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Short Communication

Isolation of Influenza A H1N2 Viruses from an Outbreak in Yokohama City during the 2001 - 2002 Influenza Season in Japan

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SUMMARY: Emergence of Influenza A H1N2 viruses was documented worldwide during the 2001 - 2002 influenza season. In Japan, H1N2 viruses were isolated from two students of a junior high school in an influenza outbreak in Yokohama City, February 2001. Genetic and antigenic analyses demonstrated that the H1N2 viruses isolated in Japan shared common features with those isolated in other countries.

Influenza A virus causes upper respiratory infections during winter in the temperate zones. The low fidelity of viral polymerase complex as well as host immune selection account for the accumulation of point mutation in hemagglutinin (HA) and neuraminidase (NA) genes (14), resulting in the antigenic drift of these surface glycoproteins of the virus. The antigenic drift plays pivotal role in year-to-year epidemics of influenza. Since 1977 and 1968, recent epidemic strains of influenza A virus have been the H1N1 and the H3N2 subtypes, respectively. Another mechanism which gives rise to antigenic variations of influenza is the antigenic shift. The segmented nature of the influenza virus genome allows genetic reassortment between two influenza viruses with different genetic compositions. Antigenic shift is observed when a reassortment between two viruses with different surface antigen subtypes results in a novel composition of surface antigen subtypes. Pandemic influenza viruses, such as the H3N2 subtype in 1968, and the H2N2 subtype in 1957, were caused by reassortments between an avian influenza virus and a circulating epidemic strain in human beings at the time (14).

Emergence of H1N2 reassortant viruses was recorded worldwide during the 2001 - 2002 influenza season in the northern hemisphere (7,15). The worldwide spread of the viruses drew the attention of national health authorities, including the World Health Organization (WHO), from the viewpoint of the effectiveness of the current available vaccine (12). Genetic characterizations revealed that these viruses acquired the HA gene from a contemporary A/New Caledonia/20/99 (H1N1)-like virus and the rest of the genes from an A/Moscow/10/99 (H3N2)-like virus (7,15). Moreover, antigenic analysis indicated that the HA and the NA proteins of theses viruses were closely related to those of A/New Caledonia/20/99-like viruses and A/Moscow/10/99-like viruses, respectively, and suggested that the current vaccine should provide effective protection against the H1N2 viruses (7,15).

A respiratory illness outbreak occurred among the first grade students at a junior high school in Izumi Ward, Yokohama City, on 5th of February 2002. Yokohama City is located on the east side of Kanagawa Prefecture, south of Tokyo Metropolis, Japan. Its population is about 3,500,000 and city area is about 435 km^2. Among 316 students of the first grade in a junior high school, 210 students showed influenza-like illness and 112 students were absent. All eight classes of the first grade were closed for 4 days. Gargles and paired-serum were collected from five students with high fevers (38 - 40°C), cough, and pharyngitis. One of them showed diarrhea. Virus isolation was carried out in Madin-Darby Caine Kidney (MDCK) cells and serological diagnosis was determined using the hemagglutination inhibition (HI) test.

Two out of the five gargle specimens yielded influenza A viruses of the H1 subtype, each designated as A/Yokohama/22/2002 and A/Yokohama/47/2002 (Table 1). The NA subtype was determined as the N2 by RT-PCR with RNA extracted from viruses in culture supernatants. No PCR product was amplified with an N1-specific primer pair from the same RNA preparation. Plaque isolation was done directly from the gargle specimens that yielded H1N2 viruses. Each virus from isolated plaques was confirmed to be of the H1N2 subtype, excluding the possibility that patients shed both the H1N1 and H3N2 viruses. These experiments were primarily carried out at the laboratory of Yokohama City Institute of Health. In order to exclude the possibility of laboratory contamination, those two original specimens were also subjected to virus isolation at the laboratory of the National Institute of Infectious Diseases. The same specimens also yielded influenza A viruses of the H1N2 subtype. These results confirmed the isolation of influenza A (H1N2) virus from an outbreak at a junior high school in Yokohama City. In addition, although virus isolation was not successful, it was demonstrated that one of the gargles contained genes of the H1 and the N2 glycoproteins, and not those of the H3 and N1, by direct RT-PCR examination (Table 1). The specimens which yielded the H1N2 viruses were collected from students belonging to the same class, and the specimen which was RT-PCT-positive but did not yield a virus was collected from a student of the other class.

