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Characterization of antigenically unique influenza C virus strains isolated in Yamagata and Sendai Cities, Japan, during 1992–1993

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Three influenza C virus strains (C/Yamagata/1/92, C/Yamagata/1/93 and C/Miyagi/5/93) isolated in Yamagata and Sendai Cities, Japan, between June 1992 and May 1993 were found to possess haemagglutinin–esterase glycoproteins that were antigenically indistinguishable from one another but were clearly different from any previous Japanese isolates. To investigate the origin of the 1992/1993 strains, their antigenic and genetic properties were compared with those of eight strains isolated outside Japan between 1967 and 1982. The results showed that the 1992/1993 isolates were closely related to a virus isolated in Brazil in 1982 (C/SaoPaulo/378/82) and that these viruses (including C/SaoPaulo/378/82) are re-assortants that had obtained PB1 and NP genes from a C/Yamagata/26/81-like parent and the other genes from another as yet unidentified parent.

Although influenza C virus usually causes a mild upper-respiratory illness characterized by fever and long-lasting nasal discharge (Katagiri *et al.*, 1983), it can also cause lower respiratory tract infections such as bronchitis and pneumonia (Moriuchi *et al.*, 1991; Y. Matsuzaki, K. Mizuta and K. Nakamura, unpublished observations). As the genome of this virus consists of seven RNA segments, reassortment characterized by exchange of genome segments between two different strains occurs *in vitro* at a high frequency (Nishimura *et al.*, 1994). Earlier studies on the RNA genomes of various isolates demonstrated that the extent of genetic difference did not correlate with the time of virus isolation, suggesting that multiple evolutionary lineages may co-circulate at any one time (Buonagurio *et al.*, 1985, 1986). More recently, we compared the antigenicity of the haemagglutinin–esterase (HE) glycoprotein as well as the HE gene sequence among 25 virus strains isolated between 1964 and 1988 and revealed the existence of four distinct virus groups represented by Aomori/74 (AO/74), Yamagata/26/81 (YA/26/81), Aichi/1/81 (AI/81) and Mississippi/80 (MS/80), three of which (YA/26/81-, AI/81- and MS/80-related lineages) co-circulated in Japan in the 1980s (Muraki *et al.*, 1996).

The surveillance of influenza C virus infections initiated in Yamagata City in 1988 and in the adjacent Sendai City in 1990 has succeeded in isolating a large number of viruses, almost all of which have been shown to possess HE genes belonging to one of the three lineages described above (Matsuzaki *et al.*, 1994; Peng *et al.*, 1996; Kimura *et al.*, 1997). We noticed, however, that three virus strains [Yamagata/1/92 (YA/1/92), Yamagata/1/93 (YA/1/93) and Miyagi/5/93 (MI/5/93)] isolated between June 1992 and May 1993 were closely similar to one another in both antigenic and genetic structures but dissimilar to any of the 60 strains isolated in Japan before June 1992, raising the possibility that an influenza C variant, belonging to an evolutionary lineage distinct from any identified previously, had been newly introduced into Japan shortly before June 1992 and then circulated in the country up to at least May 1993.

In this communication, to obtain information about the origin of the 1992/1993 isolates from Yamagata and Sendai, we compared their antigenic and genetic properties with those of eight foreign isolates from France [Paris/1/67 (PA/67)], the Republic of South Africa [Johannesburg/4/67 (JHG/67)], the United States [Georgia/1/69 (GA/69), NewJersey/1/76

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank databases with the accession numbers AB035357–AB035377.

Table 1. Comparison of the antigenicity of the three 1992/1993 isolates with that of eight foreign isolates

Antigenicity was compared by HI tests with anti-HE MAbs and chicken anti-virus sera. Bold values represent HI titres obtained with antiserum directed against the homologous strain.

