypes of the isolates were 1 H1N1, 1 H5N3, 1 H5N4, 1 H6N2, 1 H6N7, 1 H8N1, 1 H8N3, 2 H9N2 and 1 H11N9. These results indicate that a variety of subtypes of influenza virus are maintained by ducks nesting in Siberia. Two H5 influenza viruses were isolated from the fecal samples of ducks flew from Siberia in 1996, 4 months before the highly pathogenic H5N1 influenza viruses appeared in Hong Kong.

Phylogenetic analysis of influenza virus isolates

The NP genes of 19 present isolates were partially sequenced and analyzed by neighbor-joining method with those of influenza viruses isolated from birds at different places in the world, pigs in Hong Kong in 1982 and Germany in 1981, a horse in China in 1989, a mink in Sweden in 1982, and humans in Hong Kong in 1997. The evolutionary tree indicates that the NP genes of the viruses isolated from ducks in Siberia and Hokkaido were divided into two groups within the Eurasian lineage of avian influenza viruses (Fig. 2). The first group comprised all of the isolates in Siberia and 3 isolates in Hokkaido. Dk/Sib/145/96 (H11N9) and four other H11N9 viruses, which were isolated in Kobyaysky area in 1996, had sister-group relationship with the H5N1 viruses isolated from humans and chickens in Hong Kong in 1997 and also with Qa/HK/G1/97 (H9N2). DK/Hok/33/96 (H6N7), Dk/Hok/137/96 (H8N1), and Dk/Sib/1566/97 (H3N8), an isolate from the samples collected in Yakutsk, were allied to influenza virus strains isolated from domestic birds in southern China, such as Dk/Nan/1749/92 (H11N2) and Gs/Gua/1/96 (H5N1). The H4N6 and H11N6 viruses isolated in Kobyaysky area in 1996 and 1997, and Dk/Hok/55/96 (H1N1) formed another subgroup together with Dk/HK/Y439/97 (H9N2). The second group included the NP genes of Dk/Hok/4/96 (H5N3), Dk/Hok/51/96 (H8N3), and Dk/Hok/67/96 (H5N4), which were phylogenetically most related to that of Ck/HK/G9/97 (H9N2). Their common branch had Sw/HK/126/82 (H3N2) and Sw/Ger/2/81 (H1N1) close to the root. These results indicate that influenza viruses maintained by ducks nesting in Siberia are closely related to the highly pathogenic H5N1 influenza viruses isolated in Hong Kong in 1997 as well as to those isolated from pigs and domestic birds in southern China.

Discussion

The present results provide the first evidence that influenza viruses are maintained by aquatic birds in their nesting areas in Siberia. Influenza virus isolation was made only from fecal samples of ducks collected in the basin of the Lena River, in higher latitudes than 62°. On the other hand, any virus was not isolated from the samples of birds collected in the South of 55° North latitude in summer. In our previous surveillance study conducted in Alaska, most of the viruses were isolated from fecal samples of ducks collected in areas located in 65–68° North latitude in their breeding season [6]. The present results, thus, confirm that influenza viruses