Serological examination of paired sera demonstrated 16-fold or more elevation in the HI titer against A/New Caledonia/20/99 with the four patients (Table 1). Of two patients with

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Table 1. Results of virological and serological examinations

Patient No.	Virus isolation[1]	RT-PCR[2]	Serum HI titiers against[3]				
			A/New Caledonia/20/99	A/Moscow/13/98	A/Panama/2007/99	B/Johannesburg/5/99	B/Akita/27/2001
			(H1)		(H3)	(B)	
1	Negative	H1N2	**10/640**	**40/>1280**	<10/<10	10/10	<10/<10
2	Negative	Negative	<10/10	20/40	20/20	40/40	<10/<10
3	Negative	Negative	**10/160**	160/320	20/20	<10/<10	<10/<10
4	A/Yokohama/22/2002 (H1N2)	H1N2	**10/1280**	**80/>1280**	640/640	40/40	<10/<10
5	A/Yokohama/47/2002 (H1N2)	H1N2	**10/320**	**160/>1280**	<10/10	10/10	<10/<10

[1] Virus isolation was done with MDCK cells in the presence of 1.5 μg/ml of trypsin.
[2] Detection of the viral glycoproteins were done by the subtype-specific primers for H1, H3, N1, and N2. RT-PCR was carried out with one step RNA PCR kit (TAKARA, Shiga). Primer sequences and reaction protocol are available upon request.
[3] titers of acute/convalescent phase serum.

Table 2. Antigenic analysis of the H1N2 viruses

Virus antigens	HI titers with the postinfection ferret sera			
	A/Beijing/262/95	A/New Caledonia/20/99	A/Fukuoka-C/86/2000	A/Yokohama/24/2000
Reference H1N1 viruses				
A/Beijing/262/95	**1280**	640	80	10
A/New Caledonia/20/99	80	**640**	160	<10
A/Fukuoka-C/86/2000	40	160	**1280**	<10
A/Yokohama/24/2000	40	<10	160	**5120**
01-02 season isolates				
A/Yokohama/22/2002 (H1N2)	160	320	80	10
A/Yokohama/47/2002 (H1N2)	80	320	40	10
A/Yokohama/62/2002 (H1N1)	40	320	80	<10

the elevated HI titer, H1N2 viruses were isolated from their gargles and another one was positive for H1N2 by RT-PCR. The virological and serological evidence suggested that the H1N2 virus was a causative agent of the respiratory illness outbreak at the junior high school in Yokohama. Since A/H1N1 and A/H3N2 viruses were also co-circulating in the Yokohama area at that time, we could not rule out the possibility that H1N1 and H3N2 influenza viruses as well as other respiratory infectious agent might be involved in this outbreak.

Through the nation-wide influenza virus surveillance program in Japan, 3,261 A/H1 strains were isolated by local health institutes during the 2001-2002 season. Among them, 128 strains were sent to the National Institute of Infectious Diseases, and were screened for the N2 gene. None of them turned out to be the H1N2 subtype.

The antigenicity of the HA of A/Yokohama/22/2002 and A/Yokohama/47/2002 was examined using a panel of post-infection ferret serum (Table 2). Both were shown to be antigenically similar to A/New Caledonia/20/99, the vaccine strain of the 2001-2002 and 2002-2003 seasons (13).

The evolutionary features of the H1 gene of A/Yokohama/22/2002 and A/Yokohama/47/2002 were analyzed by the N-J method (Fig. 1). As show in Fig. 1, these two viruses formed a cluster with H1N2 viruses isolated in the European continent (7). Recently, Xu et al. (15) also reported that the H1 genes of the H1N2 viruses isolated in the Northern American continent and Asian countries also shared the same origin. Sequence analysis of the other gene segments indicated that the Japanese H1N2 strains were also a sigle-gene reassortant which derived their genome, except for the HA gene, from a recent human H3N2 epidemic strain (Table 3). During the 2001-2002 influenza season of the northern hemisphere, the H1N2 influenza viruses were widely isolated. The present report supported the idea of the intercontinental circulation of the reassortant virus during this season. The genetical identity among the H1N2 viruses isolated worldwide suggested that the reasortment between H1N1 and H3N2 viruses that gave rise the H1N2 reassortant might have occurred once before the 2001-2002 season (15).