	10^{-1} × HI titre (HI units/ml) of anti-HE MAbs				HI titre (HI units/ml) of antiserum to:				
Strain	J14	Q5	U4	MS2	YA/7/81*	AI/81	MS/80	YA/1/92	PA/67
Reference strains									
YA/26/81	12800	1280	8	–	**2560**	1280	2560	640	2560
AI/81	102400	16	512	–	320	**5120**	1280	640	640
MS/80	40960	–†	–	25600	80	640	**20480**	320	320
1992/1993 isolates									
YA/1/92	12800	6400	2560	–	320	1280	2560	**2560**	1280
YA/1/93	6400	1600	640	–	320	1280	1280	1280	1280
MI/5/93	6400	3200	640	–	320	1280	2560	2560	2560
Foreign isolates									
YA/26/81 group									
NJ/76	12800	2560	8	–	2560	1280	2560	640	2560
AI/81 group									
JHG/67	204800	64	512	–	320	5120	5120	1280	2560
GA/69	204800	64	512	–	320	5120	2560	1280	1280
KAN/1/79	102400	16	512	–	320	5120	2560	1280	1280
KAN/2/79	102400	16	512	–	320	5120	2560	1280	1280
MS/80 group									
GR/79	204800	–	–	51200	40	640	20480	320	320
Unassigned									
PA/67	102400	64	–	–	640	1280	2560	1280	**10240**
SP/82	25600	12800	1280	–	320	1280	2560	2560	2560

* The HE antigenicity of strain YA/7/81 was identical to that of YA/26/81 (Kawamura *et al.*, 1986; Matsuzaki *et al.*, 1994).
† Titre below 4.

(NJ/76), Kansas/1/79 (KAN/1/79) and Kansas/2/79 (KAN/2/79)], Greece [Greece/1/79 (GR/79)] and Brazil [SaoPaulo/378/82 (SP/82)] that have not as yet been well characterized. The results show that the 1992/1993 isolates are closely related to the Brazilian isolate (SP/82) and that these viruses (including SP/82) emerged through a reassortment event from a YA/26/81-like parent and another parent that has not yet been identified, having acquired the PB1 and NP genes from the former and the remaining five genes from the latter.

Strains YA/1/92, YA/1/93 and MI/5/93 were isolated from throat swab specimens collected on 4 June 1992, 30 April 1993 and 6 May 1993, respectively, from paediatric patients with acute respiratory illness by inoculating the strains into the amniotic cavity of 9-day-old embryonated hen's eggs. The viruses were each cloned by means of the limiting dilution method and then propagated in eggs. The eight foreign isolates used for comparison, all of which were kindly provided by N. J. Cox (CDC, Atlanta, USA), were also cloned and propagated in eggs.

A total of 14 influenza C strains were isolated between June 1992 and May 1993 from patients living in Yamagata or Sendai Cities. Antigenic analysis with monoclonal antibodies (MAbs) to the HE glycoprotein showed that 11 of the 14 isolates belonged to one of the three known antigenic groups represented by YA/26/81, AI/81 and MS/80 (Peng *et al.*, 1996; Kimura *et al.*, 1997). The remaining three (YA/1/92, YA/1/93 and MI/5/93), on the other hand, displayed antigenicities that were identical to one another but were clearly different from that of any of the reference strains (YA/26/81, AI/81 and MS/80), as shown in Table 1. Table 1 summarizes the results of antigenic analysis performed by haemagglutination-inhibition (HI) tests (Matsuzaki *et al.*, 1994), utilizing four different anti-HE MAbs characterized previously (Sugawara *et al.*, 1988, 1993). The reactivity patterns of the three 1992/1993 strains were also dissimilar to those of nine strains isolated outside Japan [Taylor/47 (TAY/47), AnnArbor/1/50 (AA/50), GreatLakes/54 (GL/54), Johannesburg/1/66 (JHG/66), California/78 (CAL/78), Pig/Beijing/10/81, Pig/Beijing/115/81 (P/B/115/81), Pig/Beijing/439/82 and England/83 (EG/83)] (data not shown),

