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Characterization of antigenically and genetically similar influenza C viruses isolated in Japan during the 1999–2000 season

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SUMMARY

Between October 1999 and May 2000, a total of 28 strains of influenza C virus were isolated in four Japanese prefectures: Yamagata, Miyagi, Saitama and Hiroshima. Antigenic analysis showed that the 28 isolates were divided into three distinct antigenic groups, and viruses belonging to different antigenic groups were co-circulating in each of the four prefectures. Phylogenetic analysis of the seven protein genes demonstrated that the viruses having a similar genome composition spread in various areas of Japan during the same period. Furthermore, phylogenetic analysis showed that most of the influenza C viruses isolated in various areas of the world between the 1970s and 1980s were closely related to the contemporary Japanese viruses in all gene segments. These observations suggest that the influenza C viruses cause epidemics in some communities during the same season and that antigenically and genetically similar influenza C viruses spread throughout Japan and may be circulating worldwide.

INTRODUCTION

The genome of influenza C virus consists of seven RNA segments which encode three polymerase proteins (PB2, PB1 and P3), haemagglutinin–esterase (HE) glycoprotein, nucleoprotein (NP), matrix (M1) protein, CM2 protein, and two non-structural proteins (NS1 and NS2) (reviewed in ref. [1]). Sero-epidemiological studies reveal that influenza C virus is widely distributed throughout the world [2–6] and that recurrent infection with this virus occurs frequently in children as well as in adults [4, 7]. However, outbreaks of this virus have rarely been detected, probably because influenza C viruses only cause mild respiratory illness [7, 8]. Thus, most of the influenza C viruses have been isolated only by accident during outbreaks of influenza A or B viruses [3, 9, 10]. To obtain more information about influenza C virus epidemiology, we developed a tissue culture method for primary virus isolation and then initiated surveillance for influenza C virus infections in Yamagata City, Japan in 1988 and the adjacent city of Sendai in 1990 [8]. We obtained more than 100 influenza C virus isolates during this surveillance study and demonstrated that there were some differences in the evolution between influenza A and C viruses. Antigenic analysis and sequence analysis of the HE genes of various isolates revealed the existence of six distinct virus groups, represented by Taylor/47, Kanagawa/1/76 (KA176), Yamagata/26/81 (YA2681), Aichi/1/81 (AI181), Sao Paulo/378/82 (SP82), and Mississippi/80 (MS80) [11], and the influenza C viruses belonging

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to the different antigenic groups were co-circulating in a community [12, 13]. It was also observed that there was a dominant antigenic group and the replacement of the dominant group in a community had occurred [13]. Characteristically, there was little antigenic change among the influenza C viruses belonging to the same antigenic groups for more than 10 years [13]. These observations suggest that the production of antigenic variants by immune selection has not occurred for influenza C viruses. This is clearly different from influenza A viruses which cause epidemics every year due to the emergence of antigenic variants by immune selection [14, 15]. In addition, mixed infection with influenza C virus belonging to different groups occur in humans, resulting in the emergence of reassortment viruses characterized by the exchange of genome segments between two different strains [16]. We reported the frequent occurrence of reassortment among influenza C viruses [17] and its importance. Most of the circulating viruses isolated in Sendai City are reassortants [13]. Outbreaks of influenza C virus occurred with the reassortant virus with antigenicity similar to that of YA2681 in Yamagata City in 1996 and 1998 [18]. A virus strain antigenically similar to KA176 re-emerged in 1996 for the first time in 20 years, which acquired the same internal genes from epidemic strains mentioned above through a reassortment event [13]. These observations led us to the idea that the genome composition of influenza C viruses influences their ability to spread in humans and that reassortment events were the means of evolution for influenza C viruses.

One important question concerning influenza C virus epidemiology remains unanswered: Does reassortant influenza C virus spread all over the world? New variants of influenza A virus spread rapidly worldwide, but the propagation of influenza C viruses appears to be slower than influenza A viruses [19]. We investigated whether the frequent reassortment such as observed in Sendai City was occurring in every area independently or whether the similar reassortant viruses spread in various areas. It has been difficult, however, to answer the question, because only a limited number of influenza C viruses have been isolated throughout the world. Between October 1999 and May 2000, 28 influenza C viruses were isolated in various areas of Japan. In this paper, we show that influenza C viruses having a similar antigenicity and a similar genome composition spread in various areas of Japan during the same period.

METHODS

Viruses and cells

A total of 28 strains of influenza C virus were isolated from children (<15 years of age) with acute respiratory illness in Yamagata, Miyagi, Saitama and Hiroshima prefectures of Japan between October 1999 and May 2000 [20, 21]. All these viruses were initially isolated from throat-swab specimens using Madin–Darby canine kidney (MDCK) cells as a host according to procedures described previously [8]. For this study, all these viruses were re-isolated from throat swabs and passaged and propagated by inoculating them into the amniotic cavity of 9-day-old embryonated hen's eggs and these amniotic fluids were used in the following experiments. MDCK cells were cultured in Eagle's minimal essential medium supplemented with 10% foetal calf serum.

Seven older strains [YA2681, KA176, MS80, New-Jersey/76 (NJ76), California/78 (CAL78), Kansas/1/79 (KAN179) and Greece/79 (GR79)] were also used.

