Jpn. J. Vet. Res. 48(4): 177-186(2001)

FULL PAPER

Antigenic Variation Among Equine H 3 N 8 Influenza Virus Hemagglutinins

Hiroichi Ozaki, Ayako Shimizu-Nei, Shigeo Sugita[1], Takeo Sugiura[1], Hiroshi Imagawa[1], and Hiroshi Kida*

(Accepted for publication : January 11, 2001)

Abstract

To provide information on the antigenic variation of the hemagglutinins (HA) among equine H 3 influenza viruses, 26 strains isolated from horses in different areas in the world during the 1963−1996 period were analyzed using a panel of monoclonal antibodies recognizing at least 7 distinct epitopes on the H 3 HA molecule of the prototype strain A/equine/Miami/ 1 /63 (H 3 N 8). The reactivity patterns of the virus strains with the panel indicate that antigenic drift of the HA has occurred with the year of isolation, but less extensively than that of human H 3 N 2 influenza virus isolates, and different antigenic variants co-circulate. To assess immunogenicity of the viruses, antisera from mice vaccinated with each of the 7 representative inactivated viruses were examined by neutralization and hemagglutination-inhibition tests. These results emphasize the importance of monitoring the antigenic drift in equine influenza virus strains and to introduce current isolates into vaccine. On the basis of the present results, equine influenza vaccine strain A/equine/Tokyo/ 2 /71 (H 3 N 8) was replaced with A/equine/La Plata/ 1 /93 (H 3 N 8) in 1996 in Japan. The present results of the antigenic analysis of the 26 strains supported the results of a phylogenetic analysis[5], that viruses belonging to each of the Eurasian and American equine influenza lineages have independently evolved. However, the current vaccine in Japan consists of two American H 3 N 8 strains ; A/equine/Kentucky/ 1 /81 and A/equine/La Plata/ 1 /93. It is also therefore recommended that a representative Eurasian strain should be included as a replacement of A/equine/Kentucky/ 1 /81.

Department of Disease Control, Hokkaido University Graduate School of Veterinary Medicine, Sapporo060-0818,Japan

[1]*Division of Molecular Biology, Tochigi Laboratory of Equine Research Institute, Japan Racing Association, Tochigi329-0412,Japan*

*Corresponding author

Department of Disease Control,
Hokkaido University Graduate School of Veterinary Medicine,
Sapporo060-0818, Japan
Phone : (+81-11) 706-5207 Fax : (+81-11) 706-5273.
E-mail : kida@vetmed.hokudai.ac.jp

Introduction

Equine influenza is one of the most important contagious respiratory diseases of horses. A/equine/Plague/56 (H 7 N 7) was the first influenza virus isolated from a horse in Czechoslovakia in 1956[23]. In 1963, a virus of different subtype, A/equine/Miami/1/63 (H 3 N 8) (Eq/Miami/63), was isolated from a race horse in Miami[25]. Influenza epidemics caused by H 3 N 8 viruses have occurred continuously worldwide. H 7 N 7 viruses, on the other hand, have not been isolated since the outbreaks in Mongolia and Yugoslavia in 1980. However, serological studies suggest that H 7 N 7 influenza virus is still circulating among horses in some areas of the world[26].

It is believed that equine H 3 influenza viruses originated from avian influenza viruses. A phylogenetic analysis indicated that, since 1987, equine H 3 N 8 influenza viruses have been diverging into two lineages: the Eurasian and the American lineages[4]. Antigenic and genetic variations of equine influenza viruses are less extensive than those of human influenza viruses[6,8]. On the other hand, the HA genes of H 3 influenza viruses isolated from the case in China in1989, belonged to a different lineage from that of known equine H 3 influenza viruses, indicating that the HA gene of the isolate was newly introduced from an avian source[5].

