Journal of General Virology (1999), **80**, 3167–3171. Printed in Great Britain SHORT COMMUNICATION

Transmission of Eurasian avian H2 influenza virus to shorebirds in North America

N. V. Makarova,[1,2] N. V. Kaverin,[1] S. Krauss,[2] D. Senne[3] and R. G. Webster[2]

[1] The D. I. Ivanovsky Institute of Virology, Gamaleya Str. 16, Moscow 123098, Russia

[2] St Jude Children's Research Hospital, Department of Virology and Molecular Biology, 332 N. Lauderdale, Memphis, Tennessee 38105, USA

[3] Avian Viruses Section, Diagnostic Virology Laboratory, National Veterinary Service Laboratories, APHIS, United States Department of Agriculture, PO Box 844, Ames, Iowa 50010, USA

Influenza A virus of the H2 subtype caused a serious pandemic in 1957 and may cause similar outbreaks in the future. To assess the evolution and the antigenic relationships of avian influenza H2 viruses, we sequenced the haemagglutinin (HA) genes of H2 isolates from shorebirds, ducks and poultry in North America and derived a phylogenetic tree to establish their interrelationships. This analysis confirmed the divergence of H2 HA into two geographical lineages, American and Eurasian. One group of viruses isolated from shorebirds in North America had HA belonging to the Eurasian lineage, indicating an interregional transmission of the H2 gene. Characterization of HA with a monoclonal antibody panel revealed that the antigenicity of the Delaware strains differed from the other avian strains analysed. The data emphasizes the importance of avian influenza surveillance.

Influenza is a re-emerging epidemic disease, and during the last century there have been several human pandemics. Three influenza virus subtypes, H1N1, H2N2 and H3N2, were associated with these pandemics. Seroarcheological data suggest that H2 influenza virus was responsible for the influenza pandemic of 1889–1890 (Mulder & Masurel, 1958). There are no indications that influenza A viruses of the H2 subtype circulated in the human population during the 20th century until 1957, when there was a serious H2 influenza pandemic. The pandemic virus had haemagglutinin (HA), NA and PB1 genes derived from an H2N2 avian precursor; the remainder of its genes were derived from an H1N1 virus that circulated in the human population before 1957 (Kawaoka *et al.*, 1989; Scholtissek *et al.*, 1978).

Author for correspondence: Natalia Makarova (at The D. I. Ivanovsky Institute of Virology).
Fax +7 095 5165314. e-mail finmed@glasnet.ru

Influenza viruses of the H2 subtype circulated in the human population until 1968, after which they continued to be detected in avian populations (Tumova *et al.*, 1975; Shortridge, 1979; Sinnecker *et al.*, 1983; Wright *et al.*, 1992). Most H2N2 viruses in wild and domestic birds are antigenically closely related to the viruses that circulated in the human population between 1957 and 1968, suggesting that viruses similar to the progenitor of the Asian/57 pandemic are still circulating in birds (Schafer *et al.*, 1993). Serological data showed that the percentage of detection of anti-H2 antibodies in the sera of adults was low, and no anti-H2 antibodies were detected in the sera of children (Govorkova *et al.*, 1993). These facts emphasize the importance of periodical surveillance of avian H2 influenza viruses and elucidation of the phylogenetic and evolutionary relationships among present and previously known avian H2 influenza viruses.

The two distinct lineages of avian H2 influenza viruses, American and Eurasian, are distinguished by their geographical origins (Schafer *et al.*, 1993). One virus isolated in the USA, A/Herring gull/Delaware/677/88, has been shown to belong to the Eurasian lineage, but that single observation could not be conclusively interpreted. To elucidate the evolutionary characteristics of recently isolated avian viruses of the H2 subtype, we investigated the antigenic and phylogenetic relationships among recent and previously described avian influenza H2 viruses.

