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Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs

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Summary. To provide information on the mechanism of perpetuation of influenza viruses among waterfowl reservoirs in nature, virological surveillance was carried out in Alaska during their breeding season in summer from 1991 to 1994. Influenza viruses were isolated mainly from fecal samples of dabbling ducks in their nesting places in central Alaska. The numbers of subtypes of 108 influenza virus isolates were 1 H2N3, 37 H3N8, 55 H4N6, 1 H7N3, 1 H8N2, 1 H10N2, 11 H10N7, and H10 N9. Influenza viruses were also isolated from water samples of the lakes where they nest. Even in September of 1994 when the most ducks had left for migration to south, viruses were still isolated from the lake water. Phylogenetic analysis of the NP genes of the representative isolates showed that they belong to the North American lineage of avian influenza viruses, suggesting that the majority of the waterfowls breeding in central Alaska migrate to North America and not to Asia. The present results support the notion that influenza viruses have been maintained in waterfowl population by water-borne transmission and revealed the mechanism of year-by-year perpetuation of the viruses in the lakes where they breed.

Introduction

Since 1956 numerous influenza A viruses have been isolated from a variety of avian species [1]. All of the different subtypes of influenza A viruses (H1 to H14 and N1 to N9) are circulating in aquatic birds, particularly in migratory waterfowls [11]. Waterfowls are, therefore, rated as a major reservoir of influenza A viruses in nature [34].

Influenza A viruses of avian origin have been implicated in outbreaks of influenza in mammals, such as seals [12, 32, 33], whales [14], pigs [25], minks [20], and horses [9] as well as in domestic poultries [3, 10]. These evidences indicate that a vast influenza virus gene pool for future mammalian influenza exists in avian sources. Since each pandemic of human influenza first appeared in China [34], the possibility has been raised that southern China is an influenza epicenter [27]. New human pandemic strains are believed to arise by genetic reassortment between

human and non-human viruses [6, 21, 31]. It has been shown that H2N2 Asian and H3N2 Hong Kong pandemic influenza viruses are genetic reassortants between strains of human and avian origin [16, 24, 30]. Antigenic and genetic analyses of H3 influenza viruses isolated from wild ducks captured on the Asian Pacific flyway in Japan and domestic ducks in southern China have shown that the hemagglutinins (HAs) of these viruses were closely related to those of the earliest human H3 viruses [18, 35]. These findings indicated that the HA gene of the human pandemic strain, A/Hong Kong/68 (H3N2) was derived from such an avian virus [19]. Therefore, epidemiological surveillance of avian influenza would provide information on the next epidemics for other animal species, including human pandemics.

Waterfowls such as ducks and geese in the northern hemisphere migrate to south in autumn, back to north in spring, and breed in summer in the nesting places in northern territories such as Alaska, Canada, or Siberia. Extensive surveillance of influenza in migratory waterfowls was carried out in marshaling areas, where they were gathering in preparation of migration to south, in Canada from 1978 to 1983 [13]. However, little is known about how influenza viruses are maintained in duck population in their breeding places.

It was suggested that some ducks (e.g. Northern pintail) should migrate from Alaska through Aleutian Islands to Asia, although the majority of migratory ducks in Asia are from Siberia [36]. Distinct lineages of the genes of influenza viruses isolated from avian species in different regions of the world (e.g. North America and Eurasia) were demonstrated [4, 5, 7, 8, 15, 22]. Phylogenetic analysis of the genes of influenza virus isolates, therefore, would make it possible to presume the flyways of the host birds.

To provide information how influenza viruses are maintained in duck population in their breeding areas and the mechanism of perpetuation of the viruses, virological surveillance of avian influenza was made in Alaska from 1991 to 1994 and phylogenetic analyses of the genes of the isolates were carried out.

Materials and methods

Sample collection

In each July from 1991 to 1994, 3 120 fecal materials of birds, comprising 2 345 ducks, mainly mallards (*Anas platyrhynchos*) and pintails (*Anas acuta*), 663 Canada geese (*Branta canadensis*) 6 Tundra swans (*Cygnus columbianus*), 7 dunlins (*Calidris alpina*), and 10 Mew gulls (*Larus canus*), were collected at 15 different points in Alaska of the United States (Fig. 1 and Table 1). In 1992–1994, 81 water samples were also collected from 11 different ponds and lakes were ducks and geese were nesting. In September 1994, after almost ducks migrated to south, 21 water samples of Big Minto Lake where the viruses were isolated from ducks in the summer were collected. Samples were put into cryotubes, the lids sealed, and transported with dry ice to the Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee and then stored at −80 °C until assayed.