Haemagglutination inhibition (HI) test

Four anti-HE MAbs characterized previously [22, 23] were used. HI tests were performed in microtitre plates with 0·5% chicken erythrocytes as described previously [18].

Nucleotide sequencing and phylogenetic analysis

Nucleotide sequencing and analysis were carried out as described previously [13, 18]. Briefly, viral RNA was extracted from 200 μl of the virus-containing amniotic fluid by using an RNeasy mini kit (Qiagen, Hilden, Germany). The viral RNA was then transcribed into cDNA with AMV reverse transcriptase XL (Life Science, St Petersburg, FL, USA) and a universal primer complementary to positions 1–12 at the 3′-end of all influenza C virus RNA segments. By using the resulting cDNA as a template, the individual RNA segments were amplified by PCR through 35 cycles of the thermocycler programme as described previously [24]. The PCR products were purified by a rapid gel filtration with a Chroma spin column (Clontech, Palo Alto, CA, USA) and then sequenced by using BigDye Terminator cycle sequencing FS ready reaction kit on a ABI Prism 310 (Applied Biosystems, Foster City, CA, USA) automatic sequencer. Nucleotide sequences of the oligonucleotide primers used for PCR amplification and sequencing are available from the authors upon request. Sequence data were analysed with the

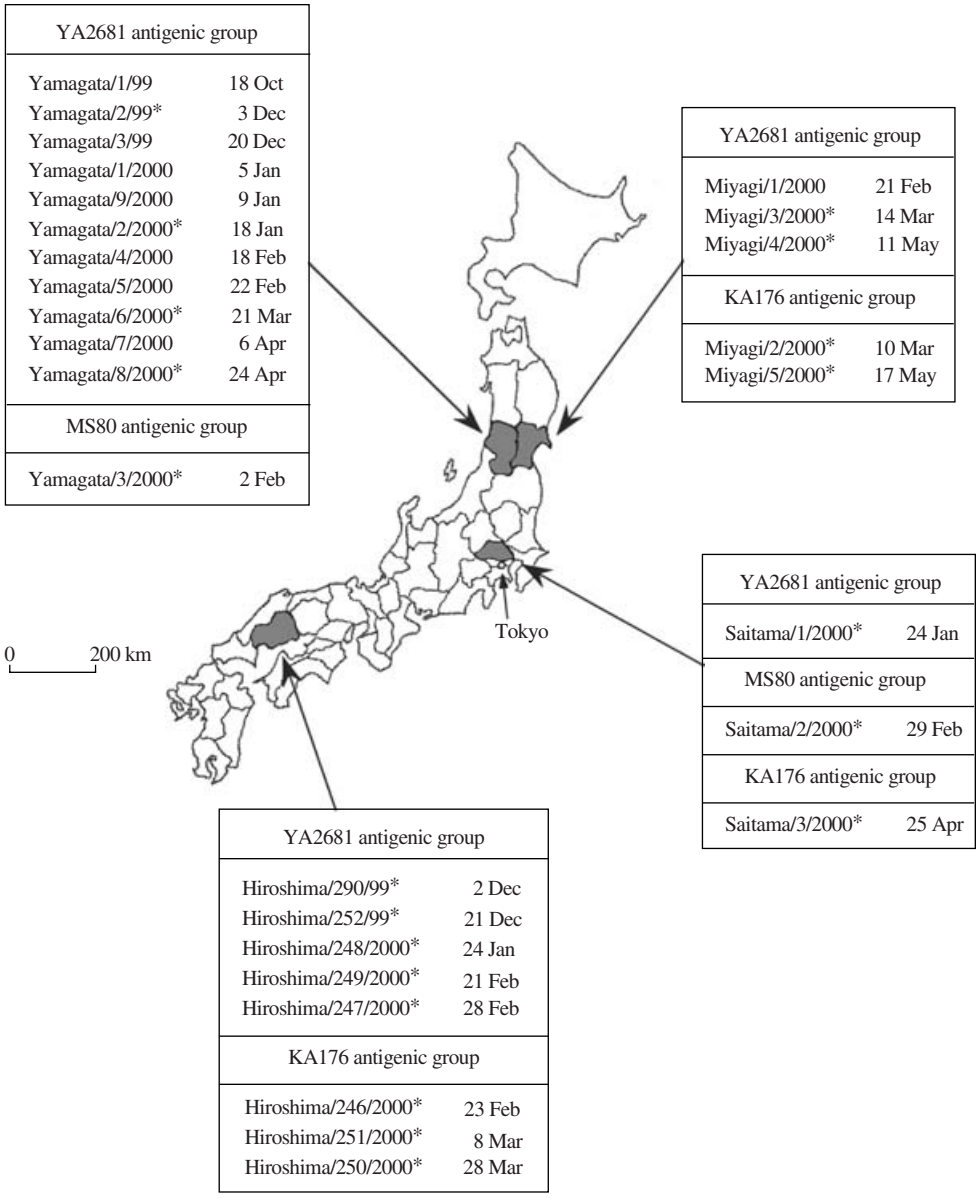

Fig. 1. Map showing the prefectures where influenza C viruses were isolated in 1999 and 2000. Antigenic group of the isolates and the date of the sample collection are also shown in the boxes. The strains marked by asterisks were sequenced in this study.

PHYLIP program (version 3.573c), and phylogenetic trees were constructed by the neighbour-joining method [25] using the same software. The nucleotide sequences determined in this study have been submitted to the DDBJ/EMBL/Genbank databases and assigned the accession numbers AB099410 to AB099480 and AB099530 to AB099627.