The viruses used in these studies (Table 1) were obtained as part of surveillance programs in the northeastern part of the USA and in Alberta, Canada, or from the virus repository at St Jude Children's Research Hospital, Memphis, TN, USA. The shorebird and gull viruses were isolated from samples collected annually in mid-May from the Delmarva Peninsula region of the USA between 1985 and 1998 (Kawaoka *et al.*, 1988). Influenza viruses from migratory ducks were isolated between 1977 and 1997 from samples collected annually in late July to early September in Alberta, Canada (Sharp *et al.*, 1993). Viruses

0001-6456

Table 1. Influenza A viruses used for phylogenetic analysis

Strain	Abbreviation
AF116203 A/Pintail duck/Alberta/293/77 (H2N3)*	pin/Alb/77
AF116204 A/Pintail duck/Alberta/211/80 (H2N3)*	pin/Alb/80
AF116202 A/Mallard/New York/66861/78 (H2N3)*	md/NY/78
AF116201 A/Laughing gull/New Jersey/75/85 (H2N9)*	lgl/NJ/85
AF116198 A/Chicken/New York/29878/91 (H2N2)*	ck/NY/91
AF116200 A/Guinea fowl/New York/20221-11/95 (H2N2)*	gf/NY/95
AF116197 A/Chicken/New York/13828-3/95 (H2N2)*	ck/NY/95
AF116199 A/Silky chicken/Pennsylvania/9600897/96 (H2N3)*	ck/PA/96
A/Blue-winged teal/Alberta/16/97 (H2N9)*	bwt/Alb/97
AF116208 A/Ruddy turnstone/Delaware/81/93 (H2N1)*	rt/DE/81/93
AF116207 A/Ruddy turnstone/Delaware/34/93 (H2N1)*	rt/DE/34/93
AF116210 A/Shorebird/Delaware/111/97 (H2N1)*	sb/DE/111/97
AF116211 A/Shorebird/Delaware/138/97 (H2N1)*	sb/DE/138/97
AF116209 A/Shorebird/Delaware/24/98 (H2N1)*	sb/DE/98
AF116206 A/Ruddy turnstone/Delaware/142/98 (H2N8)*	rt/DE/98
AF116205 A/Black duck/New Jersey/1580/78 (H2N3)	bdk/NJ/78
L11130 A/Gull/Maryland/19/77 (H2N8)	gl/MD/77
L11138 A/Mallard/Ontario/56/76 (H2N3)	ml/Ont/76
L11131 A/Guinea fowl/New Jersey/3070/91 (H2N2)	gf/NJ/91
L11135 A/Mallard/Alberta/353/88 (H2N3)	ml/Alb/88
L11137 A/Mallard/New York/6750/78 (H2N2)	ml/NY/78
L11129 A/Duck/GDR/72 (H2N9)	dk/GDR/72
L11136 A/Mallard/MT/Y61 (H2N2)	ml/MT/Y61
L11140 A/Peking duck/Potsdam/1689-4/85 (H2N3)	pdk/Pot/85
L11132 A/Herring gull/Delaware/677/88 (H2N8)	hgl/DE/88
L11139 A/Mallard /Potsdam/178-4/83 (H2N2)	ml/Pot/83
L11127 A/Chicken/Potsdam/4705/84 (H2N2)	ck/Pot/84
L11128 A/Duck/Hong Kong/273/78 (H2N2)	dk/HK/78
L11141 A/Pintail/Primorie/695/76 (H2N2)	pin/Prim/76
L20407 A/Japan/305-/57	Japan/57
L20410 A/Singapore/1/57	Singapore/57
L20409 A/RI/5-/57	RI/57
L11134 A/Krasnodar/101/59	Krasnodar/59
L11133 A/Korea/426/68	Korea/68
L11126 A/Berlin/3/64	Berlin/64
D13579 A/Izumi/5/65	Izumi/65
L11125 A/Berkeley/1/68	Berkeley/68

* Virus sequenced in this study.

were propagated at low m.o.i. in the allantoic cavity of 11-day-old embryonated chicken eggs.