Table 3. Percent similarity of nucleotide sequences with A/Yokohama/22/2002 (H1N2)[1]

Gene	A/New Caledonia/20/99 (H1N1)	A/Moscow/10/99 (H3N2)
PB2	84.5	96.0
PB1	78.3	99.5
PA	90.3	98.9
H1	99.1	–
NP	88.7	98.2
N2	–	99.0
M	91.3	98.2
NS	88.9	99.9

[1] Comparisons were made on the sequences of nucleotides 1-503 (PB2), 376-785 (PB1), 37-873 (PA), 34-1015 (H1), 988-1422 (NP), 1-1447 (N2), 127-860 (M), and 55-800 (NS).

Prevalence of the H1N2 viruses during the 2001-2002 season in Japan appeared very low, since no H1N2 virus was detected by the screening for the N2 gene among 4% of all the H1 isolates in Japan. Nevertheless, it is interesting to note that the H1N2 viruses were associated with the junior high school outbreak. During the 1999-2000, 2000-2001, and 2001-2002 influenza season in Yokohama City, 19 outbreaks of virologically confirmed influenza occurred. Most of them were either in kindergartens or elementary schools. Only two outbreaks during this period were recorded among junior high school children. One of them was caused by the H1N1 virus during the 1999-2000 season and the other was the case reported in this communication caused by the H1N2 viruses. The Public Health Laboratories Service in the United King-