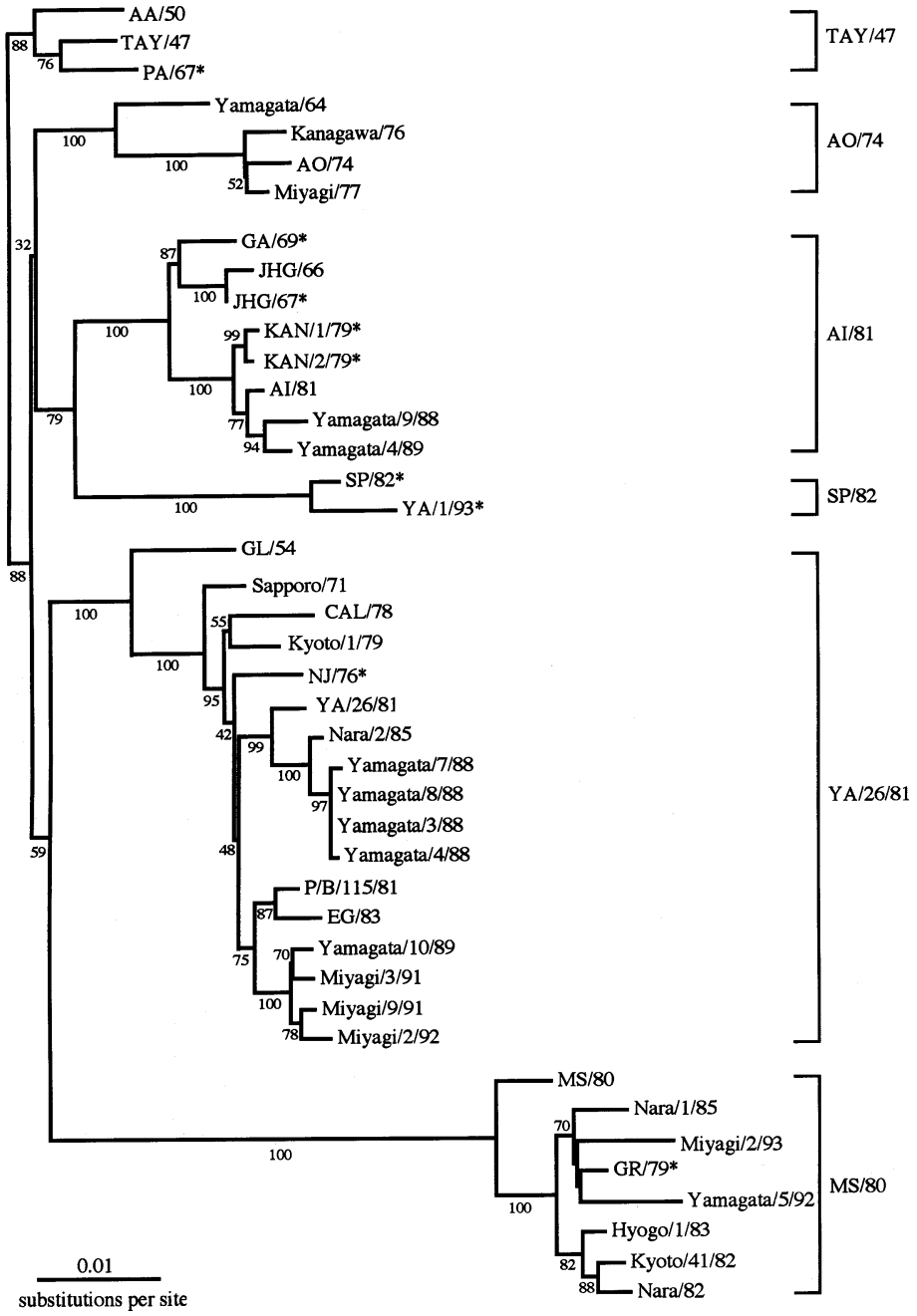

Fig. 1. Phylogenetic tree of influenza C virus HE genes. The region from nucleotides 64 to 1989 was used for analysis. The HE genes of the isolates marked by asterisks were sequenced for this study and the other sequences have been reported previously (Buonagurio *et al.*, 1985; Adachi *et al.*, 1989; Ohyama *et al.*, 1992; Umetsu *et al.*, 1992; Peng *et al.*, 1994, 1996; Matsuzaki *et al.*, 1994, 1995; Muraki *et al.*, 1996; Asahi *et al.*, 1997; Kimura *et al.*, 1997). Horizontal distances are proportional to the minimum number of nucleotide differences needed to join the gene sequences. Numbers below or above the branches are the bootstrap probabilities (%) of each branch.

