Arch Virol (1996) 141: 1349–1355

Archives of Virology

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Printed in Austria

Evolutionary characterization of recent human H3N2 influenza A isolates from Japan and China: novel changes in the receptor binding domain

Brief Report

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Accepted February 9, 1996

Summary. Recent human H3N2 influenza viruses isolated in Japan and China were characterised from an evolutionary point of view. They appeared to have divided into three minor branch clusters, including 1992–1993, 1993–1994 and 1994–1995 isolates. It was of particular interest to reveal that in addition to amino acid substitutions in the antigenic sites of the HA molecule, amino acid changes occurred at position 226 of the receptor binding site from lysine or glutamine to isoleucine in all strains belonging to the 1994–1995 branch cluster. This is the first evidence of human H3N2 influenza isolates, or any other influenza HA serotypes, to contain a conserved amino acid residue other than lysine or glutamine at this key position.

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Since the first appearance of human H3N2 viruses in China in 1968, a number of antigenic variants have been isolated in many parts of China [12, 20, 24]. Indeed, a number of vaccine strains for Hong Kong influenza which have been recommended by WHO have been Chinese isolates [29–34]. The WHO recommended virus for 1994–1995 epidemic season, A/Johannesburg/33/94 (H3N2), is an exception to this, however, even this virus is antigenically indistinguishable from

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an earlier Chinese strain, A/Guandong/25/93 [35]. In the present study, sequence and phylogenetic analyses were done on the HA of Japanese and Chinese human H3N2 influenza strains to determine the evolutionary relationship between isolates of the above two countries from the same epidemic season.

Phylogenetic analysis was done on the HA1 domain of the HA gene by constructing an evolutionary tree as described previously [10, 19] (Fig. 1). Virus strains used for analyses in the present study and their abbreviations are shown in the legend to Fig. 1. A total of 31 strains were used in phylogenetic analysis of the HA gene. Twenty-seven of their HA genes were sequenced in this study. In agreement with previous reports [1, 2, 7, 9, 23, 24], human H3N2 viruses have essentially evolved in a single lineage, although recent strains since 1991 tended to form several branch clusters. The evolutionary tree showed, for example, that Bei/352/89-like viruses such as Was/15/91, Bra/2/91, and Shi/2/91 are phylogenetically similar, belonging to the same branch cluster. Bei/352/89-like viruses circulated in the human population of Japan until the latter part of the 1992–1993 epidemic season before being displaced by Bei/32/92-like viruses. Bei/32/92-like viruses, which were isolated in China as early as 1990, were subsequently isolated in many parts of the world and found to cocirculate with Bei/352/89-like viruses in the same epidemic season [6, 7, 33]. Bei/32/92-like viruses, therefore, appear to have evolved independently of circulating epidemic strains for some time before becoming the predominant epidemic strain. Indeed, the evolutionary tree revealed that the Bei/32/92-like lineage appears to have diverged from a putative virus around 1989. Viruses from 1993–1995 were divided further into two branch clusters which distinguished the most recent Japanese and Chinese strains of 1994–1995 from the previous strains of 1993–1994. Japanese and Chinese epidemic strains of the same epidemic season were generally clustered very close to one another which demonstrated that if new antigenic variants are isolated in China there is likely very little time before they are also isolated in Japan.

Analyses of predicted amino acid sequences revealed isolate Ban/139/90 contained seven potential asparagine-linked glycosylation sights located at positions 22, 38, 63, 126, 165, 246, and 285. Nine sequences (Sic/2/87, Bei/352/89, Shi/2/91, Was/15/91, Bra/2/91, Bei/32/92, Tia/33/92, Yok/73/92 Kit/159/93 Aic/69/94, Heb/19/95, Aki/1/95 and Sen/373/95) contained an eighth potential glycosylation site at position 8, while seven of the strains (Sha/9/93, Heb/12/93, Gua/25/93, Gua/28/94, Aki/1/94, Sen/384/94 and Heb/41/94) contained a ninth potential glycosylation site at location 276. It has been demonstrated that glycosylation of position 63 can stericly hinder binding of monoclonal antibodies to antigenic site C [22]. Thus, the presence of a carbohydrate sidechain bound to an asparagine residue near, or in, an antigenic sight appears to prevent binding of neutralizing antibodies. As mentioned above, Sha/9/93, Heb/12/93, Gua/25/93, Gua/28/94, Aki/1/94, Sen/384/94 and Heb/41/94 contain a potential glycosylation sight at amino acid position 276, which is located in antigenic site C. Glycosylation of this residue, therefore, could effectively mask this site from neutralizing antibodies.