Bryans *et al*. (1966)[3] established an equine influenza vaccine containing two prototype strains, A/equine/Prague/1/57 (H 7 N 7) and Eq/Miami/63 (H 3 N 8). Since antigenic variation of equine H 3 N 8 viruses has been detected, although the extent of the variation was limited[13,18,24], it has been recommended that the vaccine should contain current H 3 N 8 equine influenza virus strains. In addition, it was shown that H 3 N 8 influenza viruses antigenically different from the prototype Eq/Miami/63 were cocirculating in the horse population[6]. According to these findings, virus strains isolated from 1979 to 1981 were introduced into the equine influenza vaccine in Japan. In countries in Europe and America, two H 3 N 8 viruses, A/equine/Miami/1/63 and A/equine/Fontainebleau/79 are used as vaccine strains. However, it was revealed that equine influenza spread even to vaccinated herds[2,15,17] and that viruses currently isolated were antigenically different from these vaccine strains[2,17].

In Japan, the outbreak of equine influenza caused by H 3 N 8 virus first occurred in December, 1971. The causative virus originated from five horses imported from New Zealand. Since then, vaccines containing Eq/Miami/63 and A/equine/Tokyo/2/71 (H 3 N 8) (Eq/Tokyo/71) had been used. In1986, Eq/Miami/63 strain was replaced with A/equine/Kentucky/1/81 (H 3 N 8) (Eq/Kentucky/81) in the vaccine. Although equine influenza has not occurred since the1971 outbreak in Japan, recent international horse trade raises the possibility of introduction of influenza virus into horses. In the present study, antigenicity and cross immunogenicity of influenza viruses including strains currently isolated from horses in the world were analyzed.

Materials and methods

Viruses

Influenza virus strains isolated in the USA, Japan, Switzerland, France, UK, Sweden, Chile, Spain, Argentina, and South Africa from 1963 to 1996 were used (Table 1). These viruses were propagated in the allantoic cavity of 10-to 11-day-old embryonated chicken egg at 35℃ for 48 hr and then purified from the allantoic fluid[12].

Monoclonal antibodies

Hybridoma cells producing monoclonal

Table 1. H 3 N 8 virus strains used in this study

Viruses		Place of origin	Referrence
A/equine/Miami/1/63	(Eq/Miami/63)	U.S.A	Waddell *et al.*, 1963
A/equine/Tokyo/2/71	(Eq/Tokyo/71)	Japan	Kumanomido *et al.*, 1972
A/equine/Kentucky/1/76	(Eq/Kentucky/76)	U.S.A	Hinshaw *et al.*, 1983
A/equine/Switzerland/2225/79	(Eq/Switzerland/79)	Switzerlan	Burrows *et al.*, 1981
A/equine/Fountainebleau/79	(Eq/Fountainebleau/79)	France	Piateau *et al.*, 1979
A/equine/California/1/80	(Eq/California/81)	U.S.A	Hinshaw *et al.*, 1983
A/equine/Kentucky/1/80	(Eq/Kentucky/81)	U.S.A	Hinshaw *et al.*, 1983
A/equine/Kentucky/1/81	(Eq/Kentucky/81)	U.S.A	Hinshaw *et al.*, 1983
A/equine/New York/500/83	(Eq/New York/83)	U.S.A	Kawaoka *et al.*, 1989
A/equine/Santiago/1/85	(Eq/Santiago/85)	Chile	Kawaoka *et al.*, 1989
A/equine/Cordoba/5/85	(Eq/Cordoba/85)	Spain	
A/equine/Santa Fe/1/85	(Eq/Santa Fe/85)	U.S.A	
A/equine/La Plata/1/85	(Eq/La Plata/85)	Argentina	
A/equine/Johannesberg/85	(Eq/Johannesberg/86)	South Africa	Kawaoka and Webster, 1989
A/equine/Kentucky/692/88	(Eq/Kentucky/88)	U.S.A	
A/equine/Kentucky/12717/90	(Eq/Kentucky/90)	U.S.A	
A/equine/Alaska/29759/91	(Eq/Alaska/91)	U.S.A	
A/equine/Kentucky/1/91	(Eq/Kentucky/91)	U.S.A	
A/equine/Idaho/37875/91	(Eq/Idaho/91)	U.S.A	
A/equine/Kentucky/1/92	(Eq/Kentucky/92)	U.S.A	
A/equine/Lambourn/22778/92	(Eq/Lambourn/92)	United Kingdom	
A/equine/Avesta/1/93	(Eq/Avesta/93)	Sweeden	
A/equine/La Plata/1/93	(Eq/La Plata/93)	Argentina	
A/equine/Kentucky/1/94	(Eq/Kentucky/94)	U.S.A	
A/equine/La Plata/1/95	(Eq/La Plata/95)	Argentina	
A/equine/La Plata/1/96	(Eq/La Plata/96)	Argentina	