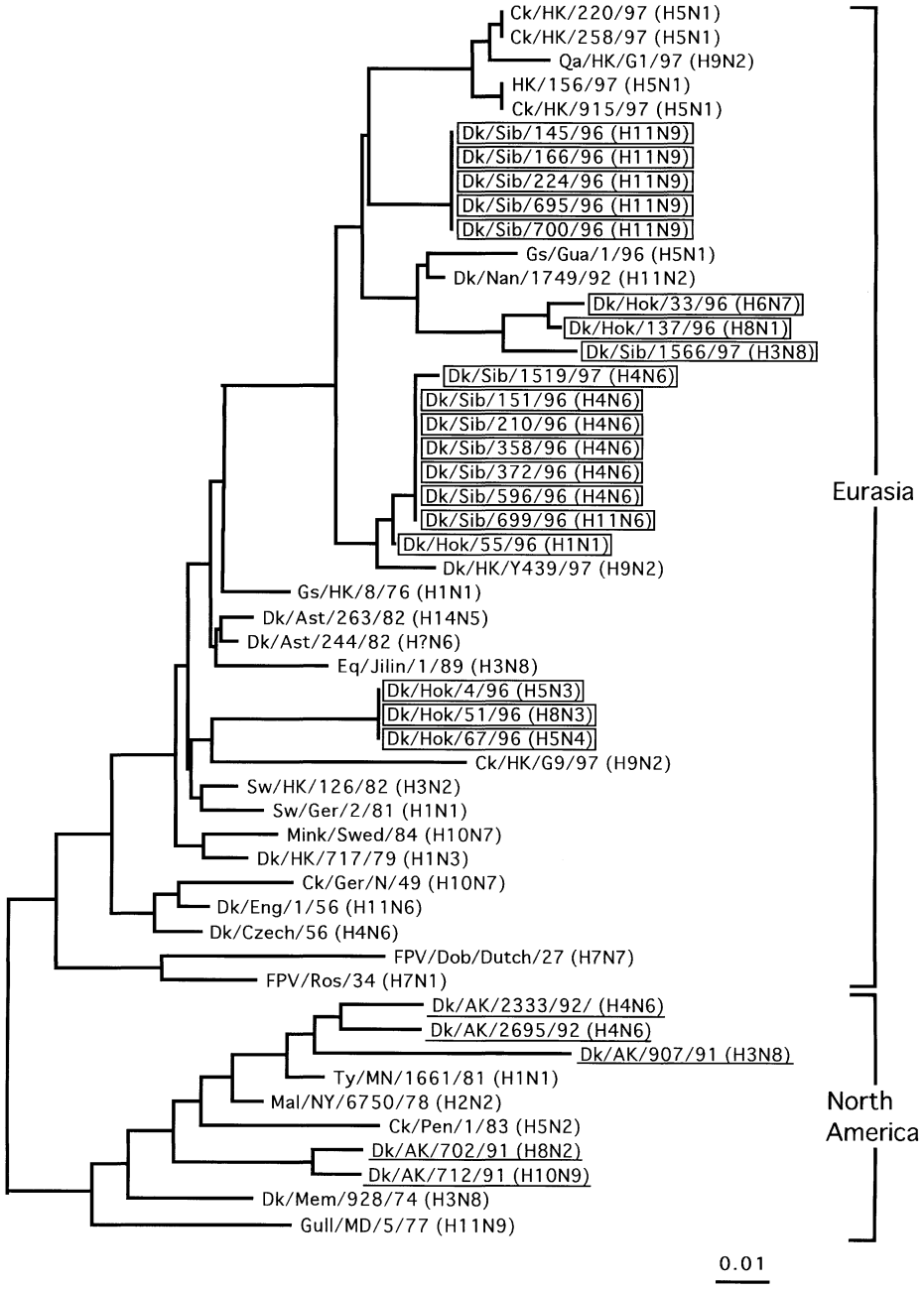

Fig. 2. Phylogenetic tree for avian-like influenza virus NP genes. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Viruses isolated from waterfowls in this study and previously in Alaska [6] are enclosed and underlined, respectively. Abbreviations: Ck (chicken), HK (Hong Kong), Qa (quail), Dk (duck), Sib (Siberia) Gs (goose), Gua (Guangdong), Nan (Nanchang), Hok (Hokkaido), Ast (Astrakhan), Eq (equine), Sw (swine), Ger (Germany), Swed (Sweden), Eng (England), Czech (Czechoslovakia), FPV (fowl plague virus), Dob (Dobson), Ros (Rostock), Ty (turkey), Mal (mallard), Mem (Memphis)

are maintained in duck population in their nesting areas close to the Arctic Circle in Siberia as well as in Alaska.

In our previous study in Alaska it was suggested that viruses are preserved in frozen lake water in winter [6]. The NP gene of Dk/Sib/1519/97 (H4N6) was most closely related to those of H4N6 viruses isolated in the preceding year in the same area. This finding also suggests that influenza viruses have been perpetuated in the lakes where they breed.

Ten influenza virus strains were isolated from 819 fecal samples collected in Hokkaido, 45° North latitude, where ducks migrating from Siberia congregate. The NP gene of Dk/Hok/55/96 (H1N1), Dk/Hok/33/96 (H6N7), and Dk/Hok/137/96 (H8N1) were closely related to those of the viruses isolated from ducks in the basin of the Lena River, indicating that the host ducks migrated from the basin of the Lena River. Three isolates, Dk/Hok/4/96 (H5N3), Dk/Hok/51/96 (H8N3), and Dk/Hok/67/96 (H5N4), were distantly related to other 16 isolates tested. This finding suggests that birds harboring these 3 isolates might fly from some other nesting area than the basin of the Lena River in Siberia. Limited combinations of HA and NA subtypes were found in each nesting area, whereas a variety of combinations were found in Hokkaido. The results indicate that frequent genetic reassortment must occur in the lakes where ducks congregate in autumn.

It is noted that the NP genes of the viruses isolated in the basin of the Lena River formed a unique cluster with those of highly virulent H5N1 influenza viruses isolated from chickens and humans in Hong Kong in 1997 as well as Qa/HK/G1/97 (H9N2), which is regarded as a donor of the internal genes of the H5N1 viruses [4]. The genes of Dk/Hok/33/96 (H6N7), Dk/Hok/137/96 (H8N1), and Dk/Sib/1566/97 (H3N8) were also closely related to that of Gs/Gua/1/96 (H5N1), which is a possible source of the H5 HA gene in the highly virulent H5N1 viruses [23]. These findings indicate that not only the NP genes but also the HA genes of the H5N1 influenza viruses originate from the viruses maintained by ducks nesting in Siberia.

The NP genes of Dk/Hok/4/96 (H5N3), Dk/Hok/51/96 (H8N3), and Dk/Hok/67/96 (H5N4) were also related to those of avian-like influenza viruses isolated from pigs [2, 3, 9, 12] as well as to those of isolates from domestic birds in southern China. The findings together with the proposed hypothesis for the emergence of the pandemic viruses [9, 10, 15], indicate that pandemic influenza virus strains should inherit the genes from the viruses maintained by ducks nesting in Siberia.