RESULTS

Isolation of influenza C viruses

A total 28 influenza C viruses were isolated in four different prefectures of Japan between October 1999 and May 2000. The number of nasopharyngeal swab specimens collected during this period were 1164, 2096, 599 and 859, and the number of influenza C virus isolates were 12, 5, 3 and 8 in Yamagata, Miyagi, Saitama and Hiroshima prefectures respectively. The highest isolation rate of the month was 2·3% (December 1999 in Yamagata). Yamagata and Miyagi prefectures are neighbouring and both prefectures are located approximately 300 km north of Tokyo. Saitama prefecture is only 25 km from Tokyo but Hiroshima prefecture is approximately 700 km to the west of Tokyo (Fig. 1). Therefore, it was demonstrated that the influenza C viruses

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Table 1. *Antigenic characterization of representative influenza C isolates*

Antigenic group	Virus strain	HI titre (HI units/ml) of anti-HE MAbs			
		J14	Q5	U1	MS2
	Yamagata/26/81 (YA2681)	64 000	6400	1600	<
	Kanagawa/1/76 (KA176)	256 000	1280	<	<
	Mississippi/80 (MS80)	204 800	<*	64 000	12 800
YA2681	Yamagata/2/99	32 000	6400	400	<
	Miyagi/1/2000	32 000	12 800	1600	<
	Saitama/1/2000	32 000	12 800	1280	<
	Hiroshima/290/99	16 000	12 800	320	<
KA176	Miyagi/2/2000	256 000	640	<	<
	Saitama/3/2000	128 000	320	<	<
	Hiroshima/246/2000	128 000	160	<	<
MS80	Yamagata/3/2000	256 000	<	128 000	256 000
	Saitama/2/2000	128 000	<	32 000	64 000

* Titre below 20.

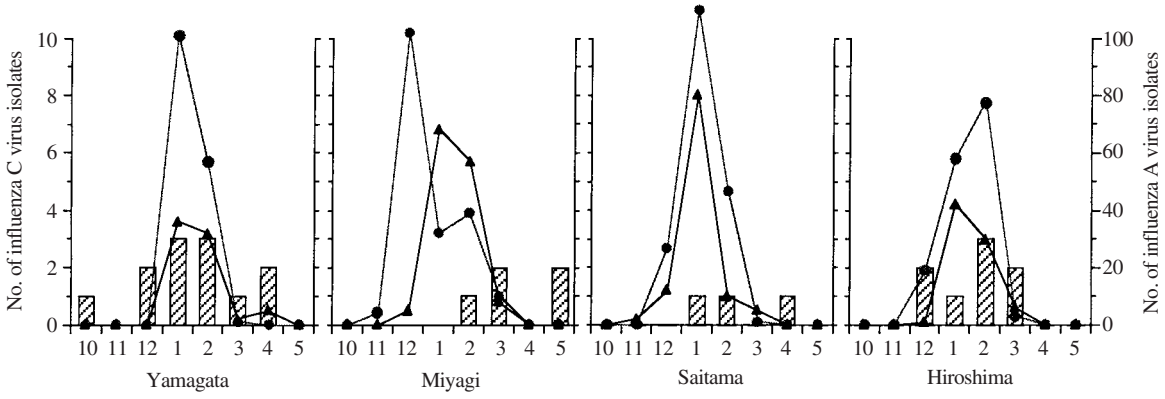

Fig. 2. Monthly distribution of influenza A (H1 and H3) and C virus strains isolated in Yamagata, Miyagi, Saitama and Hiroshima prefectures between October 1999 and May 2000. ▨, Flu C; –●–, Flu A (H1); –▲–, Flu A (H3).

were prevalent simultaneously in these separate areas of Japan. During this period, H1 and H3 influenza A viruses were prevalent in all these prefectures (Fig. 2). Of the 28 throat swabs from which influenza C viruses were isolated, H1 influenza A virus was isolated from one sample (Yamagata/5/2000 throat swab). Most children infected with influenza C virus had fever, cough and nasal discharge, and the clinical manifestations of the patients were difficult to distinguish from those with influenza A virus.

Antigenic analysis of influenza C viruses

The 28 influenza C virus strains isolated in Japan between 1999 and 2000 were examined in HI tests for reactivity with four different anti-HE MAbs characterized previously [22, 23]. The reactivity patterns of all the isolates were similar to one of the three antigenic groups represented by YA2681, KA176 and MS80 among the five antigenic groups (AI181, YA2681, KA176, MS80 and SP82) reported previously [11]. Antigenic characterization of the representative strains of each prefecture and antigenicity of all the isolates are shown in Table 1 and Figure 1 respectively.