antibodies to the HA of A/equine/Miami/1/63 (H 3 N 8) influenza viruses were prepared following fusion of myeloma cells SP 2/O Ag 14[22] with spleen cells from mice immunized with each virus[10]. The spleen donor was a BALB/c mouse immunized with two intraperitoneal injections of each of the purified influenza viruses (~10μg hemagglutinin protein) given 3 weeks apart. Fusion was carried out 2 - 3 months later, 4 days after an intravenous booster injection of the purified virus. The hybridoma cells producing antibodies were cloned in soft agar and grown intraperitoneally to produce ascites in BALB/c mice. Monoclonal antibodies to the HA of A/duck/Hokkaido/8/80 (H 3 N 8) and A/Aichi/2/68 (H 3 N 2) described previously[11] were confirmed to bind to the HA of A/equine/Miami/1/63 and used in this study.

Serological tests

Hemagglutination and hemagglutination -inhibition (HI) tests were performed by the microtiter methods[21]. Viral antigens for the HI test were prepared by disruption of the purified influenza viruses with 0.1% Tween 20 and an equal volume of ether for 30 min at 20℃. Enzyme-linked immunosorbent assay

(ELISA) was done as described previously[10]. Goat anti-mouse IgG antibody conjugated with horseradish peroxydase was used (BIO RAD) for ELISA. Neutralization tests were performed by a plaque reduction assay using MDCK cells[10].

Selection of antigenic variants

Antigenic variants were selected as described previously[11]. Briefly, monoclonal antibody was mixed with serially diluted parent virus, incubated for 30 min at 20℃, and the mixture was inoculated into 10-to 11-day old embryonated chicken eggs. Antigenic variants that grew in the presence of the monoclonal antibody were plaque-cloned on MDCK cells.

Sequencing of the HA genes

Viral RNA was extracted from purified virus[1]. cDNA was synthesized by using reverse transcriptase[9] and random 6 -base synthetic primer (Promega). The HA genes of equine H 3 influenza viruses were amplified by polymerase chain reaction (PCR)[19] using synthetic primers as follows; MIHA 20 : 5 '-TCTGTCAATCATGAAGACAACC- 3 ' and MIHAR1058 : 5' -GCTATTGCTCCAAAGATTCCT CTG-3' . Direct sequencing of the PCR products were performed with the above primers and H 3 -785R : 5' -TATTGTCAAATAGATGC TTA-3' [20].

Immunization of mice

The potency of the vaccine was determined by titrating the neutralizing antibodies produced in the immunized mice by egg method. Briefly, four-week-old male ddY mice (Nippon SLC) were intraperitoneally immunized with 0. 5ml of the five-fold serial dilutions of 0. 1%formalin-inactivated vaccine in PBS. Three weeks later, sera were obtained from the mice and pooled. Each pool was diluted twofold and subjected to HI and neutralization tests. Equal volumes of each dilution and the $10^{4.0}$ EID_{50} of challenge virus suspension were mixed and inoculated in embryonated chicken eggs.

Animal experimentations were performed according to the Guidelines for Animal Experimentation at Hokkaido University Graduate School of Veterinary Medicine.

Results

Characterization of monoclonal antibodies to A/equine/Miami/ 1 /63 (H3N8) influenza virus HA

In this study, we prepared 10 monoclonal antibodies which reacted to Eq/Miami/63HA at high titers by ELISA (Table 2). Seven mon-