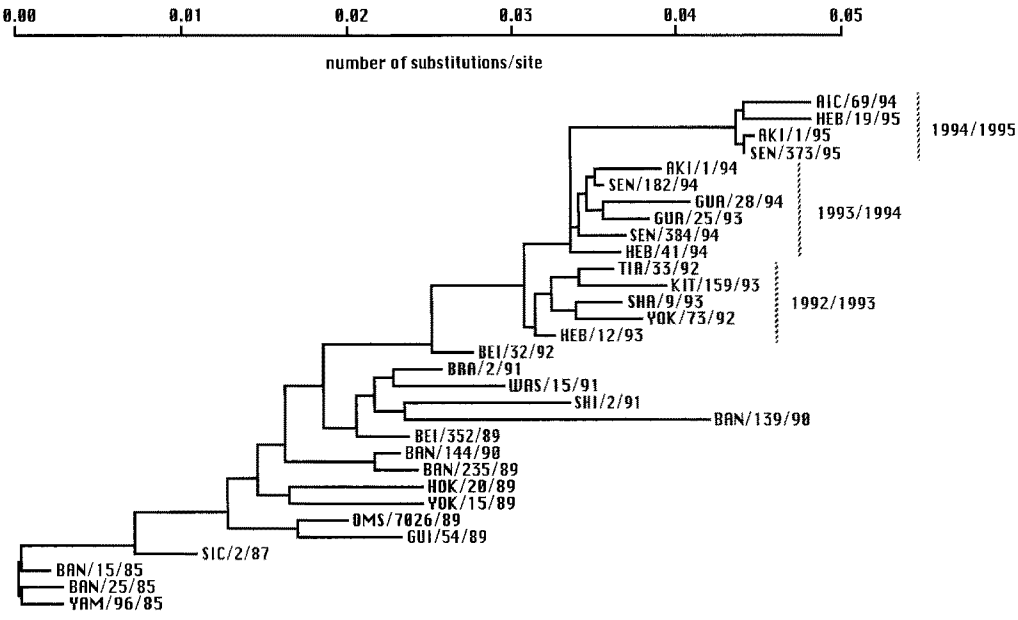

Fig. 1. The phylogenetic tree of the HA1 domain coding region of the HA gene of H3N2 influenza virus isolates. The following 24 Hong Kong (H3N2) influenza strains were propagated in 11 day old embryonated hen's eggs for analysis of the H3HA gene: A/Hebei/19/95 (Heb/19/95), A/Akita/1/95 (Aki/1/95), A/Guandong/28/94 (Gua/28/94), A/Akita/1/94 (Aki/1/94), A/Sendai/c384/94 (Sen/384/94), A/Sendai/c182/94 (Sen/182/94), A/Kitakyushu/159/93 (Kit/159/93), A/Hebei/12/93 (Heb/12/93), A/Brazil/2/91 (Bra/2/91), A/Washington/15/91 (Was/15/91), A/Tianjin/33/92 (Tia/33/92), A/Yokohama/73/92 (Yok/73/92), A/Bangkok/139/90 (Ban/131/90), A/Shiga/2/91 (Shi/2/91), A/Beijing/352/89 (Bei/352/89), A/Bangkok/235/89 (Ban/235/89), A/Bangkok/144/90 (Ban/144/90), A/Guizhou/54/89 (Giu/54/89), A/OMS/7026/89 (OMS/7026/89), A/Hokkaido/20/89 (Hok/20/89) and A/Yokohama/15/89 (Yok/15/89) with the exception of A/Hebei/41/94 (Heb/41/94), A/Sendai/c373/95 (Sen/373/95), and A/Aichi/69/94 (Aic/69/94), which were passaged in MDCK cells. Viral RNA was purified from allantoic fluid or cell supernatant using methods described previously [4]. The HA1 region of segment 4 was amplified in overlapping cassettes using the reverse transcription-polymerase chain reaction (RT-PCR) [18] and their nucleotide sequences determined. All nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under the following accession numbers: D30662–D30665, D30668–D30669, D49959–D49967, U48439–U48447. Phylogenetic analysis included a total of 31 haemagglutinin HA1 sequences, of which seven sequences, A/Yamagata/96/85 (Yam/96/85), A/Bangkok/2746/89 (Ban/25/89), A/Bangkok/2465/89 (Ban/15/89), A/Sichuan/2/87 (Sic/2/87), A/Beijing/32/92 (Bei/32/92), A/Shandong/9/93 (Sha/9/93), and A/Guandong/25/93 (Gua/25/93) have been published previously [7, 9, 13]. Calculation was done using the neighbor-joining (N–J) method [10, 19] based on the number of total substitutions in the HA1 domain. The tree was drawn as described previously [19]