In Yamagata prefecture, the 11 isolates were antigenically similar to YA2681 but only Yamagata/3/2000 had similar antigenicity to that of MS80. However, only Yamagata/3/2000 was isolated in a neighbouring city which was approximately 50 km from Yamagata City. Thus, it was revealed that a single group of influenza C viruses closely related to YA2681 were circulating in Yamagata City during

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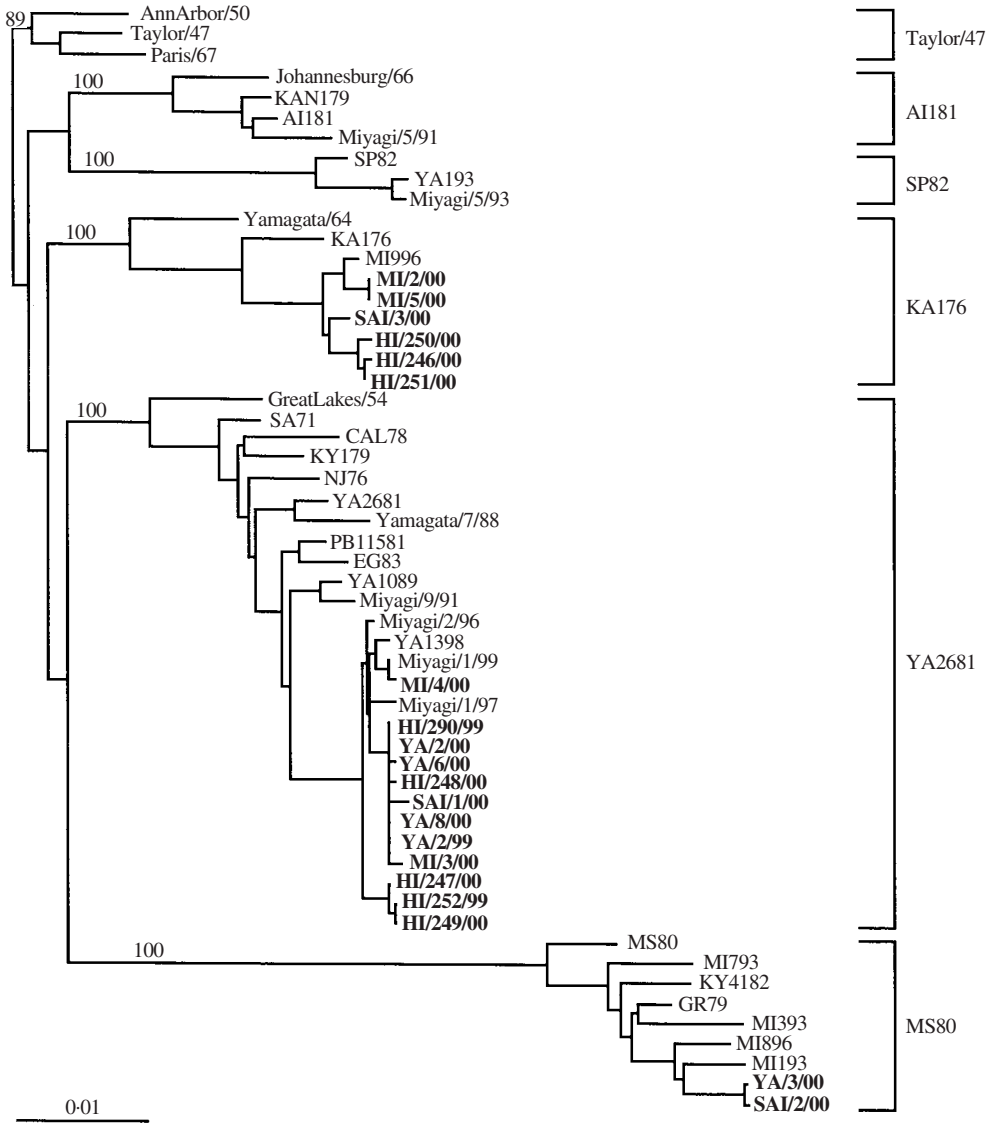

Fig. 3. Phylogenetic tree of influenza C virus HE genes. The region from nucleotides 64–1989 was used for analysis. Numbers above the branches are the bootstrap probabilities (%) of each branch determined by the PHYLIP program (version 3.573c). Viruses isolated in this study are indicated in bold type. Abbreviations used in viruses isolated in this study are as follows: YA, Yamagata; MI, Miyagi; SAI, Saitama; HI, Hiroshima.

this period. In Miyagi and Hiroshima prefectures, influenza C viruses belonging to the two antigenic groups of YA2681 and KA176 were co-circulating. The three strains isolated in Saitama prefecture were similar to YA2681, MS80 and KA176.

Phylogenetic analyses of the individual RNA segments of influenza C virus strains

To ascertain that the genetic similarity of the influenza C viruses which were isolated in different prefectures and had similar antigenicity, the sequence of the HE gene (nucleotides 64–1989) was determined for 20 strains (those listed in Fig. 1) and the six internal gene segments of a representative 11 strains were sequenced in the partial region of PB2, PB1, P3 and NP genes and in the complete coding region of M and NS genes. The phylogenetic trees of individual genes were constructed by using them in addition to the previously reported sequences [11–13, 17, 18, 24, 26–35] (Figs 3 and 4). The sequences of four strains (NJ76, CAL78, KAN179 and GR79) isolated in previous years and determined here were used for the construction of six internal gene trees. The