Virus isolation

Each fecal sample was put into phosphate-buffered saline (PBS; pH 7.2) containing antibiotics (2 000 units of penicillin, 400 μg of streptomycin, 40 μg of gentamycin and 200 units of polymyxin B

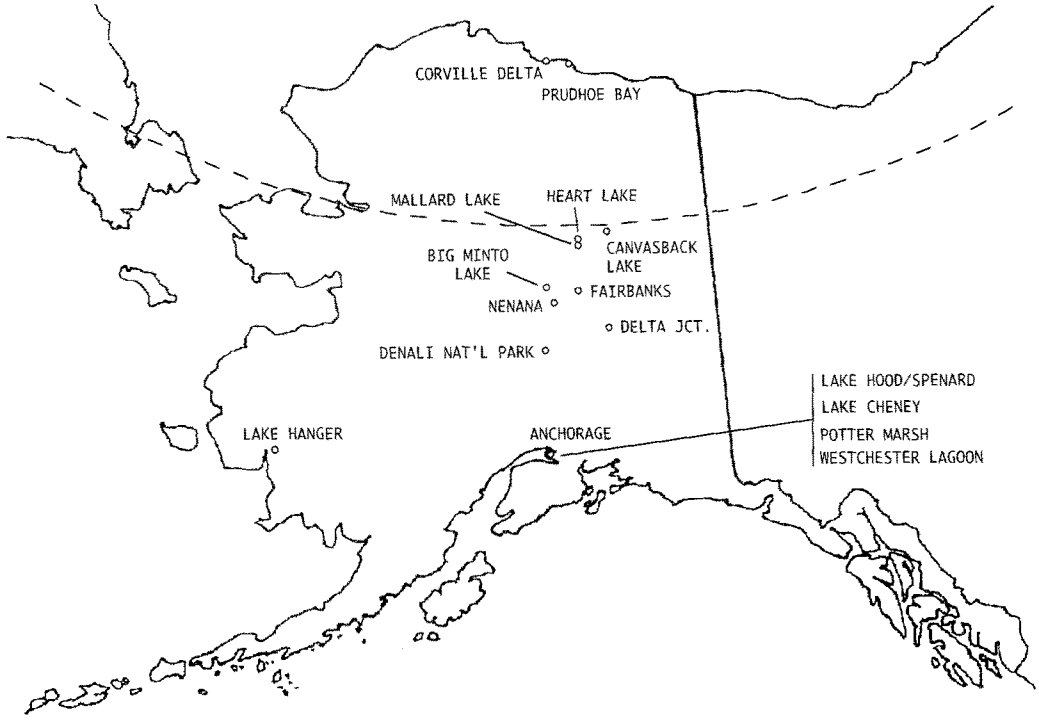

Fig. 1. Location of 15 different points where fecal materials of waterfowls were collected in Alaska. ---- Arctic circle

per ml) to give 10–20% suspension. The suspension was centrifuged at 2500 rpm for 20 minutes. One tenth ml of the supernatant was inoculated into allantoic cavities of two 9–11-day-old fertile hen's eggs. The eggs were incubated at 35 °C for 2 to 3 days unless death of the embryo was detected. At the end of the incubation period or upon embryo death, the allantoic fluids were tested for hemagglutinating activity.

Water samples were concentrated using formalin-treated chicken erythrocytes as follows: two ml of 5% formalin-treated chicken erythrocytes in 30 × PBS was added to each 50 ml of water sample and mixed. After incubation for 1 h at 4 °C, samples were centrifuged at 1500 rpm for 5 minutes. After removing the supernatant, 0.2 ml of PBS containing antibiotics were added and the erythrocytes were resuspended. One tenth ml of the resuspended erythrocytes was injected into allantoic cavity of 2 eggs per sample. Each unconcentrated water sample was also inoculated.

Identification of isolates

All hemagglutinating agents were identified in hemagglutination-inhibition and neuraminidase-inhibition tests using specific antisera to the reference strains of influenza viruses [2, 26]. The antisera were used as previously prepared and described [17].

Phylogenetic analysis of the NP genes

Phylogenetic analysis was based on partial nucleotide sequences of the NP genes (positions 1291–1430) of influenza viruses, determined by reverse transcription and PCR direct sequencing using viral RNA as a template and oligonucleotide primers. Sequence data of each gene, together with sequences from GenBank, were analyzed by Neighbor-joining method [23] using a computer software, ODEN version 1.1.1 (Yasuo Ina, National Institute of Genetics, Mishima, Japan).