Predicted amino acid sequences of the HA1 region from 21 H3N2 viruses were aligned and the amino acids of their antigenic sites and the receptor binding domain are illustrated in Fig. 2. Comparisons of deduced amino acid sequences of the HA1 domain of the HA genes from recent H3N2 isolates identified

	Antigenic Site A			Antigenic Site B		Antigenic Site C		Receptor Binding							
	133	137	140-146	155-160	185-197	51-54	276-280	98	134-138	153	155	183	190	194	224-228
Sichuan/2/87	S	Y	KRGSVNS	HKSEYK	RVTDREQTNLYVR	ICDS	TCSSE	Y	GGSYA	W	H	H	E	L	RGLSS
Beijing/352/89	*	*	*****K*	*E****	PS******K****	****	*****	*	*E***	*	*	*	*	*	*****
Bangkok/139/90	*	*	*****K*	*E**N*	PI******S****	****	*****	*	*D***	*	*	*	*	*	*****
Shiga/2/91	*	*	*******	*E****	PI******S****	****	*****	*	*****	*	*	*	*	*	*****
Brazil/2/91	*	*	*****K*	*E****	PI******S****	****	***N*	*	*E***	*	*	*	*	*	*****
Washington/15/91	*	*	*****K*	*E*D**	PI******S****	****	*****	*	*E***	*	*	*	*	*	*****
Beijing/32/92	D	*	*******	******	PS***D**S****	****	*****	*	*****	*	*	*	D	*	**Q**
Tianjin/33/92	D	*	*******	******	PS**SD**S****	****	*****	*	*****	*	*	*	D	*	**Q**
Yokohama/73/92	D	*	*******	**L***	PS**SD**S****	****	K****	*	*****	*	*	*	D	*	**Q**
Shangdong/9/93	D	*	*******	**L***	PS**SD**S****	**G*	N****	*	*****	*	*	*	D	*	**Q**
Hebei/12/93	D	*	*******	**L***	PS**SV**S****	****	N****	*	*****	*	*	*	V	*	**Q**
Kitakyushu/159/93	D	*	*******	******	PS**SD**S***Q	****	***F*	*	*****	*	*	*	D	*	**Q**
Guandong/25/93	D	*	*******	**L***	PS**SD**S****	****	N****	*	*K***	*	*	*	D	*	**Q**
Hebei/41/94	D	*	*******	**L***	PS**SD**S****	****	N****	*	*K***	*	*	*	D	*	*****
Sendai/c182/94	D	*	*******	**L***	PS**SD**S****	****	N****	*	*K***	*	*	*	D	*	**Q**
Sendai/c384/94	D	*	*******	**L***	PS**SD**S****	****	N****	*	*K***	*	*	*	D	*	**Q**
Akita/1/94	D	*	*****K*	**L***	PS**SD**S****	****	N****	*	*K***	*	*	*	D	*	**Q**
Guandong/28/94	D	*	*******	**L***	PS**SD**S****	****	N****	*	*K***	*	*	*	D	*	**Q**
Aichi/69/94	D	*	*******	**L***	PS**SD**S***Q	****	N*K**	*	*K***	*	*	*	D	*	**I**
Hebei/19/95	D	*	*****K*	**L***	PS**SD**S***Q	****	N*N**	*	*K***	*	*	*	D	*	**I**
Akita/1/95	D	*	*******	**L***	PS**SD**S***Q	****	N*N**	*	*K***	*	*	*	D	*	**I**
Sendai/c373/95	D	*	*******	**L***	PS**SD**S***Q	****	N*N**	*	*K***	*	*	*	D	*	**I**