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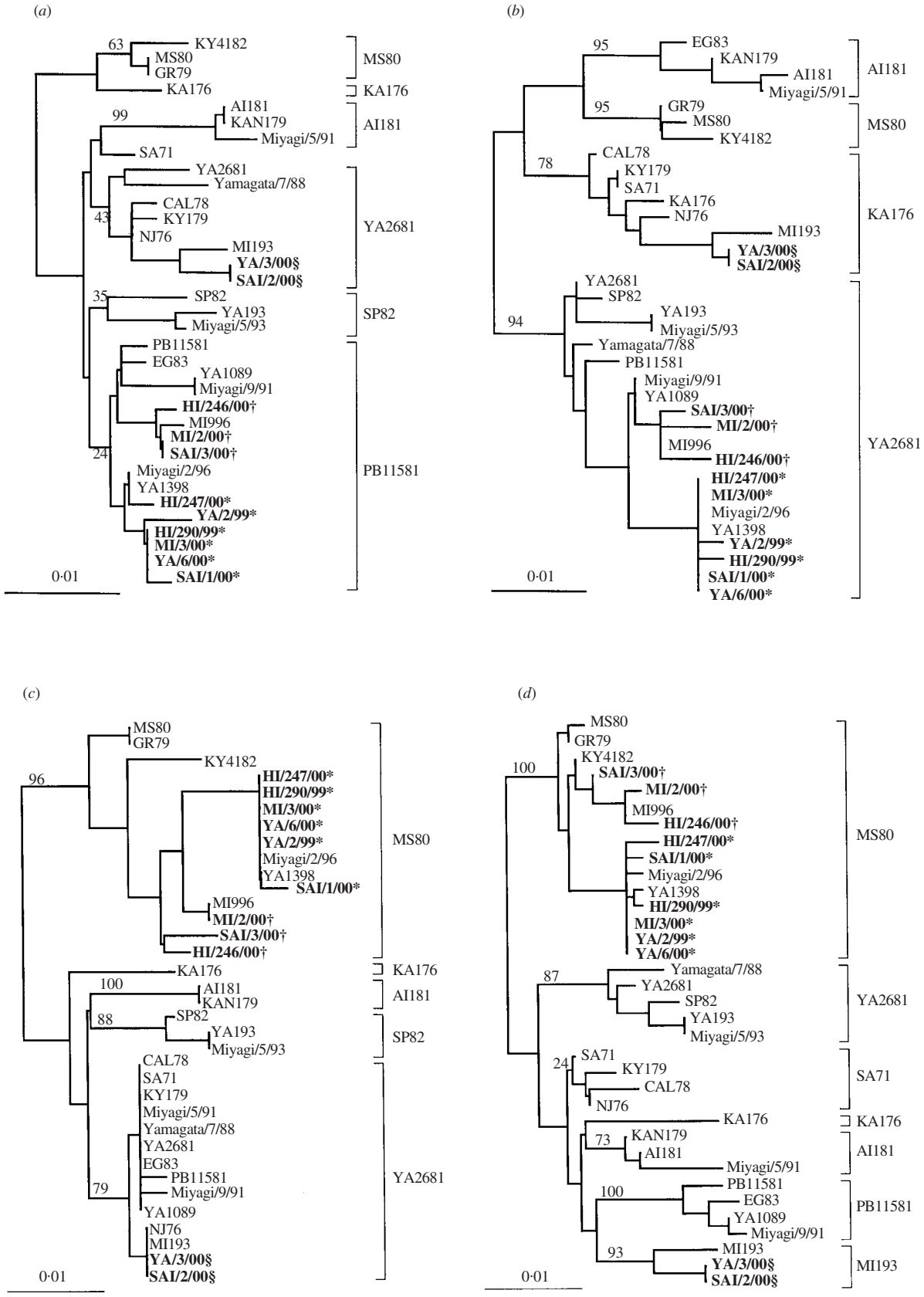

Fig. 4. For legend see opposite.

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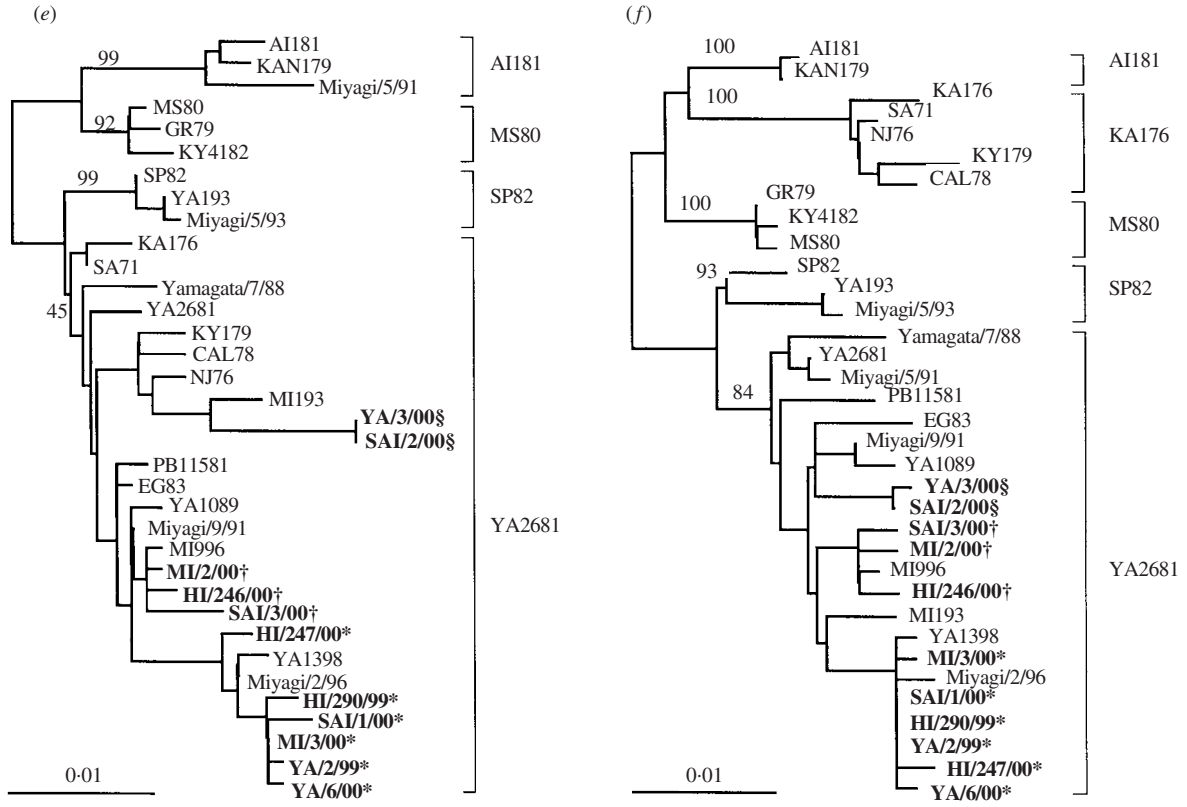