the HE antigenicity of which had been characterized previously (Matsuzaki *et al.*, 1994; our unpublished results). The additional eight foreign isolates (listed in Table 1) were therefore examined for reactivity with anti-HE MAbs and their reactivity patterns were compared with those of the three 1992/1993 strains. The results showed that six of the eight isolates belonged to one of the three known antigenic groups: NJ/76 fell within the YA/26/81 virus group; JHG/67, GA/69, KAN/1/79 and KAN/2/79 within the AI/81 virus group; and GR/79 within the MS/80 virus group. In contrast, the patterns of PA/67 and SP/82 were clearly different from that of any of the three reference strains. It was evident, however, that strain

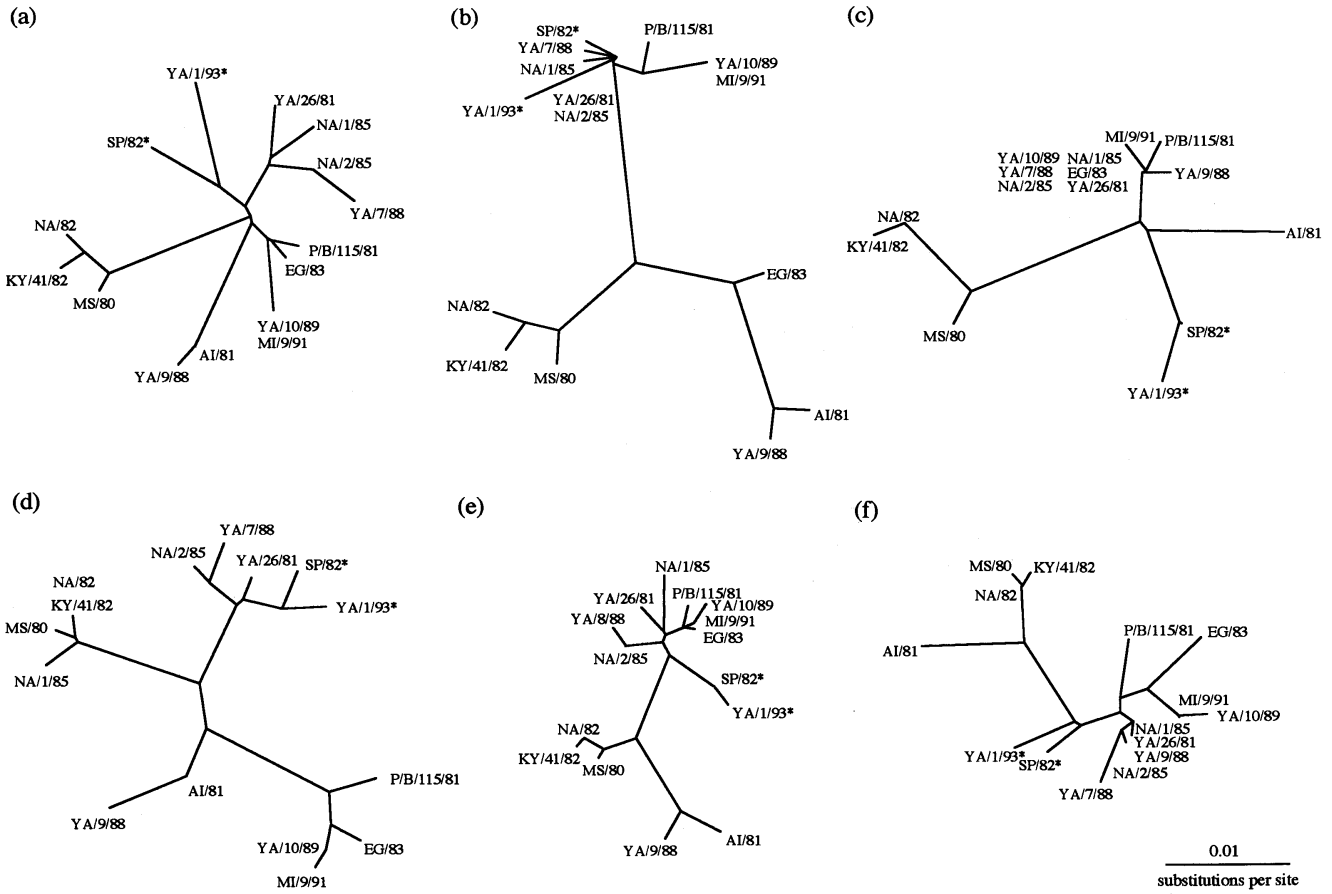

Fig. 2. Phylogenetic trees for the PB2 (*a*), PB1 (*b*), P3 (*c*), NP (*d*), M (*e*) and NS (*f*) genes of influenza C isolates. The sequences marked by asterisks were sequenced in this study and the other sequences have been reported previously (Peng *et al.*, 1996; Kimura *et al.*, 1997; Tada *et al.*, 1997). Virus abbreviations: NA/82, Nara/82; KY/41/82, Kyoto/41/82; NA/1/85, Nara/1/85; NA/2/85, Nara/2/85; YA/7/88, Yamagata/7/88; YA/8/88, Yamagata/8/88; YA/9/88, Yamagata/9/88; YA/10/89, Yamagata/10/89; MI/9/91, Miyagi/9/91.

SP/82 had a remarkably similar antigenicity to that of the three 1992/1993 isolates.

Strains YA/1/92, YA/1/93 and MI/5/93 and the eight foreign isolates were examined further by HI tests for reactivity with chicken antisera to five different strains. The results (Table 1) support the conclusion stated above that the 1992/1993 strains possessed antigenic properties indistinguishable from those of SP/82. It was also confirmed that six of the foreign isolates (JHG/67, GA/69, NJ/76, KAN/1/79, KAN/2/79 and GR/79) were antigenically similar to one of the three reference strains, whereas PA/67 was distinguishable from all of them.