Table 2 . Characterization of monoclonal antibodies to Eq/Miami/63 HA molecule

MAbs	ELISA titers (x100)	HI titers		Neutralizing antibody titers	Isotype of immunoglobulin
		intact virus	HA rosset		
M 6 / 1	1, 024	1, 280	3, 200	2, 400	IgG 2 a
M 1 / 4	65, 536	25, 600	25, 600	5, 600	IgG 1
D58/ 2	65, 536	2, 560	3, 200	400	IgG 2 a
A13/ 1	4, 096	1, 280	1, 600	400	IgG 2 a
A32/ 2	16, 384	25, 600	51, 200	25, 600	IgG 3
M18/ 1	16, 384	<80	1, 280	2, 100	IgG 1
M12/ 3	16, 384	<80	1, 600	800	IgG 1
D22/ 3	4, 096	<80	<80	<100	IgG 2 a
M 9 / 2	1, 024	3, 200	3, 200	1, 400	IgG 3
M41/ 2	4, 096	25, 600	25, 600	16, 900	IgG 3

oclonal antibodies inhibited hemagglutination of intact virus but the other three antibodies (D22/3, M12/3, and M18/1) did not. Since it was shown that some of monoclonal antibodies to avian influenza virus HA failed to inhibit hemagglutination of intact virus but inhibited that of HA rossets[10], HA rossets of Eq/Miami/63 were prepared as antigen for HI test. As shown in Table 2, M12/3 and M18/1 inhibited the hemagglutination of the HA rossets. Nine monoclonal antibodies neutralized infectivity of Eq/Miami/63 but D22/3 did not.

Epitope mapping of the HA molecule of equine influenza virus with antigenic variants

To establish the epitope specificity of each monoclonal antibody, a series of antigenic variants were selected in the presence of the monoclonal antibodies and their reactivity patterns with each antibody were analyzed by ELISA (Table 3). The antigenic variants selected in the presence of each monoclonal antibody did not react with the homologous antibody. Out of 10 monoclonal antibodies, 3antibodies (M6/1, M18/1, and M12/3) recognized independent epitopes. While the other 7 antibodies recognized two distinct overlapping epitope regions. One was recognized with M1/4, A13/1, D58/2, and A32/2. The other was recognized with D22/3, M9/2, and M41/2. Therefore, five different antigenic sites were defined on the equine H3 HA using the present 10 monoclonal antibodies.

To define the location of the epitopes recognized by the panel of monoclonal antibodies on the HA molecule, the HA genes of the anti-

Table 3. Reactivity patterns of antigenic variants selected with monoclonal antibodies

	Reactivity of antigenic variants									
MAbs	vM6/1	vM1/4	vD58/2	vA13/1	vA32/2	vM18/1	vM12/3	vD22/3	vM41/2	vM9/2
M6/1	−									
M1/4		−	−							
D58/2		−	−	−						
A13/1			−	−						
A32/2				−	−					
M18/1						−				
M12/3							−			
D22/3								−	−	
M41/2								−	−	−
M9/2								−	−	−

Each of the monoclonal antibodies was tested by ELISA with the variants; no entry indicates ≧1,600, ; −indicates no binding (<400).

Table 4. Positions of nucleotide substitutions on the HA genes of variants selected with monoclonal antibodies and the deduced amino acid

	Nucleotide change		Amino acid change	
Antigenic variants	Location	Nucleotide	Location	Amino acid
vM6/1	595	G→T	198	Asn→Asp
vM1/4	424	C→A	141	Arg→Met
vA13/1	399	T→C	133	Asn→Asp
vA32/2	472	G→A	157	Ser→Tyr
vM41/2	495	T→C	165	Asn→Asp
vM9/2	496	T→G	165	Asn→Thr