Fig. 4. Phylogenetic trees for the PB2 (*a*), PB1 (*b*), P3 (*c*), NP (*d*), M (*e*) and NS (*f*) genes of influenza C isolates. The nucleotide sequences of the following regions were used for analysis: nucleotides 52–520 for PB2 genes; nucleotides 50–425 for PB1 genes; nucleotides 49–420 for P3 genes; nucleotides 71–670 for NP genes; nucleotides 26–1147 for M genes, and nucleotides 28–889 for NS genes. Numbers below or above the branches are the bootstrap probabilities (%) of each branch determined by the PHYLIP program (version 3.573c). Viruses isolated in this study (bold type) having the HE genes of the YA2681-, KA176- and MS80-related lineage are marked by *, † and § respectively. Abbreviations are as given in the legend to Figure 3.

genome compositions of the isolates in this study determined by phylogenetic analyses are summarized in Table 2.

YA2681-related lineage

The HE genes were divided into six discrete lineages, represented by Taylor/47, AI181, SP82, KA176, YA2681 and MS80. The 12 isolates with antigenicity similar to that of YA2681 were all within the YA2681-related lineage. The nucleotide sequences of the HE genes of 12 isolates were remarkably similar, with sequence identities from 99·4 to 100%. Furthermore, these nucleotide sequences were highly homologous (99·5–99·8% nucleotide identity) to those of the strains which were circulating in Yamagata and Sendai cities since 1996 [Yamagata/13/98 (YA1398), Miyagi/2/96, Miyagi/1/97 and Miyagi/1/99 in Fig. 3]. In the six internal gene trees (Fig. 4), six isolates (YA/2/99, YA/6/00, MI/3/00, SAI/1/00, HI/290/99 and HI/247/00) possessed the PB2 gene on the lineage represented by pig/Beijing/115/81 (PB11581), the PB1, M and NS genes on the lineages represented by YA2681 and the P3 and NP genes on the lineages represented by MS80. This genome composition was similar to those of YA1398, a representative strain of the outbreak in Yamagata City. We previously reported that two outbreaks of influenza C virus which occurred in Yamagata City in 1996 and 1998 were caused by a reassortant virus, which had inherited PB2, PB1, HE, M and NS genes from a PB11581-like virus and P3 and NP genes from an MS80-like virus [18]. Therefore, it seems that the reassortants between PB11581-like virus and MS80-like virus continued to circulate and caused epidemics in Yamagata, Miyagi, Saitama and Hiroshima prefectures during the same period.

KA176-related lineage

In the HE gene tree, the six strains (MI/2/00, MI/5/00, SAI/3/00, HI/246/00, HI/251/00 and HI/250/00)

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Table 2. *Genome compositions of previous isolates and representative influenza C viruses isolated in this study*

Virus strain	RNA segment*							Virus isolated in	
	PB2	PB1	P3	HE	NP	M	NS	Country	Year
AI181-related lineage									
KAN179	A	A	A	A	A	A	A	USA	1979
AI181	A	A	A	A	A	A	A	Japan	1981
SP82-related lineage									
SP82	S	Y	S	S	Y	S	S	Brazil	1982
YA193	S	Y	S	S	Y	S	S	Japan	1993
YA2681-related lineage									
SA71	SA	K	Y	Y	SA	Y	K	Japan	1971
NJ76	Y	K	Y	Y	SA	Y	K	USA	1976
CAL78	Y	K	Y	Y	SA	Y	K	USA	1978
KY179	Y	K	Y	Y	SA	Y	K	Japan	1979
YA2681	Y	Y	Y	Y	Y	Y	Y	Japan	1981
PB11581	P	Y	Y	Y	P	Y	Y	China	1981
YA1089	P	Y	Y	Y	P	Y	Y	Japan	1989
EG83	P	A	Y	Y	P	Y	Y	England	1983
YA/2/99	P	Y	M	Y	M	Y	Y		
YA/6/00	P	Y	M	Y	M	Y	Y		
MI/3/00	P	Y	M	Y	M	Y	Y		
SAI/1/00	P	Y	M	Y	M	Y	Y		
HI/290/99	P	Y	M	Y	M	Y	Y		
HI/247/00	P	Y	M	Y	M	Y	Y		
KA176-related lineage									
KA176	K	K	K	K	K	Y	K	Japan	1976
MI/2/00	P	Y	M	K	M	Y	Y		
SAI/3/00	P	Y	M	K	M	Y	Y		
HI/246/00	P	Y	M	K	M	Y	Y		
MS80-related lineage									
GR79	M	M	M	M	M	M	M	Greece	1979
MS80	M	M	M	M	M	M	M	USA	1980
KY4182	M	M	M	M	M	M	M	Japan	1982
YA/3/00	Y	K	Y	M	MI	Y	Y		
SAI/2/00	Y	K	Y	M	MI	Y	Y		

