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Genetic Analysis of HA1 Gene of Influenza A (H3N2) Viruses Isolated from Returning Travelers at Chubu International Airport in Aichi Prefecture

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Since the pandemic of 1968, the H3N2 subtype of influenza A virus (AH3) continues to circulate and has caused significant morbidity and mortality worldwide. Continuously accumulated mutations on the hemagglutinin (HA) gene of this virus have generated antigenically drifted strains that cause annual epidemics. Despite the recent dissemination of information regarding the evolution of circulating influenza viruses (1), questions remain about newly drifted viral emergence and how such viruses spread globally. However, it is generally accepted that frequent air travel plays an important role in the rapid and extensive spread of influenza (2). In Aichi Prefecture, we conducted a surveillance program to investigate influenza viruses carried by incoming travelers from abroad. The analysis of the influenza viruses isolated from travelers during the period of 1996 to 1999 revealed the possibility that viruses imported by air travelers may have influenced domestic influenza epidemics (3). In this report, we present a molecular epidemiological analysis of the HA1 (immunogenic subunit of HA) gene of the influenza viruses isolated from travelers during the period from 2006 to 2008, and the results were compared with those of analyses of domestic isolates obtained from residents of Aichi Prefecture.

Throat-swab specimens were collected from travelers who reported flu-like symptoms to the Chubu International Airport Quarantine Branch Office (Tokoname, Japan). Specimens from domestic patients in Aichi Prefecture were also collected. All specimens were inoculated onto Madin-Darby canine kidney (MDCK) cells and the cultures were observed for characteristic cytopathic effects (CPEs) for up to 2 weeks. Serological types and subtypes were determined for each isolate by hemagglutination inhibition (HI) testing of CPE-positive MDCK culture supernatants using type-specific sera against influenza viruses (provided by the National Institute of Infectious Diseases, Japan). Viral RNA was extracted from the culture supernatants using the High Pure Viral RNA kit (Roche Applied Science, Penzberg, Germany). A region containing the complete HA1 gene was amplified from the viral RNA using the One-step RT-PCR kit (Invitrogen, Carlsbad, Calif., USA) and primers designed before (4). Sequencing of the amplified DNA fragments was carried out with a Model-4200 automated DNA sequencer (Li-Cor, Lincoln, Nebr., USA). Fragments with a length of 987-nt of the AH3 virus HA1 gene were used for phylogenetic analysis by GENETYX (ver.7; Genetyx Corp., Tokyo, Japan).

From the 26 specimens collected at the airport during two winter seasons (2004/2005 to 2006/2007), 11 influenza AH3 viruses and 2 influenza B viruses were isolated. The AH3 viruses were isolated from travelers who visited Singapore, Thailand, China, Hawaii, Italy, and Australia (Table 1). Among the regions visited, 6 (55%) samples were from people returning from Asia, and 3 (27%) were from people returning from Hawaii. We also analyzed 32 domestic strains of AH3 virus

Table 1. Information of virus isolates from travelers returning from abroad

Strain	Country visited	Traveling period	Onset date	Date of sample collection
A/Aichi/165/2005	China	2005.6.10-6.18	2005.6.16	2005.6.18
A/Aichi/198/2006	Australia	2006.2.3-2.7	2006.2.6	2006.2.7
A/Aichi/46/2007	Hawaii	2007.3.8-3.14	2007.3.11	2007.3.14
A/Aichi/66/2007	Singapore	2007.3.21-3.24	2007.3.23	2007.3.25
A/Aichi/67/2007	Thailand	2007.3.26-3.29	2007.3.28	2007.3.29
A/Aichi/68/2007	Hawaii	2007.4.7-4.11	2007.4.10	2007.4.11
A/Aichi/69/2007	Thailand	2007.4.22-4.27	2007.4.27	2007.4.27
A/Aichi/71/2007	China	2007.4.29-5.1	2007.5.1	2007.5.1
A/Aichi/70/2007	Italy	2007.4.26-5.3	2007.5.2	2007.5.3
A/Aichi/72/2007	Thailand	2007.4.14-5.4	2007.5.3	2007.5.4
A/Aichi/79/2007	Hawaii	2007.5.18-5.22	2007.5.21	2007.5.22

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isolated from residents of Aichi Prefecture during the 2005/2006 to 2007/2008 seasons.