In order to clarify the results of antigenic analysis, the nucleotide sequence of the HE gene was determined for one of the three 1992/1993 isolates (YA/1/93) as well as for all of the eight foreign isolates by sequencing PCR products directly according to procedures described elsewhere (Kimura *et al.*, 1997). A phylogenetic tree was then constructed by the neighbour-joining method (Saitou & Nei, 1987), utilizing these nine sequences as well as 33 sequences reported previously, including those of two prototype strains, TAY/47 and AA/50. In our previous phylogenetic studies (Muraki *et al.*, 1996; Kimura *et al.*, 1997), these latter two strains were excluded from the analysis, since numerous passages in embryonated eggs may have introduced many additional mutations not present in the original isolates. As expected from the results of antigenic analysis, six of the eight foreign isolates were found to belong to one of the three lineages: NJ/76 was within the YA/26/81-related lineage, JHG/67, GA/69, KAN/1/79 and KAN/2/79 within the AI/81-related lineage and GR/79 within the MS/80-related lineage (Fig. 1). Strain PA/67, together with two oldest strains (TAY/47 and AA/50), formed a lineage (designated the TAY/47-related lineage) that was separate from the four lineages identified previously. More importantly, the HE gene sequence of YA/1/93 showed a much higher degree of identity to SP/82 (99·1%) than to any of the other 40 strains (92·3–96·9%), and strains YA/1/93 and SP/82 made up a separate sixth lineage (designated the SP/82-related lineage). Furthermore, comparison of the predicted amino acid sequence showed that the

HE protein of YA/1/93 differed from that of SP/82 by only two amino acids (residues 134 and 331), yet it differed from those of the other seven foreign isolates by as many as 17–23 amino acids.