* Abbreviations: A, AI181-like strain; S, SP82-like strain; Y, YA2681-like strain; SA, SA71-like strain; K, KA176-like strain; P, PB11581-like strain; M, MS80-like strain; MI, MI193-like strain.

having antigenicity similar to that of KA176, which were isolated in Miyagi, Saitama and Hiroshima prefectures, were within the KA176-related lineage. The nucleotide sequences of HE genes of these six strains were highly homologous to each other (99·3–100% nucleotide identity) and showed a much higher degree of homology with Miyagi/9/96 (MI996) (99·4–99·7% nucleotide identity) than with KA176 of the reference strain (98·4–98·7% nucleotide identity). In the trees of the six internal gene segments, MI/2/00, SAI/3/00 and HI/246/00 were closely related to MI996. MI996 isolated in Sendai City in 1996 was a strain that emerged for the first time in 20 years in Japan, receiving the same internal genes as those obtained by epidemic strains in Yamagata City through a reassortment event [13]. This result showed that these three strains were reassortant viruses that inherited only the HE gene from a KA176-like virus and the remaining six genes from a PB11581-like virus (PB2, PB1, M and NS) and an MS80-like virus (P3 and NP).

MS80-related lineage

The two strains isolated in Yamagata and Saitama prefectures (YA/3/00 and SAI/2/00) were located on the MS80-related lineage in the HE gene tree. The nucleotide sequences of these two isolates were also

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strikingly similar with sequence identities of 99·9%. The phylogenetic position of YA/3/00 and SAI/2/00 were identical in all of the six internal gene trees and similar to Miyagi/1/93 (MI193). YA/3/00 and SAI/2/00 were located on the YA2681 lineage in the trees of the PB2, P3, M and NS gene sequences, on the KA176 lineage in the PB1 gene tree and on the MI193 lineage in the NP gene tree. We previously documented that the influenza C viruses with HE genes in an MS80-related lineage isolated in Yamagata and Sendai cities from 1992 to 1996 could be divided into four groups based on their genome composition, which were represented by MI193, Miyagi/3/93 (MI393), Miyagi/7/93 (MI793) and Miyagi/8/96 (MI896) [13]. It was confirmed in this study that YA/3/00 and SAI/2/00 were reassortant viruses having the same genome composition as MI193, which acquired PB2, PB1, P3 and M genes from a Kyoto/1/79 (KY179)-like virus and the NS gene from a PB11581-like virus.

Phylogenetic analysis of foreign isolates in the 1970s and comparison with contemporary Japanese influenza C viruses

In order to examine the possibility that the genetically similar influenza C viruses spread worldwide, the nucleotide sequences of the six internal protein genes were determined for foreign isolates in 1970s (NJ76, CAL78, KAN179 and GR79) and compared with those of the contemporary Japanese strains. Phylogenetic trees are shown in Figure 4 and the genome composition is summarized in Table 2 with the results of isolates in the 1980s reported previously [11, 17, 24]. In the trees of PB1, NP and NS genes, NJ76 and CAL78 with HE genes belonging to the YA2681-related lineage are located on a different lineage from that of YA2681 of the reference strain but on a lineage similar to that of Sapporo/71 (SA71) and KY179 that were Japanese isolates with HE genes on a YA2681-related lineage in the 1970s. Including the analysis of the other gene segments, NJ76 and CAL78, isolated in the United States in 1976 and 1978 respectively, are more closely related to KY179 which was isolated in Japan in 1979. KAN179 with an HE gene belonging to the AI181-related lineage was located on the AI181 virus lineage in all of the six internal gene trees. Thus, KAN179, isolated in the United States in 1979, has the same genome composition with AI181, isolated in Japan in 1981. GR79 isolated in Europe, having an HE gene belonging to the MS80-related lineage, is closely related to MS80 and Kyoto/41/82 (KY4182) in the trees of six internal genes. This shows that the MS80-like viruses were circulating in Europe, the United States and Japan around 1980.

DISCUSSION

We have demonstrated here that the influenza C viruses with similar antigenicity and similar genome composition spread in various areas of Japan during the same period. Influenza A and B viruses cause frequent epidemics worldwide, but influenza C viruses have been isolated only occasionally. This is the first report demonstrating that influenza C viruses cause epidemics in some communities during the same season.