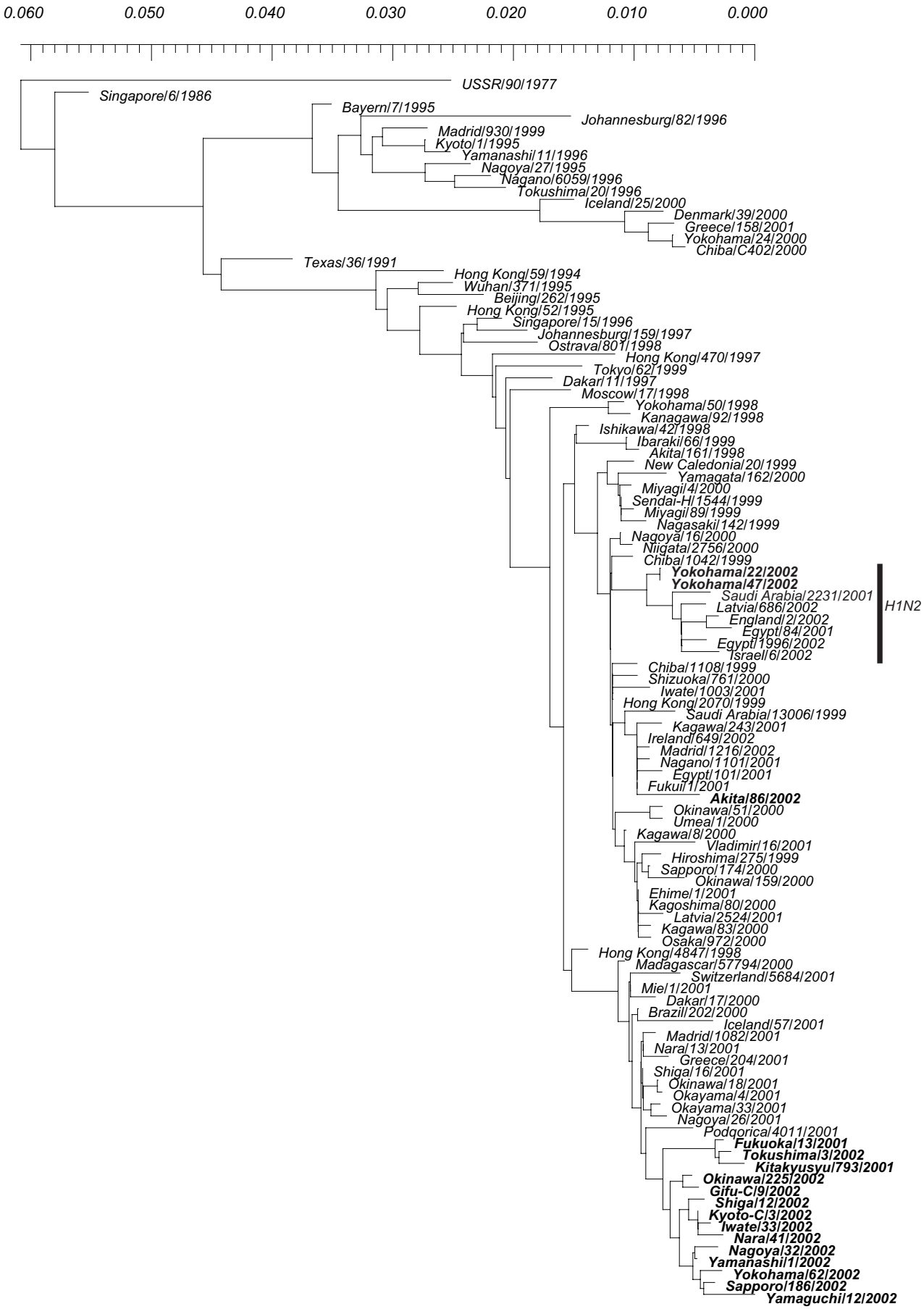

Fig. 1. Phylogenetic tree of the nucleotide sequence of the HA1 region of the H1 genes. The tree was generated by the N-J method. Japanese isolates obtained during the 2001-2002 season, including the H1N2 isolates, are shown in bold italic.

dom (U. K.) reported that eight out of 12 influenza outbreaks during the 2001 - 2002 season were caused by the H1N2 viruses in the U.K. (3). Although the H1N2 virus affected mainly children younger than 5 years of age, the H1N2 virus also caused outbreaks among school-aged children. The H1N2 outbreaks in the U.K. also showed attack rates as high as those of the Yokohama case. While no unique symptom was observed regarding the H1N2 infection, such a high attack rate and age distribution of outbreaks might be a characteristic of the H1N2 infection.

It was estimated that the reassortant arose in early 2001 or earlier (15). During the 2000 - 2001 season, A/H1 virus was predominant worldwide and A/H3 viruses had been isolated only sporadically in the U.K. (5) and the United States (U. S.) (2). However, a substantial number of H3 strains, 16% of the total influenza A and B isolates, were recorded in Japan. On the contrary, H3 viruses almost exclusively dominated during the 1999 - 2000 season in the U.K. (4) and the U. S. (1). Interestingly, the H1 and the H3 viruses co-circulated (60% versus 40%) in Japan during the 1999 - 2000 season. Epidemiological profile of influenza may be common within Asian countries including Japan. Therefore, as described above for Japan, both H1 and H3 viruses might have been prevalent to some extent during both the 1999 - 2000 and the 2000 - 2001 seasons in Asian countries. Thus, it is plausible to speculate that the reassortment event may have occurred in South Asia during either season, as Xu et al. suggested (15). An H1N2 virus was certainly isolated sporadically in Japan during the 1999 - 2000 season, although further transmission was not noted (unpublished data).

H1N2 viruses have been circulating in the swine population in Japan as well as in the U.S. and Europe (6,8 - 10). Genetic analysis of these viruses demonstrated that pigs indeed serve as a mixing vessel (11) for human, avian, and swine viruses. However, none of these swine H1N2 viruses are genetically related to the H1N2 viruses isolated from humans during the 2001 - 2002 season. Thus, when and where the reassortment that gives rise to the human H1N2 virus took place remains to be elucidated.

Influenza vaccines for the 2002 - 2003 season contain A/New Caledonia/20/99 (13), whose HA protein is antigenically and genetically closely related to the HA of the currently circulating H1N2 virus. Thus, as far as no additional antigenic drift would occur, the vaccine should provide good protection against the H1N2 virus (12). Determining whether the H1N2 virus will replace the current epidemic subtypes, will co-circulate with the current ones, or will disappear, will be one of the points of focus for the international surveillance of the influenza in the upcoming influenza season.

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