Hemagglutinins of the avirulent H5 influenza viruses isolated in Hokkaido were antigenically and genetically related to those of the highly virulent H5N1 viruses in Hong Kong [5]. Recently we demonstrated that formalin-inactivated virions of Dk/Hok/4/96 (H5N3) was induced enough protective immunity in mice against lethal dose of the virulent virus, which was equivalent to those of an H5N1 influenza virus possessing modified HA derived from the virulent HK/156/97 (H5N1) [19]. Since none of the 15 HA subtypes can be ruled out as potential candidates for future pandemics [7], each HA subtype of influenza virus isolate from aquatic bird could be a vaccine strain candidate for a future pandemic.

The present findings indicate that the precursor genes of pandemic influenza viruses are perpetuated in ducks nesting in Siberia. For the prediction of possible HA subtypes of future pandemic strains, it is important to expand virological surveillance of avian influenza especially in Siberia and Asia. Influenza virus strains isolated from avian species in the surveillance should be thoroughly characterized, stored, and be provided for the use of diagnosis and vaccine preparation for the prevention and control of pandemic influenza.

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References

1. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG (1998) Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351: 472–477
2. Gammelin M, Mandler J, Scholtissek C (1989) Two subtypes of nucleoproteins (NP) of influenza A viruses. Virology 170: 71–80
3. Gorman OT, Bean WJ, Kawaoka Y, Donatelli I, Guo YJ, Webster RG (1991) Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. J Virol 65: 3 704–3 714
4. Guan Y, Shortridge KF, Krauss S, Webster RG (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: 9 363–9 367
5. Imai M, Takada A, Okazaki K, Kida H (1999) Antigenic and genetic analyses of H5 influenza viruses isolated from wild ducks in Asia. Jpn J Vet Res 46: 171–177
6. Ito T, Okazaki K, Kawaoka Y, Takada A, Webster RG, Kida H (1995) Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch Virol 140: 1 163–1 172
7. Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka Y, Webster RG (1994) Potential for transmission of avian influenza viruses to pigs. J Gen Virol 75: 2 183–2 188
8. Kida H, Kawaoka Y, Naeve CW, Webster RG (1987) Antigenic and genetic conservation of H3 influenza virus in wild ducks. Virology 159: 109–119
9. Kida H, Shortridge KF, Webster RG (1988) Origin of the hemagglutinin gene of H3N2 influenza viruses from pigs in China. Virology 162: 160–166
10. Kida H, Webster RG, Kawaoka Y, Shortridge KF, Naeve CW, Ito T, Itakura C, Mori F, Shimizu Y (1989) Origin of the hemagglutinin gene of A/Hong Kong/68 (H3N2) influenza virus. In: Chen YC, Doraisingham S, Liang AE (eds) Current topics in medical virology. World Scientific, Singapore London, pp 365–376
11. Kida H, Yanagawa R (1979) Isolation and characterization of influenza A viruses from wild free-flying ducks in Hokkaido, Japan. Zentralbl Bakteriol Mikrobiol Hyg I Abt Orig A 244: 135–143
12. Ludwig S, Stitz L, Planz O, Van H, Fitch WM, Scholtissek C (1995) European swine virus as a possible source for the next influenza pandemic? Virology 212: 555–561

13. Röhm C, Zhou N, Süss J, MacKenzie J, Webster RG (1996) Characterization of a novel influenza hemagglutinin, H15: criteria for determination of Influenza A subtypes. Virology 217: 508–516
14. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425
15. Scholtissek C, Rhode W, Von Hoyningen V, Rott R (1978) On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87: 13–20
16. Shortridge FK, Stuart-Harris CH (1982) An influenza epicentre? Lancet 2: 812–813
17. Suarez DL, Perdue ML, Cox N, Rowe T, Bender C, Huang J, Swayne DE (1998) Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 72: 6 678–6 688
18. 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
19. Takada A, Kuboki N, Okazaki K, Ninomiya A, Tanaka H, Ozaki H, Itamura S, Nishimura H, Enami M, Tashiro M, Shortridge KF, Kida H (1999) Avirulent avian influenza virus as a vaccine strain against a potential human pandemic. J Virol 73: 8 303–8 307
20. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56: 152–179
21. Webster RG, Laver WG (1972) Studies on the origin of pandemic influenza. I. Antigenic analysis of A2 influenza viruses isolated before and after the appearance of Hong Kong influenza using antisera to the isolated hemagglutinin subunits. Virology 48: 433–444
22. Yasuda J, Shortridge KF, Shimizu Y, Kida H (1991) Molecular evidence for a role of domestic ducks in the introduction of avian H3 influenza viruses to pigs in southern China, where the A/Hong Kong/68 (H3N2) strain emerged. J Gen Virol 72: 2 007–2 010
23. Xu X, Subharao, Cox NJ, Guo (1999) Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 261: 15–18

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