Although the number of the isolates was less than one tenth compared to that of influenza A viruses, it is interesting that influenza C viruses were isolated during the same period when influenza A viruses (H1 and H3) were being isolated all over the country (Fig. 2). However, it was revealed by our long-term surveillance that the influenza C viruses were not always isolated during the same period that the influenza A or B viruses were isolated [12, 13, 18]. Furthermore, there are some differences in the evolution of influenza C and A viruses. The emergence of antigenic variants by immune selection is a common means of evolution for influenza A virus [15]. But we previously observed that we failed to detect the antigenic differences by HI tests with anti-HE MAbs and polyclonal antisera among influenza C viruses belonging to the same antigenic groups during the last three decades [13]. In this study, antigenic analysis with anti-HE MAbs showed that there was little antigenic difference between the isolates in this study and the reference strains, which had been isolated 19–24 years ago (Table 1). These results suggest that there has been little antigenic change among the influenza C viruses belonging to the same antigenic groups during this time period. Therefore, it appears likely that the production of antigenic variants does not contribute to the evolution of influenza C viruses. We had proposed alternatively that reassortment might be the means of evolution for the influenza C viruses [13].

In the 1999–2000 season, viruses belonging to one of three antigenic groups; YA2681, KA176 and MS80, were co-circulating in the Yamagata, Miyagi, Saitama and Hiroshima prefectures of Japan. Viruses

in the YA2681 antigenic group were isolated in all four prefectures and were reassortants between PB11581-like and MS80-like viruses. This reassortant virus emerged in Yamagata and Sendai cities in 1996 [13, 18] and caused two outbreaks in 1996 and 1998, and continued to circulate dominantly in Yamagata City until 2000. This observation indicates that influenza C viruses can be maintained without antigenic change in a community for a long time, presumably because of the prolonged time until the majority of the residents in a community are infected with this virus and the annual supply of susceptible children.

A virus in the KA176 antigenic group was isolated in 1996 for the first time in 20 years in Sendai City (MI996) [13], and spread to various areas of Japan during 2000. This finding, together with the fact that these viruses are reassortants which acquired only the HE gene from a KA176-like virus and the other internal genes from epidemic strains of the Yamagata/26/81 antigenic group mentioned above, strongly suggests that viruses in the KA176 antigenic group acquired selective advantage and spread over the country, receiving the same internal genes from the epidemiologically dominant strains through a reassortment event. Therefore, it appears that reassortment events play an important role in the survival of influenza C viruses. It was questioned why the viruses in the KA176 antigenic group had not been isolated in Yamagata prefecture in spite of their epidemiological advantage. Very recently, we obtained evidence that viruses in the KA176 antigenic group spread as the dominant strain in Yamagata City during 2002 (Y. Matsuzaki, unpublished results). This observation suggests that the propagation of newly introduced influenza C virus throughout the country does not necessarily occur simultaneously.

We previously documented that most of the influenza C viruses isolated by our surveillance work, which were divided into five lineages by HE gene sequence (AI181, YA2681, SP82, KA176 and MS80-related lineage), were reassortant viruses genetically differentiated from the reference strain [13]. Moreover, we pointed out that the influenza C viruses isolated in Japan since 1970 had reassorted frequently and the production of the dominant strain seemed to succeed by changing its genome composition. In this study, it was revealed that the foreign isolates in the 1970s and 1980s had genome compositions similar to those of contemporary isolates of Japan (Table 2): KAN179 isolated in the United States in 1979 with an HE gene in an AI181-related lineage had a genome composition identical to that of AI181. SP82 isolated in Brazil in 1982 with a HE gene in an SP82-related lineage had a genome composition identical to that of Yamagta/1/93 (YA193). GR79 isolated in Greece in 1979 and MS80 isolated in the United States in 1980, which had HE genes of an MS80-related lineage, had a genome composition similar to that of KY4182. Among influenza C viruses having HE genes in the YA2681-related lineage, only England/83 (EG83) isolated in England in 1983 has a unique genome composition. NJ76 and CAL78 isolated in the United States in 1976 and 1978 respectively, possessed a genome composition similar to that of KY179, isolated in Japan in 1979. PB11581 isolated from a pig in China in 1981 was closely related to Yamagata/10/89 (YA1089), isolated in Japan in 1989. These observations suggested that the reassortants of influenza C virus were newly introduced into Japan from abroad and propagated all over the country replacing the pre-existing viruses. It is likely that the reassortant viruses were also exported abroad. In any case, it is strongly suspected that antigenically and genetically similar influenza C viruses are widespread all over the world. In Japan, taking the case of the viruses in the YA2681-related lineage, SA71-like viruses were isolated in the 1970s, YA2681-like viruses were dominant in the 1980s, PB11581-like viruses were isolated from 1989 to 1992 and the reassortant viruses acquired the NP and P3 genes from an MS80-like virus that emerged in 1996 [13]. Thus, it is certain that the influenza C viruses having the same genome composition continue to circulate in a community for several years. It is likely that influenza C viruses spread slowly over the world throughout this period, whereas new variants of influenza A virus spread rapidly worldwide annually [14, 19].

Although influenza C viruses are sometimes isolated from pigs [36], the viruses seem to be maintained within the human population [24] and the reassortment event may occur in a patient infected with two different strains of influenza C viruses. In this study, only children <15 years of age have been sampled. Infection of influenza C viruses in adults may contribute to the occurrence of frequent reassortment and the spread of this virus. If the surveillance of influenza C virus in adults as well as in children could be performed in the various regions of the world, it would become clear where a reassortant virus emerges first and how it is spread throughout the world.

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