Fig. 2. Predicted amino acid differences in the antigenic sites and receptor binding domain of the HA1 domain of the H3 HA gene of recent influenza A isolates [26, 28]. Amino acids of Sic/2/87 are given at the top as the reference strain with only the amino acid differences of the other strains indicated below. The sequences of Sic/2/87, Bei/32/92, Sha/9/93 and Gua/25/93 have been published previously [7, 13]. The predicted amino acid sequences of the HA1 domain of the HA protein of A/Beijing/353/89 (Bei/353/89) and Was/15/91 have been published previously [15]. However, their nucleotide sequences were not available for phylogenetic analysis which required us to sequence them independently. In our laboratory, we use A/Bei/352/89 for antigenic characterization which is the antigenic equivalent of Bei/353/89. The HA1 domain of Bei/352/89 but was found to contain two amino acid differences at positions 148 and 193 when compared to Bei/353/89 [15]

a significant number of amino acid differences located in three of the characterized antigenic sites and the receptor binding domain [3, 5, 8, 26, 28]. It was interesting to note amino acid differences found between viruses isolated prior to, and following, the aforementioned antigenic drift in the 1992–1993 epidemic season. Amino acid differences found at position 145 of antigenic site A and 156 and 186 of antigenic site B of viruses isolated from 1989–1991, when compared to Shi/2/91, are not found in viruses isolated after 1992. Furthermore viruses isolated since 1992 contained additional amino acid changes in antigenic sites A, B, and C which have since been conserved [26, 28]. Similarly, current Chinese and Japanese strains Heb/19/95, Aic/69/94, Aki/1/95, and Sen/373/95 were found to contain an additional two differences in antigenic sites B and C when compared to earlier isolates.

It was particularly noteworthy that changes in the receptor binding pocket of the HA protein of recent H3N2 viruses were confirmed [25, 28]. For example, Bei/352/89, Bra/2/91, and Was/15/92 contain a glycine to glutamic acid conversion at position 135 when compared to Sic/2/87. However, most viruses from 1992 and 1993, contained glycine at this site while isolates from 1994 and 1995 contained lysine. Furthermore, all viruses from 1992–1995 contained a glutamic acid to aspartic acid change at position 190 when compared to Sic/2/87, with the exception of Heb/12/93 which contained a valine. Amino acid position 226 has been previously reported to be involved in the binding specificity to host cell sialic acid receptors [14, 16, 17, 25, 27]. Most strains isolated from 1992–1994 contain a glutamine at position 226 which suggests a preference to bind Neu α-2-3 Gal over Neu α-2-6 Gal [11, 16, 17]. It has been reported that passaging of influenza viruses in embryonated eggs may select for viruses containing glutamine at position 226 [21, 27]. This is supported by the fact that of the isolates since 1992 to be sequenced in this study, only Heb/41/94, which was passaged in MDCK cells, contains leucine at position 226. Interestingly, however, viruses isolated from 1989–1991 sequenced in this study contained leucine at this position despite being passaged repeatedly in eggs. Most interestingly, isolates from 1994–1995 influenza season from China and Japan were found to contain isoleucine at residue 226. All HA serotypes of naturally circulating human and animal type A influenza viruses reported contain either leucine or glutamine at position 226 [1, 14–16]. Thus, this is the first report in which such a conserved change has been demonstrated at this position in any natural influenza isolate of any HA serotype. The influence of such a change on the receptor binding properties of the HA has not yet been characterized. However, it is unlikely that a change from leucine to isoleucine which would require a CUG to AUC double mutation, or from glutamine to isoleucine which would require an CAG to AUC triple mutation, is due to selection by passing in eggs or MDCK cells as isoleucine was found in viruses passaged using both systems.

Amino acid changes in the receptor binding domain of the HA protein may affect the binding specificity of the virus to host cell sialic acid residues, thus, resulting in pathogenic changes of epidemic influenza viruses. Furthermore,

decreased affinity of the HA molecules to chicken red blood cells, which has been observed with recent H3 viruses, may be explained by subsequent characterisation of the cell receptor binding specificity of recent human H3N2 influenza viruses.

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Received August 21, 1995