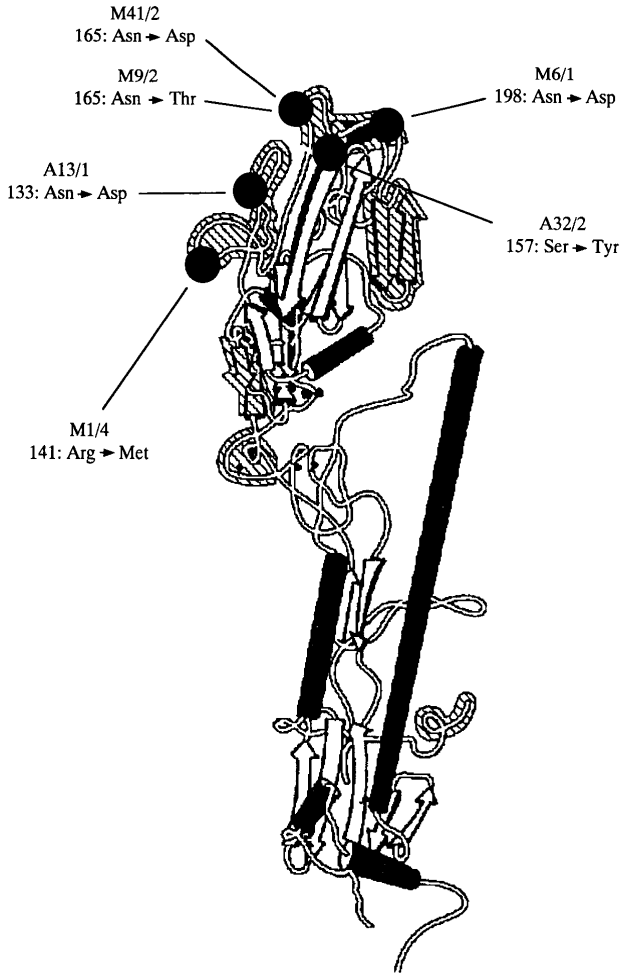

Fig. 1. Positions of amino acid substitution in the HA of monoclonal variants of A/equine/Miami/1/63 (H3N8) placed on the A/Aichi/2/68 (H3N2) HA (Wiley *et al.*, 1981) [27]. •indicates the position of amino acid substitution in the HA of monoclonal variant.

genic variants selected with the antibodies were sequenced. The deduced amino acid sequence of the HA molecule of antigenic variants revealed a single amino acid substitution in each variant (Table 4). These substitutions were located in the regions corresponding to the antigenic sites A (M1/4 and A13/1), AB (A32/2), and B (M6/1, M41/2, and M9/2) on the A/Aichi/2/68 (H3N2) HA molecule[28] (Fig. 1).

Antigenic comparison of H3N8equine influenza viruses

Antigenecities of 26 equine influenza viruses including vaccine strains and recent isolates were analyzed by ELISA using the panel of monoclonal antibodies to the H3 HA (Table 5). The results indicate that equine influenza viruses showed antigenic drift with the year of isolation. Antigenic analysis of the strains with the panel of monoclonal antibodies also revealed that antigenicity of recent isolates was different from that of Eq/Tokyo/71 and Eq/Kentucky/81.

Comparison of immunogenicity of vaccine strains and recent isolates from horses

We examined the cross immunogenicity between the recent epidemic and vaccine strains according to the vaccine standard[14]. The sera of mice vaccinated with each of the 7 strains showed high HI antibody titers against the homologous strain. Mice immunized with Eq/Tokyo/71 showed high HI antibody titer to Eq/Alaska/91 and Eq/Idaho/91, but significantly lower titers to Eq/Kentucky/92. On the other hand, mice immunized with Eq/Kentucky/81 showed high HI antibody titers to Eq/Idaho/91, but significantly lower titers to Eq/Alaska/91 and Eq/Kentucky/92 (data not shown). These sera were then examined by neutralization test (Table 6). The sera of mice immunized with the previous vaccine strain (Eq/Tokyo/71 or Eq/Kentucky/81) neutralized infectivity of Eq/Idaho/91and Eq/Alaska/91 ($>5^{4.0}$), but not that of Eq/Kentucky/92 ($<5^{1.5}$). The sera of mice immunized with ether Eq/Idaho/91 or Eq/Alaska/91 neutralized infectivity of the homologous strains ($>5^{4.5}$), but not that of Eq/Kentucky/92 ($<5^{1.5}$). In contrast, when mice were immunized with Eq/Kentucky/92, the sera of these mice neutralized infectivity not only of resent strains ($5^{4.0}$, $5^{4.5}$, and $5^{4.25}$, respectively) but also of vaccine strains ($5^{3.5}$and $5^{4.5}$, respectively). The results indicate that the vaccine does not induce anti-Eq/Kentucky/92 neutralizing anti-