Phylogenetic analysis was based on the nucleotide sequences of 15 newly sequenced HA genes (HA1 subunit) and the previously sequenced HA genes of avian H2 viruses (Table 1). The sequences were determined by reverse transcription (Huddleston & Brownlee, 1982), and PCR direct sequencing was performed by the Centre for Biotechnology at St Jude Children's Research Hospital by using dye-terminator Cycle-Sequencing Ready-Reaction kits with AmpliTaq DNA polymerase FS (Perkin-Elmer, Applied Biosystems). Sequence data of each gene were analysed by the Wisconsin Package version 9.0, Genetics Computer Group, Madison, WI, USA. Phylogenetic analysis of new sequences, together with sequences from GenBank, was performed by a combination of the Neighbour-Joining method (Saitou & Nei, 1987) for detection of distances and the Maximum-Parsimony method (Fitch, 1970) for generation of a phylogenetic tree using PHYLIP (the PHYLogeny Inference Package) version 3.57c. The phylogenetic tree is rooted to the H5 HA1 sequence from A/Chicken/Pennsylvania/1/83 (H5N1) (J04325) because of the high similarity (74%) between H2 and H5 serotypes (Air, 1981; Nobusawa *et al.*, 1991).

The phylogenetic tree (Fig. 1) confirms the divergence of H2 influenza A viruses into two distinct geographical lineages: American and Eurasian. All newly sequenced HA1 genes from domestic poultry were of the American lineage. However, a large group of recent American isolates from shorebirds in

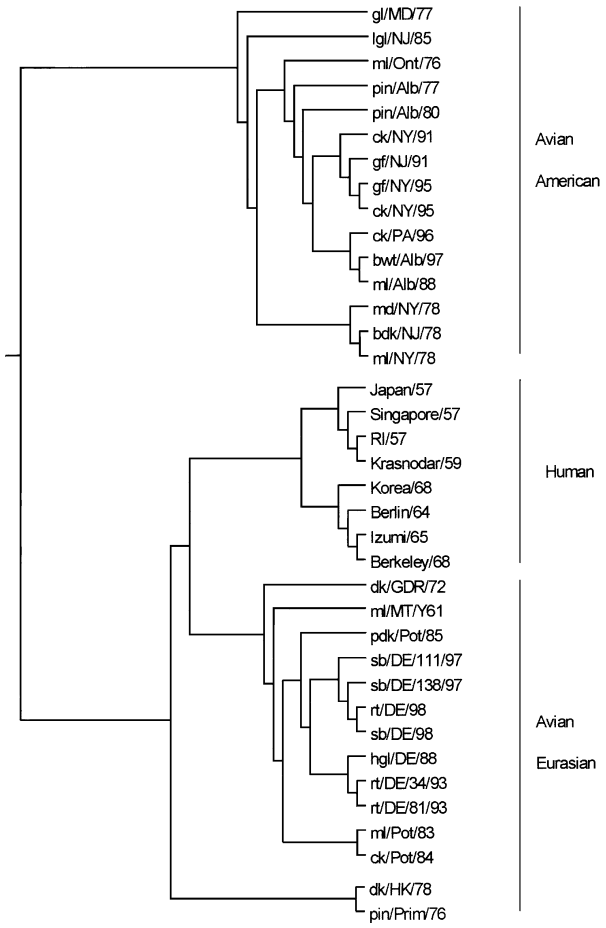

Fig. 1. Phylogenetic tree of the HA1 genes of H2 influenza A viruses. The tree is rooted to the H5 HA sequence from A/Chicken/Pennsylvania/1/83 (accession no. J04325). Horizontal distances are proportional to the number of nucleotide changes in HA1. Strain abbreviations are listed in Table 1.

Delaware Bay belonged to the Eurasian lineage. As reported previously (Schafer *et al*., 1993), the A/Herring gull/Delaware/677/88 strain isolated in Delaware Bay also is of the Eurasian lineage. Together, the viruses isolated in Delaware Bay composed a divergent sublineage in the Eurasian section of the tree. All of these viruses were isolated from seabirds (gulls and shorebirds). Interestingly, two viruses previously isolated from gulls in North America (A/Gull/Maryland/19/77 and A/Laughing gull/New Jersey/75/85) were of the American lineage but diverged from other isolates of that lineage. A/Peking duck/Potsdam/1689-4/85 was the Eurasian virus most closely related to the group of American viruses with Eurasian HA. These data suggest that Eurasian H2 was transmitted from Eurasian to American avian hosts.