The phylogenetic tree of the HA1 genes of the AH3 viruses is presented in Fig. 1. The tree consists of 2 main clades, clades I and II, and the latter branches into 3 subclades: IIa, IIb, and IIc. The viruses isolated during the 2005/2006 season belonged to clades I and IIc, whereby the latter clade was predominant. A/Hiroshima/52/2005, the vaccine strain used for the 2006/2007 and 2007/2008 seasons in Japan, also belongs to clade IIc. Clades IIa and IIb included viruses isolated in the 2006/2007 to 2007/2008 seasons. A/Brisbane/10/2007, the vaccine strain for the 2008/2009 season (5), belongs to clade IIb. The viruses isolated in the 2007/2008 season were classified into 2 further subclades, IIa-i and IIb-i, which represent branched clades from IIa and IIb, respectively. The 2 viruses in clade IIa-i were isolated in September 2007, very early in the 2007/2008 season. No other viruses isolated thereafter belonged to this clade. Most virus in the 2007/2008 season belonged to clade IIb-i.

The amino acid sequences of HA1 were deduced from the nucleotide sequences. In Table 2, amino acid substitutions are shown in comparison with the sequence of strain A/California/7/2004. Each phylogenetic lineage group was characterized by the signature amino acid differences and each is represented by the strains listed in Table 2. Of the 18 substitutions listed in Table 2, 14 (77.8%) were located within antigenic sites (6,7). The finding that the majority of mutated codons were located within antigenic sites suggests that the mutation in the HA gene is a pathway for viral escape from the host immune system. Major differences between members of clade I and those of the other clades were characterized by amino acid changes at S193F and D225N. These characteristic dual mutations had previously been reported by our group as well as by other groups (4,8). The isolates in clade IIa showed amino acid changes at R142G and N144D, with the exception of 2 isolates in clade IIa-ii. The isolates in IIa-ii had not mutated at N144, but did possess R142G and additional amino acid changes at L157S and K173E in the antigenic sites. For clade IIb, most isolates had common amino acid changes at G50E and K140I. Despite belonging to clade IIb, A/Aichi/46/2007 (isolated from travelers returning from Hawaii) did not harbor these mutations, but instead possessed N144D, which is characteristic of the members of clade IIa. The isolates in the 2007/2008 season formed clade IIb-i, and had a single or more than one amino acid change, as compared to isolates from the previous 2006/2007 season. A/Aichi/102/2008, in clade IIb-i, was the most recent isolate (isolated from a specimen collected on May 2008); this isolate showed 4 unique amino acid changes at positions L3F, K83N, L157S, and K173N, which differed from the other isolates in clade IIb.

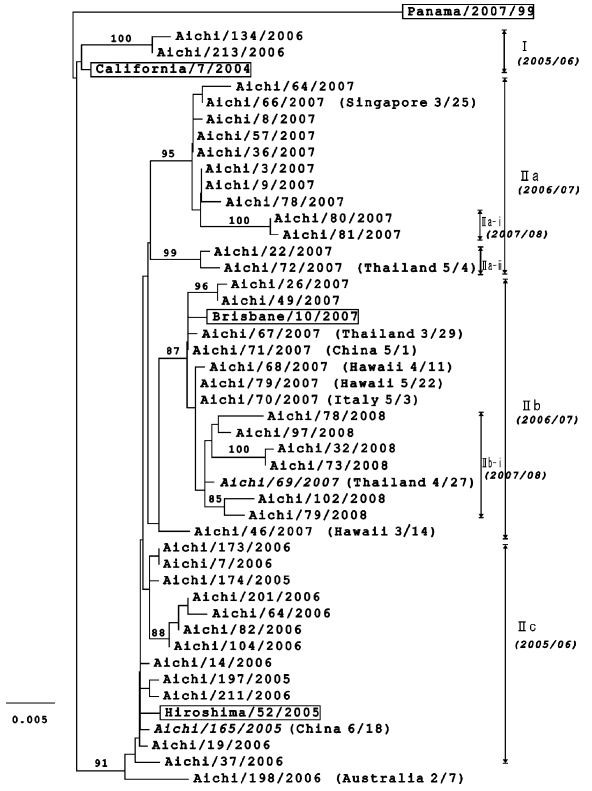

Fig. 1. Phylogenetic tree of the HA1 genes for influenza A (H3N2) virus isolates during the recent seasons. The Aichi isolates during the 2005/2006 to 2007/2008 seasons, and the reference strains (A/Panama/2007/99, A/California/7/2004, A/Hiroshima/52/2005, A/Brisbane/10/2007) were analyzed. Eleven isolates were from the passengers with flu-like symptoms at Chubu International Airport. The country or the state that each passenger visited was indicated in the parentheses. The influenza season was indicated under the code for phylogenetic clades, I, IIa, IIb, IIc, IIa-i, and IIb-i, provided that the strains in italic type (*Aichi/165/2005* and *Aichi/69/2007*) were isolated in the previous season. The out-group in the tree is A/Panama. The numbers at branching points are bootstrap values base on 100 replicates and values over 80% are shown.