Table 5. Reactivity of MAbs to the equine H 3 influenza viruses

Viruses	M6/1	M1/4	D58/2	D22/3	M18/1	A13/1	A32/2	M12/3	M9/2	M41/2
Eq/Miami/63	+	+	+	+	+	+	+	+	+	+
Eq/Tokyo/71	−	−	+	−	+	−	−	+	−	+
Eq/Kentucky/76	−	−	−	+	+	+	+	+	+	+
Eq/Switzerland/79	+	+	+	+	+	+	+	+	+	+
Eq/Fountainebleau	−	−	−	−	+	−	−	+	−	−
Eq/California/80	−	−	−	−	+	+	+	+	+	+
Eq/Kentucky/80	−	−	−	−	+	−	−	+	+	+
Eq/Kentucky/81	−	−	−	+	+	+	+	+	+	+
Eq/New York/83	−	−	−	−	−	−	−	+	+	+
Eq/Santiago/85	−	−	−	−	+	−	+	+	+	+
Eq/Cordoba/85	−	−	−	−	−	−	+	+	+	+
Eq/Santa Fe/85	−	−	−	−	+	−	+	+	−	−
Eq/La Plata/85	−	−	−	−	−	−	−	+	+	+
Eq/Johannesberg/86	−	−	−	−	+	−	−	+	+	+
Eq/Kentucky/88	−	−	−	−	−	−	−	+	+	+
Eq/Kentucky/90	−	−	−	−	−	−	+	+	+	+
Eq/Alaska/91	−	−	−	−	+	−	−	+	+	+
Eq/Kentucky/91	−	−	−	−	−	−	+	+	+	−
Eq/Idaho/91	−	−	−	−	−	−	−	−	+	+
Eq/Kentucky/92	−	−	−	−	−	−	+	−	+	+
Eq/Lambourn/92	−	−	−	−	−	+	+	+	+	+
Eq/Avesta/93	−	−	−	−	−	+	+	+	+	+
Eq/La Plata/93	−	−	−	−	−	−	−	−	+	+
Eq/Kentucky/94	−	−	−	−	−	−	−	−	+	+
Eq/La Plata/95	−	−	−	−	−	−	−	+	+	+
Eq/La Plata/96	−	−	−	−	−	−	−	+	+	+

Each of the monoclonal antibodies was tested with equine H 3 viruses by ELISA; + means significant binding to the virus (≧ 1 : 1,600), − indicates no binding to the viruses (< 1 : 400).

Table 6. Virus neutralization with vaccinated mouse sera

	Neutralization titers[a] of mouse sera immunized with the following vaccines (log_5)						
Viruses	Vaccine[b]	Eq/Miami/63	Eq/Tokyo/71	Eq/Kentucky/81	Eq/Idaho/91	Eq/Alaska/91	Eq/Kentucky/92
Eq/Miami/63	2.50	4.25	<1.50	1.85	<1.50	<1.50	<1.50
Eq/Tokyo/71	4.50	2.50	>5.50	<1.50	4.00	>5.50	3.50
Eq/Kentucky/81	4.75	<1.50	<1.90	4.50	2.50	2.85	4.50
Eq/Idaho/91	4.25	<1.50	4.50	4.50	4.50	>5.25	4.00
Eq/Alaska/91	>5.17	<1.50	>4.50	4.00	4.50	>5.50	4.50
EqKentucky/92	<1.50	<1.50	<1.50	<1.50	<1.50	<1.50	4.25

[a] Neutralization titers are expressed as log_5 of the highest vaccine dilution to give protection $10^4 EID_{50}$ challenge virus.

[b] Constructed of inactivated Eq/Tokyo/71 (H 3 N 8), Eq/Kentucky/81 (H 3 N 8), and Eq/Newmarket/77 (H 7 N 7)

bodies but Eq/Kentucky/92induced neutralizing antibodies to both 1991 isolates and vaccine strains.