The rates of evolution were determined from the nucleotide sequences of the HA1 of H2 influenza viruses using GeneDoc (Multiple sequence alignment editor and shading utility version 2.3000, 1997) for statistical analysis of the relationships between pairs of sequences. In the Delaware Bay group of viruses, the evolution rate was $2{\cdot}47 \pm 0{\cdot}23$ nucleotide substitutions per year, a rate significantly greater than that of the Eurasian viruses ($1{\cdot}57 \pm 0{\cdot}23$ substitutions per year) but less than that of the American viruses ($3{\cdot}8 \pm 0{\cdot}5$ substitutions per year). The differences are statistically significant at $P \leqslant 0{\cdot}05$. In the group of Delaware Bay viruses, 34·5% of all nucleotide substitutions led to amino acid replacements. In the American lineage, 25·6% of nucleotide substitutions resulted in amino acid replacements, and in the Eurasian lineage, 20·4% of nucleotide substitutions resulted in amino acid replacements. Thus, although the Delaware Bay group falls between the Eurasian and the American viruses in the rate at which it acquires nucleotide substitutions, it has the highest percentage of nucleotide substitutions that result in amino acid changes. In the human H2 viruses having circulated in 1964–1968 (A/Korea/426/68, A/Izumi/5/65, A/Berlin/3/64, A/Berkeley/1/68), a comparable percentage (35·7%) of nucleotide substitutions led to amino acid changes.

As shown previously, human influenza viruses evolve rapidly because of the pressure of the neutralizing antibodies of the host (Both *et al*., 1983). Avian species produce humoral and cell-mediated responses to influenza virus infection, but their antibody responses are short-lived (Kida *et al*., 1980); moreover, a large number of young susceptible birds are produced every year, eliminating the immune pressure among avian viruses. The high rate of HA evolution in Eurasian-type American H2 viruses suggests the existence of an unknown positive selection mechanism that favours amino acid changes in the transferred virus, probably connected with the transfer to the new environment. This finding is also at variance with our earlier findings of evolutionary stasis among influenza viruses in their natural reservoirs (Webster *et al*., 1992) and indicates that this may not apply to all aquatic birds.

The higher percentage of amino acid replacements in the HA of the Delaware group of viruses may lead to changes in their antigenic reactivity pattern. To investigate this possibility, haemagglutination titrations and haemagglutination inhibition (HI) assays were performed as previously described (Rogers *et al*., 1983). Ascitic fluids from mice containing monoclonal antibodies (MAbs) to the HA of the H2 influenza subtype (Yamada *et al*., 1984) were used in the HI tests. The HA of avian viruses isolated in North America demonstrated two patterns of reactivity with the panel of MAbs (Table 2). However, the antigenic and phylogenetic groups did not coincide. The American viruses react with most MAbs in the panel. In contrast, the Delaware Bay (shorebird and gull) viruses reacted with only two or three MAbs. This pattern of reactivity, which had been reported earlier for one Delaware Bay shorebird strain (A/Herring gull/Delaware/677/88), extended to all of the four shorebird viruses characterized. However, the Delaware Bay strains are not unique in this respect. One Eurasian strain, A/Duck/GDR/72, and one American strain, A/Chicken/New York/29878/91, had shown a similar pattern of reaction (Schafer *et al*., 1993).

Table 2. Haemagglutination inhibition by monoclonal antibodies

+, HI titres less than tenfold different from homologous titres; −, HI titres at least tenfold lower than homologous titres or undetectable.