To confirm that the 1992/1993 isolates in question were closely related to SP/82, partial nucleotide sequences of strains YA/1/93 and SP/82 were determined for the PB2 (positions 52–520), PB1 (positions 50–425), P3 (positions 49–420) and NP (positions 71–670) genes as well as nearly complete sequences of the M (positions 24–1147) and NS genes (positions 45–892) by direct sequencing of PCR products. The primers used for asymmetric PCR and sequencing are described elsewhere (Peng *et al*., 1994; Kimura *et al*., 1997; Tada *et al*., 1997). Fig. 2 shows phylogenetic trees of the individual genes constructed by using the sequences of YA/1/93 and SP/82, in addition to the published sequences of 13 strains isolated between 1981 and 1991 (Peng *et al*., 1996; Kimura *et al*., 1997; Tada *et al*., 1997). It was clear that, in all six RNA segments, YA/1/93 was more closely related to SP/82 than to any of the 13 reference strains. We reported previously that the PB2 genes were split into four distinct lineages represented by YA/26/81, AI/81, MS/80 and P/B/115/81 (Peng *et al*., 1996; Kimura *et al*., 1997). The PB2 genes of YA/1/93 and SP/82 seemed to make up a separate fifth lineage. Similarly, the P3 genes of these two isolates formed a fourth lineage independent of the three lineages recognized previously, represented by YA/26/81, AI/81 and MS/80. Strains YA/1/93 and SP/82 also possessed M and NS genes that were distantly related to the genes of any of the reference strains. In the trees for the PB1 and NP genes, in contrast, YA/1/93 and SP/82 were located within a branch cluster consisting of viruses that possessed HE genes within the YA/26/81-related lineage. These results, together with those of the HE gene sequence analysis, suggest strongly that YA/1/93 and SP/82 are naturally occurring reassortants that have inherited PB1 and NP genes from a YA/26/81-like virus and HE, PB2, P3, M and NS genes from another as yet unidentified parent. Additionally, T1-oligonucleotide fingerprinting analysis with total virus RNA showed that oligonucleotide maps of strains YA/1/92 and MI/5/93 were remarkably similar to that of YA/1/93, differing by only three and four oligonucleotide spots, respectively, among $\sim$ 70 spots used for comparison (data not shown), which suggests that YA/1/92 and MI/5/93 are also reassortants with the same genome composition as those of YA/1/93 and SP/82. These observations support the notion described previously that reassortment of the genome of influenza C viruses occurs frequently in nature and plays a role in generating genetic variation (Peng *et al*., 1994, 1996; Kimura *et al*., 1997; Tada *et al*., 1997).

The observations that three influenza C strains (YA/1/92, YA/1/93 and MI/5/93) related closely to SP/82 were isolated during a relatively short period (June 1992 to May 1993) and that SP/82-like viruses had not been isolated in Japan before June 1992 lead us to speculate that a virus similar to SP/82 was introduced into Japan shortly before June 1992 and began to spread in the country, although we cannot exclude the possibility that the failure to detect SP/82-like viruses before June 1992 might be due to the potential limitations of our surveillance work. After May 1993, a total of 59 influenza C strains were isolated in Yamagata and Sendai Cities but none of them had HE antigenicity similar to that of SP/82 (unpublished results), suggesting that the SP/82-like virus, even if it was imported into Japan at the time described above, did not have an epidemiological advantage over the pre-existing viruses. Previously, we presented data that suggest that an influenza C virus closely similar to swine isolates (represented by P/B/115/81) obtained in China during 1981 and 1982 was introduced into Japan around 1989 (Kimura *et al*., 1997). Very recently, we obtained evidence that this imported virus, unlike the SP/82-like virus, continues to circulate as the predominant strain in Yamagata and Sendai Cities up to the present (unpublished results). Hereafter, it is important to investigate the molecular basis for the apparent difference in the epidemiological potential between the SP/82-like and P/B/115/81-like viruses.

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