Discussion

In the present study, the antigenicity of the hemagglutinins of 26 equine H 3 N 8 influenza viruses isolated during the 1963 to 1996 period was analyzed with the panel of monoclonal antibodies. The present results of antigenic analysis indicate that antigenic drift has occurred in the equine H 3 virus HA since 1976 with the year of isolation. The extent of the antigenic drift of equine influenza virus is much more than that of duck influenza virus strains which was shown to be highly conserved, but less than that of human strains[7,11]. One of the vaccine strains, Eq/Kentucky/81reacted with 7 of the panel of monoclonal antibodies to the HA of Eq/Miami/63, while Eq/Kentucky/92, Eq/La Plata/93, Eq/Kentucky/94, Eq/La Plata/95, and Eq/La Plata/96reacted only with a few of the panel of monoclonal antibodies to the HA of Eq/Miami/63, indicating that recent isolates are antigenically different from the vaccine strain. Two recent Eurasian isolates, Eq/Lambourun/92and Eq/Avesta/93, showed different reactivity patterns from the above recent American strains, supporting the notion, based on the results of phylogenic analyses, that the Eurasian strains have evolved in a different lineage from that of the American strains.

The present results showed that neither vaccine strains (Eq/Tokyo/71 and Eq/Kentucky/81) nor the 1991 isolates tested induced sufficient neutralizing antibodies to Eq/Kentucky/92 in mice. In contrast, immunization with Eq/Kentucky/92 induced neutralizing antibodies not only to the 1991 isolates but also to the vaccine strains in mice. Thus, Eq/Kentucky/92 was more immunogenic to a wide range of equine H 3 influenza viruses than the vaccine strains. It was, therefore, recommended that Eq/Kentucky/92 or a recent American strain should be included in the vaccine, Eq/Tokyo/71being replaced with Eq/La Plata/93 in Japan in 1996.

Phylogenetic analysis revealed that there are 2 European and American lineages of equine influenza viruses[4,16]. It has been described that even vaccinated horses were infected with influenza virus belonging to the American lineage in Europe where the vaccine is prepared only from Eurasian strains[2,15,17]. Equine influenza vaccine currently used in Japan contains two H 3 N 8 strains, Eq/Kentuckey/81 and Eq/La Plata/93, both of which belong to the American lineage. On the basis of the present results of antigenic and phylogenetic analyses, it is recommended that a Eurasian strain (Eq/Lambourn/92or Eq/Avesta/93) be included as a replacement of Eq/Kentucky/81 in the equine influenza vaccine.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Equine Research Institute, Japan Racing Association

References

1) Bean, W. J., SriramM, G. and Webster, R. G. 1980. Erectrophoretic analysis of iodine-labeled influenza virus RNA segments. *Anal. Biochem.*, 102 : 228-232.

2) Binns, M. M., Daly, J. M., Chirnside, E. D., Mumford, J. A., Wood, J. M., Richards, C. M. and Daniels, R. S. 1993. Genetic and antigenic analysis of an equine influenza H 3 isolate from the 1989epidemic. *Arch. Virol.*, 130 : 33-43.

3) Bryans, J. G., Doll, E. R., Wilson, J. C. and McCollum, W. H. 1966. Immunization for equine influenza. *J. Am. Vet.*

Med. Assoc., 148 : 413-417.

4) Daly, J. M., Lai, A. C., Binns, M. M., Chambers, T. M., Barrandeguy, M., and Mumford, J. A. 1996. Antigenic and genetic evolution of equine influenza A viruses. *J. Gen. Virol.*, 77 : 661-671.

5) Guo, Y., Wang, M., Kawaoka, Y., Gorman, O., Ito, T., Saito, T. and Webster, R. G. 1992. Characterization of a new avian-like influenza A virus from horses in China. *Virology*, 188 : 245-255.

6) Hinshaw, V. S., Naeve, C. W., Webster, R. G., Douglas, A., Skehel, J. J. and Bryans, J. 1983. Analysis of antigenic variation in equine 2 influenza A viruses. *Bull. WHO*, 61 : 153-158.

7) Kawaoka, Y., Bean, W. J. and Webster, R. G. 1989. Evolution of the hemagglutinin of equine influenza viruses. *Virology*, 169 : 283-292.

8) Kawaoka, Y. and Webster, R. G. 1989. Origin of the hemagglutinin on A/equine/Johannesburg/86 (H 3 N 8) : the first known equine influenza out break in South Africa. *Arch. Virol.*, 160 : 159-164.