	MAb to Japan/57											
	33/1	79/1	61/7	41/4	121/7	67/7	137/5	112/2	L122/4	28/3	131/4	75/1
Human viruses												
Japan/57	1	128	16	64	64	64	64	2	256	16	32	64
Korea/68	−	−	+	−	−	−	−	+	−	−	−	−
Avian viruses												
pin/Alb/77	+	+	+	+	+	+	+	−	+	+	+	+
md/NY/78	+	+	+	+	−	+	+	−	+	−	−	−
pin/Alb/80	−	+	+	+	−	−	+	−	+	−	−	−
ck/NY/95	+	+	+	+	−	+	+	+	+	−	+	−
lgl/NJ/85	−	+	+	−	−	−	−	−	+	−	−	−
rt/DE/81/93	−	−	+	−	−	−	−	−	−	+	−	−
rt/DE/34/93	−	−	+	−	−	−	−	−	−	−	−	−
sb/DE/111/97	+	−	+	−	+	+	−	−	+	−	−	−
sb/DE/138/97	+	−	+	−	−	−	−	−	+	−	+	−

The disparity between the antigenic and phylogenetic groupings suggests that most of the amino acid replacements have not been affecting antigenic specificity. However, the changes at position 137, 189 and 141–144 may be relevant to the antigenic variability of the H2 subtype. These substitutions are located in antigenic sites A and B as outlined for the 3D structure of H3 (Wiley *et al.*, 1981).

Several influenza A virus HA subtypes comprise geographically separate lineages (Garcia *et al.*, 1997; Donis *et al.*, 1989). These lineages may have emerged because of nonoverlapping migration routes. Most wild birds follow North–South migration routes that are separate for each hemisphere. Further, the birds that migrate across the Atlantic do so at times when North–South migrations are over. For example, the Canadian terns and gulls that cross the Atlantic (Curry-Lindahl, 1975) arrive in Europe after the European birds have migrated to Africa. On rare occasions, unusual weather conditions may bring the two populations into contact, allowing the interregional transmission of viruses. Turnstones, which are pelagic birds and whose migration is not fully resolved, may play a role in this transmission (Curry-Lindahl, 1975). However, such occasional transmissions would be unlikely to lead in each case to circulation of the transferred virus; otherwise, the phylogenetically distinct lineages would not have arisen. The circulation of Eurasian H2 virus in the Delaware shorebirds for at least 10 years does not necessarily suggest the existence of some specific, as yet unknown, conditions in the area that favour the virus: new Eurasian-type H2 viruses may be circulating in other parts of America as well. All of the sequenced H2 viruses isolated from gulls in other parts of America were isolated earlier than 1988. The first Delaware Eurasian-type virus was isolated in 1988. If the transfer occurred not long before 1988, it is possible that the Eurasian-type viruses are in fact not restricted to Delaware Bay but are instead restricted to shorebirds and gulls (Kawaoka *et al.*, 1988).

H2 virus is the only pandemic influenza virus subtype that has not circulated in the human population for the last 30 years. Therefore, the probability that H2 viruses will reappear in humans may be high. It is generally accepted that the 1957 and 1968 influenza pandemics were produced by reassortant human strains with HA genes that originated from avian influenza viruses. Although the details of the transfer of Eurasian H2 virus strains to North America are not yet clear, the existence of this transfer has important implications. The Eurasian avian influenza virus strains are those most closely related to human H2 viruses (Schafer *et al.*, 1993) and are potential sources of genes for future pandemic influenza strains. These are compelling reasons for the further study and surveillance of avian H2 strains circulating in the Americas.

The research described in this publication was made possible in part by award RN1-412 of the U. S. Civilian Research & Development Foundation for the Independent States of the Former Soviet Union (CRDF) and by Public Health Research Grant AI29680 and Cancer Centre Support (CORE) grant CA-21765 from the National Institutes of Health, and by the American Lebanese Syrian Associated Charities. We thank Sharon Naron for editorial assistance.

References

Air, G. M. (1981). Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus. *Proceedings of the National Academy of Sciences, USA* **78**, 7639–7643.

Both, G. W., Sleigh, M. J., Cox, N. J. & Kendal, A. P. (1983). Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. *Journal of Virology* **48**, 52–60.