Results

Isolation of influenza A viruses from fecal samples of waterfowls

To assess if waterfowls harbor influenza A viruses at their breeding areas, we collected 3 120 fresh fecal samples of birds in different areas in Alaska, USA during their late breeding season from 1991 to 1994. From these samples, 205 hemagglutinating agents were isolated. Of these, 108 were serologically identified as influenza viruses and the remainings as paramyxoviruses. Most of the influenza virus isolates were obtained from fecal samples of ducks, while only 4 were isolated from those of geese (Table 1). These viruses were isolated mainly from the samples collected in the central Alaska (i.e., pond in Nenana, Big Minto Lake, and Mallard Lake on the Yukon flat), while the frequencies of influenza virus isolation were very low in north and south Alaska. These results indicate that influenza viruses have been maintained in duck population in the major breeding areas of central Alaska.

Antigenic classification of the influenza A virus isolates

As shown in Table 2, subtypes of the influenza virus isolates from ducks were H3N8, H8N2, H10N2, H10N7, and H10N9 in 1991, H4N6 and H10N7 in 1992, H3N8, H4N6, and H7N3 in 1993, and H2N3, H3N8, and H4N6 in 1994. In each of Big Minto Lake and Heart Lake, influenza viruses of different subtypes were isolated at the same time (see footnote of Table 1). Additionally, different subtypes of influenza viruses were isolated in different years in the same lake (Big Minto Lake and Mallard Lake). These results indicate that different subtypes of influenza A viruses co-circulate in duck population in their breeding areas.

Isolation of influenza A viruses from lake water

To investigate the mechanism of perpetuation of the viruses from year to year, virus isolation was carried out from water samples of the lakes where ducks were nesting. In 1992, two viruses were isolated from the samples from different lakes in Alaska, where the same subtypes of influenza virus were isolated from ducks (Table 3). In the summer of 1994, 7 viruses were isolated from lake water and out of these, 2 samples contained $10^{1.8}$ and $10^{2.8}$ EID_{50} per ml of water samples. In the autumn of 1994, after most ducks left for migration to south, virus isolation was carried out from the water samples of the Big Minto Lake where the viruses were isolated in the summer. Three viruses were isolated from the water samples. These results support the notion that influenza viruses have been maintained in waterfowls by water-borne transmission.

Phylogenetic analyses of influenza virus isolates

To investigate the flyways of waterfowls carrying influenza viruses, phylogenetic analyses of the NP genes of 18 representative strains which were isolated in different years in the different lakes in Alaska was performed. Figure 2 shows an evolutionary tree constructed on the basis of partial sequences of the NP genes together with

Table 1. Isolation of influenza viruses from fecal samples of waterfowls in Alaska in 1991–1994

Location	Species	No. of samples with virus/ total no. of samples tested			
		1991	1992	1993	1994
Lake Hood/Spenard	Duck	0/302	2[d]/317	0/26	0/15
	Goose	4[a]/197	0/39	0/2	
	Gull		0/3		
Lake Cheney	Duck	0/293	0/133	0/34	
	Goose	0/185	0/8	0/3	
Potter Marsh	Duck	0/1	0/18		
	Goose	0/130			
	Gull	0/4			
Westchester Lagoon	Duck		0/134		
	Goose		0/2		
Lake Hanger	Duck		0/44		
Fairbanks	Duck	0/156	0/116		0/54
	Dunlin	0/7			
	Gull				0/3
Nenana	Duck	14[b]/65	0/41		2[h]/2
Delta Junction	Duck	0/27			
Denali Nat'l Park	Duck			0/6	
Big Minto lake	Duck	9[c]/149		14[f]/205	4[i]/28
	Goose			0/9	
Mallard Lake	Duck		46[e]/103	7[g]/53	0/24
	Goose			0/3	
Heart Lake	Duck			0/29	6[j]/39
Canvasback Lake	Duck			0/6	
Corville Delta	Duck		0/14		
	Goose		0/16		
	Swan		0/6		
Prudhoe Bay	Goose	0/69			
Total		27/1585	48/994	21/376	12/165

No. of each antigenic subtype of the isolates is as follows: [a] 3 H10N7 and 1 H8N2, [b] 14 H3N8, [c] 7 H10 N7, 1 H10N2, and 1 H10N9, [d] 1 H4 N6 and 1 H10N7, [e] 46 H4N6, [f] 7 H3N8, 6 H4N6, and 1 H7N3, [g] 7 H3N8, [h] 2 H3N8, [i] 4 H3N8, and [j] 3 H3N8, 2 H4N6, 1 H2N3

those from GenBank. Although the sequence data of the recent isolates in old world was not available, the present approach is valid because distinct lineages between the genes of isolates from avian species in North America and Eurasia have been demonstrated previously in several reports [4, 5, 7, 8, 15, 22]. The results showed that the NP genes of the viruses isolated from ducks in Alaska belong to the North American lineage of avian influenza viruses. These results suggest that waterfowls carrying these viruses migrate through the Continent of North America to south and not through Aleutian Islands to Asia.