9) Kawasaki, E. S., 1980. Amplification of RNA. In: *PCR Protocols*, A Guide to Methods and Amplifications, pp. 21-27, Innus, M. A., Gelefand, D. H., Shinsky, J. J. and White, T. J. eds., Academic Press, New York.

10) Kida, H., Brown, L. E. and Webster, R. G. 1982. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/ 1 /80 (H 7 N 7) influenza virus. *Virology*, 122 : 38-47.

11) Kida, H., Kawaoka, Y., Naeve, C. W., and Webster, R. G. 1987. Antigenic and genetic conservation of H 3 influenza in wild ducks. *Virology*, 159 : 109-119.

12) Kida, H. and Yanagawa, R. 1979. Isolation of a new avian paramyxovirus from rock pigeon (Columba livia). *Zentralbl. Bakteriol. mikrobiol. Hyg.*, A 245 : 421-428.

13) Kono, Y., Ishikawa, K., Fukunaga, Y. and Fujino, M. 1972. The first outbreak of equine influenza in Japan. *Anim. Health Quarterly*, 12 : 183-187.

14) Kudo, H., Ohde, H., Tamanaka, T., Ohtsuka, T. and Matsumoto, H. 1986. Hemagglutinination-inhibiting antibodies to equine influenza viruses in Japanese horses and antigenic variation of the viruses. *Kitasato Arch. Exp. Med.*, 59 : 49-55.

15) Livesay, G. J., Yadav, M. P., O'neill, T. and Mumford, J. A. 1991. The outbreak of equine influenza (H 3 N 8) in Britain in1989. In: *Equine Infectious Diseases VI* : Proceedings of 6 th International Conference on Equine Iinfectious Disease, pp. 321, eds., Plowright, W., Rossdale, P. D. and Wade, J. F. R and E Publications Ltd., Newmarket.

16) Manuguerra, J. C., Zientara, S., Sailleau, C., Rousseaux, C., Gicquel, B., Rijks, I. and Van der Werf, S. 2000. Evidence for evolutionary stasis and genetic drift by genetic analysis of two equine influenza H 3 viruses isolated in France. *Vet. Microbiol.*, 74 : 59-70.

17) Oxburgh, L., Berg, M., Klingeborn, B., Emmoth, E. and Linne, T. 1993. Equine influenza virus from the1991 Swedish epizootic shows major genetic and antigenic divergence from the prototype virus. *Virus Res.*, 28 : 263-272.

18) Pereira, H. G., Takimoto, S., Piegas, N. S. and Ribeiro Dovalle, L. A. 1972. Antigenic variation of equine (Heq 2 Neq 2) influenza virus. *Bull. WHO*, 47 : 465 -469.

19) Saiki, R. K., Sharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Srlich, H. A. and Arnheim, N. 1985. Enzymatic amolification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 230 : 1350-1354.

20) Sanger, F. S., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with shain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.*, 74 : 5463-5467.

21) Sever, J. L. 1962. Application of a microtechnique to viral serological investigations. *J. Immunol.*, 88 : 320-329.

22) Shulman, M., Wilde, C. D. and Kohler, G. 1978. A better line for making hybridomas secreting specific antibodies. *Nature*, 276 : 269-270.

23) Sovinova, O., Tumova, B., Pouska, F. and Nemec, J. 1958. Isolation of a virus causing respiratory disease in horses. *Acta. Virol.*, 2 : 52-61.

24) Tomson, G. R., Mumford, J. A., Spooner, P. R., Burrows, R. and Powell, D. G. 1977. The outbreak of equine influenza in England : January1976. *Vet. Rec.*, 100 : 465-468.

25) Waddel, G. H., Teigland, M. B. and Sigel, M. M. 1963. A new influenza virus associated with equine respiratory disease. *J. Am. Vet. Assoc.*, 143 : 587-590.

26) Webster, R. G. 1993. Are equine 1 influenza viruses still present in horses? *Equine Vet. J.*, 25 : 537-538.

27) Wiley, D. C., Wilson, I. A. and Skehel, J. J. 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature*, 289 : 373-378.