Table 2. Amino acid substitutions of the HA1 subunit of AH3 viruses

Clade	Strain	Amino acid substitution in HA1[1)]																	
		3	6	45	50[C]	83[E]	121[A]	122[A]	124[A]	140[A]	142[A]	144[A]	145[A]	157[B]	173[D]	188[B]	192[B]	193[B]	225
I	California/7/2004	L	N	S	G	K	N	N	S	K	R	N	N	L	K	N	I	S	D
IIc	Hiroshima/52/2005	–	–	–	–	–	–	–	–	–	–	–	–	–	–	D	–	F	N
IIb	Brisbane/10/2007	–	–	–	E	–	–	–	–	I	–	–	–	–	–	D	–	F	N
I	Aichi/213/2006	–	–	–	–	–	–	–	–	–	–	–	S	–	E	Y	–	–	–
IIc	Aichi/19/2006	–	–	–	–	–	–	–	–	–	–	–	–	–	–	D	–	F	N
IIa	Aichi/57/2007	–	–	–	–	–	–	–	–	–	G	D	–	–	–	D	–	F	N
IIa-i	Aichi/81/2007	–	–	–	–	–	–	T	R	–	G	D	–	–	–	D	–	F	N
IIa-ii	Aichi/72/2007	–	I	N	–	–	–	–	–	–	G	–	–	S	E	D	–	F	N
IIb	Aichi/46/2007	–	–	–	–	–	–	–	–	–	–	D	–	–	–	D	–	F	N
IIb	Aichi/67/2007	–	–	–	E	–	–	–	–	I	–	–	–	–	–	D	–	F	N
IIb-i	Aichi/73/2008	–	–	–	E	–	K	–	–	I	–	–	–	–	Q	D	–	F	N
IIb-i	Aichi/79/2008	F	–	–	E	–	–	–	–	I	–	–	–	–	N	D	T	F	N
IIb-i	Aichi/102/2008	F	–	–	E	N	–	–	–	I	–	–	–	S	N	D	–	F	N

[1)]: Letters (A-E) in upper case following each amino acid number indicate the antigenic site location of the residue.

Among the 11 airport-related isolates, 9 were collected during the period from March to May in 2007, and from late in the 2006/2007 season, and the other 2 were collected in late in the 2004/2005 and 2005/2006 seasons, respectively. Seven of 9 isolates in the 2006/2007 season (Hawaii, Thailand, China, Italy) belonged to clade IIb, and the other 2 isolates, obtained from travelers returning from Singapore and Thailand, belonged to clade IIa, to which most domestic isolates in that season belonged. In the following 2007/2008 season, most of the domestic isolates belonged to IIb-i, a subclade of IIb. A/Aichi/69/2007, which was isolated from a traveler from Thailand in the previous 2006/2007 season, was also included in this clade. Similarly, as regards clade IIc, all domestic isolates were collected during the 2005/2006 season, whereas A/Aichi/165/2005, isolated from a traveler from China in the preceding 2004/2005 season, was also included in clade IIc. It appears that these 2 isolates from the returning travelers represent imported Asian strains that eventually proceeded to become domestic isolates in subsequent seasons. The presence of these isolates also substantiated the recent argument that new influenza virus strains emerge in tropical zones that include south Asian countries such as Thailand and China (9-11).

Our above findings suggest that imported viruses (i.e., those carried by returning travelers) have important public health implications in terms of predicting the following season's influenza strains. These results also emphasize the importance of conducting influenza surveillance at an airport quarantine office in order to detect new influenza viruses entering Japan.

The nucleotide sequences of the genes used in this study can be found in GenBank under the following accession numbers: A/Panama/2007/99: DQ508865, A/California/7/2004: DQ865973, A/Hiroshima/52/2005: ABX79354, A/Brisbane/10/2007: ABW23422, Aichi strains: AB243869, AB246366, AB259102-12, AB259740-41, AB289672-78, AB453349-79, AB457183-84.

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