Table 2. Antigenic characterization of influenza A virus isolates from fecal samples of waterfowls in Alaska from 1991 to 1994

Antigenic subtype	Number of influenza A viruses isolated in the following year				Total
	1991	1992	1993	1994	
H2N3				1	1
H3N8	14		14	9	37
H4N6		47	6	2	55
H7N3			1		1
H8N2	1				1
H10N2	1				1
H10N7	10	1			11
H10N9	1				1
Total	27	48	21	12	108

Table 3. Isolation of influenza viruses from water samples of lakes in Alaska in 1992–1994

Location	No. of samples with virus/ total no. of samples tested			
	1992 summer	1993 summer	1994 summer	1994 antumn
Lake Hood/Spenard	1[a]/4	0/2		
Lake Cheney	0/3	0/3		
Potter Marsh	0/4			
Westchester Lagoon	0/1			
Lake Hanger	0/4			
Fairbanks	0/5			
Big Minto Lake		0/10	7[c]/13	3[d]/21
Mallard Lake	1[b]/2	0/5	0 /17	
Heart Lake		0/5		
Canvasback Lake		0/3		
Corville Delta	0/1			
Total	2/23	0 /28	7/30	3/21

No. of each antigenic subtype of the isolates is as follows: [a] 1 H4N6, [b] 1 H4N6, [c] 4 H3N8 and 3 H4N6, [d] 2 H3N8 and 1 H7N3

Discussion

Hinshaw et al. [13] demonstrated high incidence of infection of ducks in the marshaling areas for their migratory flyway in Canada at the end of breeding season. They suggested that it was probably due to large numbers of susceptible juvenile birds in the areas. The present surveillance study of waterfowls in Alaska reveals