Curry-Lindahl, K. (1975). Faglar over land och hav. En global oversikt av faglarnas flyttning, pp. 113–114. Stockholm: Albert Bonniers Forlag.

Donis, R. O., Bean, W. J., Kawaoka, Y. & Webster, R. G. (1989). Distinct lineages of influenza virus H4 hemagglutinin genes in different regions of the world. *Virology* **169**, 408–417.

Fitch, W. M. (1970). Distinguishing homologous from analogous proteins. *Systematic Zoology* **19**, 99–113.

Garcia, M., Suarez, D. L., Crawford, J. M., Latimer, J. W., Slemons, R. D., Swayne, D. E. & Perdue, M. L. (1997). Evolution of H5 subtype avian influenza A viruses in North America. *Virus Research* **51**, 115–124.

Govorkova, E. A., Kizina, A. A., Krylov, V. F. & Smirnov, I. (1993). The level of population immunity to influenza A viruses with haemagglutinin subtype H2. *Zhurnal Mikrobiologii Epidemiologii i Immunobiologii* **6**, 58–59 (in Russian).

Huddleston, J. A. & Brownlee, G. G. (1982). The sequence of the nucleoprotein gene of human influenza A virus, strain A/NT/60/68. *Nucleic Acids Research* **10**, 1029–1038.

Kawaoka, Y., Chambers, T. M., Sladen, W. L. & Webster, R. G. (1988). Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? *Virology* **163**, 247–250.

Kawaoka, Y., Krauss, S. & Webster, R. G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *Journal of Virology* **63**, 4603–4608.

Kida, H., Yanagawa, R. & Matsuoka, Y. (1980). Duck influenza lacking evidence of disease signs and immune response. *Infection and Immunity* **30**, 547–553.

Mulder, J. & Masurel, N. (1958). Pre-epidemic antibody against 1957 strain of Asiatic influenza in serum of older people living in the Netherlands. *Lancet* i, 810–814.

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

Rogers, G. N., Pritchett, T. J., Lane, J. L. & Paulson, J. C. (1983). Differential sensitivity of human, avian, and equine influenza A viruses to a glycoprotein inhibitor of infection: selection of receptor specific variants. *Virology* **131**, 394–408.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.

Schafer, J. R., Kawaoka, Y., Bean, W. J., Suss, J., Senne, D. & Webster, R. G. (1993). Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. *Virology* **194**, 781–788.

Scholtissek, C., Rohde, W., Von Hoyningen, V. & Rott, R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* **87**, 13–20.

Sharp, G. B., Kawaoka, Y., Wright, S. M., Turner, B., Hinshaw, V. & Webster, R. G. (1993). Wild ducks are the reservoir for only a limited number of influenza A subtypes. *Epidemiology and Infection* **110**, 161–176.

Shortridge, K. F. (1979). H2N2 influenza viruses in domestic ducks [letter]. *Lancet* i, 439.

Sinnecker, R., Sinnecker, H., Zilske, E. & Kohler, D. (1983). Surveillance of pelagic birds for influenza A viruses. *Acta Virologica* **27**, 75–79.

Tumova, B., Eisengarten, H. J., Siebelist-Konstantinow, I., Stumpa, A. & Webster, R. G. (1975). A duck influenza virus with haemagglutinin related to that of A/Singapore/57 (H2N2) virus. *Acta Virologica* **19**, 261.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiological Reviews* **56**, 152–179.

Wiley, D. C., Wilson, I. A. & Skehel, J. J. (1981). Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* **289**, 373–378.

Wright, S. M., Kawaoka, Y., Sharp, G. B., Senne, D. A. & Webster, R. G. (1992). Interspecies transmission and reassortment of influenza A viruses in pigs and turkeys in the United States. *American Journal of Epidemiology* **136**, 488–497.

Yamada, A., Brown, L. E. & Webster, R. G. (1984). Characterization of H2 influenza virus hemagglutinin with monoclonal antibodies: influence of receptor specificity. *Virology* **138**, 276–286.

Received 13 May 1999; Accepted 13 August 1999