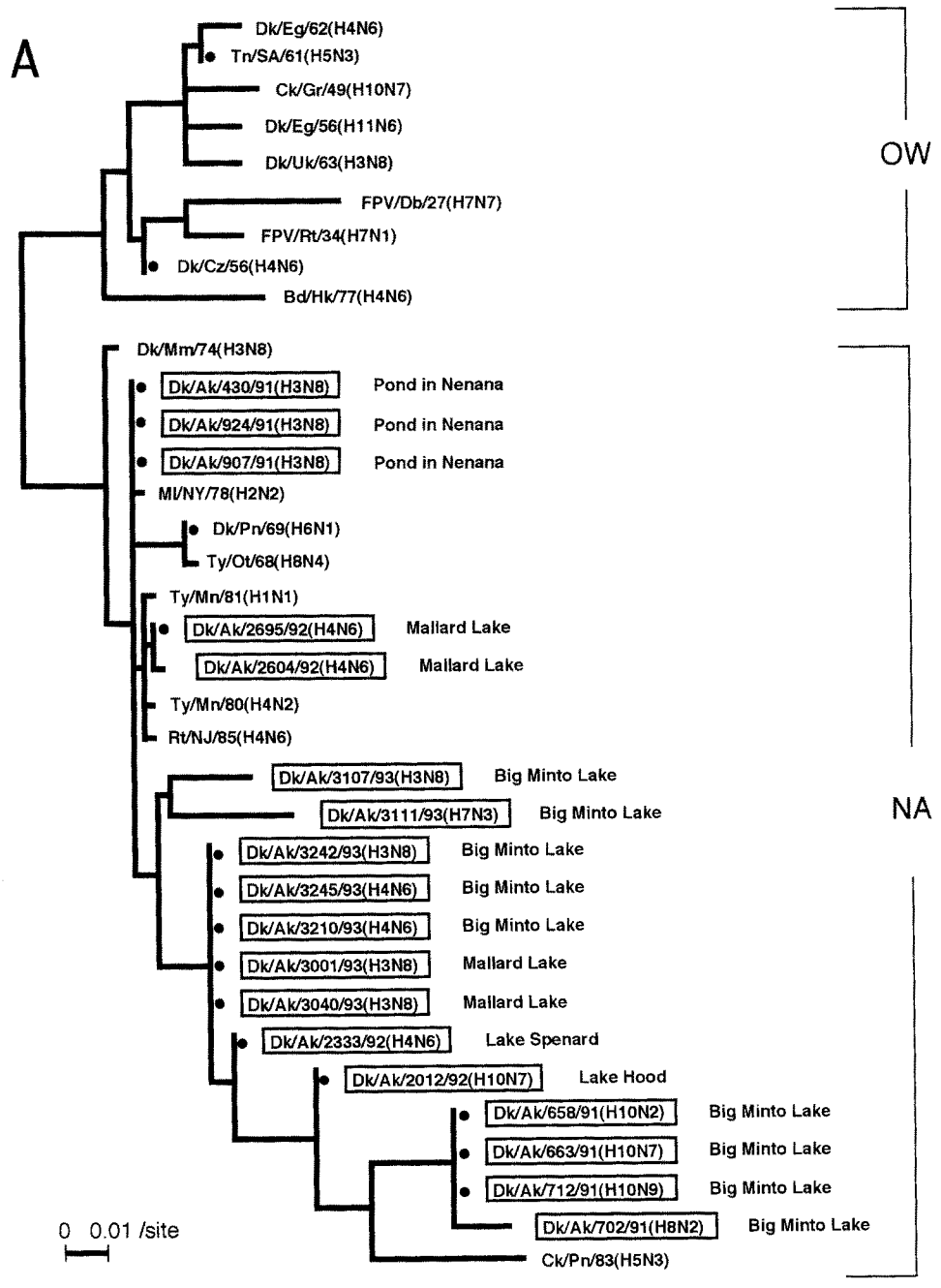

Fig. 2. Phylogenetic tree for avian influenza A virus NP genes. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Dots represent that the sequences are identical with the nodes. Vertical lines are for spacing branches and labels. Viruses isolated from waterfowls at their nesting points in Alaska are in boxes, together with the name of the lake where they were isolated. Abbreviations for viruses are found in Gorman et al. [8]. Each tree was branched into Old World (*OW*) and North American (*NA*) sublineages

that high incidence of virus infection of ducks has already occurred in the breeding places of ducks. Continuous surveillance of influenza viruses in migratory birds in Canada and USA showed that the frequency of virus isolation from migratory ducks coming from northern territories in autumn was high (more than 20% of juveniles), whereas those from south in spring was extremely low (less than 0.25%) [34], suggesting that viruses are mainly supplied when they migrate to south. The present results suggest that first amplification of the virus by juvenile birds has already occurred in the breeding areas of waterfowls and the viruses were brought to southern area by their migration.

The mechanism of perpetuation of influenza viruses in avian species year by year has not been well understood. Influenza viruses were isolated from unconcentrated lake water when migratory ducks are present [11]. In the present study, high titers of the viruses were isolated from water samples of different lakes in the breeding areas of ducks in northern high latitudes in summer (Lake Hood, Mallard lake and Big Minto Lake). Furthermore, the viruses remained viable in the lake water after that almost ducks left for migration to south. It is possible that influenza viruses are preserved in frozen lake water during winter when ducks are absent and that ducks coming back from south are infected with these viruses in spring. Survival of influenza viruses in water is dependent on the virus strain and the salinity, pH, and temperature of the water; at 17 °C some strains remain infectious for up to 207 days, and at 4 °C they remain infectious for longer period [28, 29]. The major breeding areas of ducks in Alaska located in high latitudes, 65–68 degrees north, near the arctic regions. The lake water in this area is cold enough for viruses to survive for longer period in autumn when ducks leave for migration to south. Therefore, influenza viruses could be preserved in the frozen lake water while ducks are absent.

The phylogenetic analysis of the NP genes of influenza virus isolates from ducks in Alaska showed that all of those analyzed belonged to the North American lineage (Fig. 2). Therefore, these viruses were not supposed to be the gene sources of Hong Kong pandemic strains. Alternatively, Bean et al. [4] suggested that A/duck/Hokkaido/21/82 (H3N8) which was isolated from a migratory duck captured on the Asian Pacific flyway in Japan bore the North American type HA gene. There are other major breeding areas of waterfowls in west and south Alaska such as Yukon Delta, Seward Peninsula, and St. Lawrence Island. The possibility that waterfowls in these areas migrate to Asia through Aleutian Islands was retained. Phylogenetic analysis of various virus isolates from waterfowls may make it possible to presume the migratory flyways of host birds carrying influenza viruses.

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