

Genetic Analyses of an H3N8 Influenza Virus Isolate, Causative Strain of the Outbreak of Equine Influenza at the Kanazawa Racecourse in Japan in 2007

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ABSTRACT. In August 2007, an outbreak of equine influenza occurred among vaccinated racehorses with Japanese commercial equine influenza vaccine at Kanazawa Racecourse in Ishikawa prefecture in Japan. Apparent symptoms were pyrexia (38.2–41.0°C) and nasal discharge with or without coughing, although approximately half of the infected horses were subclinical. All horses had been shot with a vaccine that contained two inactivated H3N8 influenza virus strains [A/equine/La Plata/93 (La Plata/93) of American lineage and A/equine/Avesta/93 (Avesta/93) of European lineage] and an H7N7 strain (A/equine/Newmarket/1/77). Influenza virus, A/equine/Kanazawa/1/2007 (H3N8) (Kanazawa/07), was isolated from one of the nasal swab samples of diseased horses. Phylogenetic analysis indicated that Kanazawa/07 was classified into the American sublineage Florida. In addition, four amino acid substitutions were found in the antigenic sites B and E in the HA1 subunit protein of Kanazawa/07 in comparison with that of La Plata/93. Hemagglutination-inhibition (HI) test using 16 serum samples from recovering horses revealed that 1.4- to 8-fold difference in titers between Kanazawa/07 and either of the vaccine strains. The present findings suggest that Japanese commercial inactivated vaccine contributed to reducing the morbidity rate and manifestation of the clinical signs of horses infected with Kanazawa/07 that may be antigenically different from the vaccine strains.

KEY WORDS: antigenic analysis, equine influenza virus, genetic analysis, Japan, racehorses.

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Influenza A virus, a member of family Orthomyxoviridae, is a highly infectious respiratory pathogen of birds and mammals including humans, horses and pigs. Equine influenza is caused by influenza A virus and characterized by acutely spreading signs that include pyrexia, anorexia, coughing, dyspnoea and a nasal discharge in the horses [39]. Two distinct subtypes of influenza virus, H7N7 and H3N8, have been recognized in the horse. The first equine influenza virus isolated in Czechoslovakia in 1956 was an H7N7 subtype [31]. The H3N8 subtype virus was, then, isolated from a racehorse in Miami in 1963 [34]. Although the H7N7 subtype virus has not been isolated from the horse for over three decades [35], the H3N8 viruses are prevalent among horses worldwide. In Japan, the first outbreak of equine influenza was recognized in Tokyo Race Course in December, 1971 [1, 14], and the causative virus, A/equine/Tokyo/71 (H3N8), was isolated [12]. Since then, no occurrence of equine influenza has been reported in Japan.

Recently, two genetically and antigenically distinct evolutionary lineages of H3N8 equine influenza virus, designated as American and European lineages, have been demonstrated [6, 15]. In addition, recent American lineage was further subdivided into three sublineages designed as ‘Argentina’, ‘Kentucky’, and ‘Florida’ [15, 16]. These lineages are co-circulating without geographic barriers [16]. Japanese commercial inactivated vaccine contains two

H3N8 strains, A/equine/La Plata/93 (La Plata/93) of American lineage and A/equine/Avesta/93 (Avesta/93) of European lineage and an H7N7 strain (A/equine/Newmarket/1/77), and is used widely for racing and riding horses [23]. Outbreaks of equine influenza among vaccinated horses have been reported in some countries [17, 22, 27]. The factors involved in such a vaccine failure are thought to variable vaccine potency, poor response of horses to vaccination, antigenic drift of equine influenza virus, or a combination of these [22].

In 2007, equine influenza outbreaks occurred in Japan including Kanazawa Racecourse in Ishikawa prefecture for the first time in 36 years. Equine influenza virus, designed as A/equine/Kanazawa/1/2007 (Kanazawa/07), was isolated from infected racehorse in the Kanazawa Racecourse. All horses in the Kanazawa Racecourse had been shot with Japanese commercial inactive vaccine. In the present study, to characterize the causative H3N8 equine influenza virus of the outbreak in the Kanazawa Racecourse, the genomes of Kanazawa/07 were compared to those of laboratory strains including vaccine strains and field isolates. In addition, to analyze the antigenic property of Kanazawa/07, the convalescent sera from the horses were tested with the isolate and the vaccine strains.

MATERIALS AND MEHODS

Detection of viral antigen and RNA: During August 17–21 in 2007, nasal swabs were taken from all 548 racehorses

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in the Kanazawa Racecourse. ESPLINE INFLUENZA A&B-N kit (Fujirebio, Tokyo, Japan) to detect influenza A and B specific nucleoproteins [2] was used for screening test. RNA was extracted from the nasal swabs positive in the screening test using the Trizol LS Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to instructions of the manufacturer.

PCR primers and RT-PCR: To amplify the partial genes of the hemagglutinin (HA), neuraminidase (NA), and six internal proteins (PB2, PB1, PA, NP, M and NS), we used 10 primer-pairs; HA1-specific primer-pair, sense (5'-AGC AAA AGC AGG GGA TAT TTC TG-3') and anti-sense (5'-GCT ATT GCT CCA AAG ATT C-3'), described by Newton *et al.* [22], and 9 primer-pairs, which were designed for this study, are shown in Table 1.

RT-PCR was carried out with a RNA PCR Kit (AMV) Ver.3.0 (Takara bio Inc., Shiga, Japan) according to the instructions of the manufacturer. The cycling conditions of PCR were as follows: 95°C for 5 min, then 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final incubation of 72°C for 10 min.

Nucleotide sequencing and phylogenetic analysis: The RT-PCR products were purified with a High Pure PCR product Purification Kit (Roche, Mannheim, Germany). At least two independent PCR products were purified and used as templates in sequencing reactions (Dye 6 Terminator Cycle Sequencing Chemistry Protocol; Beckman Coulter, Fullerton, CA, U.S.A.) and analyzed on a Multi Capillary DNA Analysis System CEQ2000 (Beckman Coulter). After initial forward and reverse sequencing with the amplification primers, subsequent sequencing primers were designed from the derived sequences. The sequences were first subjected to a multiple sequence alignment using GENETYX-MAC Version 10.1 (GENETYX, Tokyo, Japan). Evolutional distance was estimated by the three-parameter method [13]. The robustness of the grouping was tested by bootstrap re-sampling [8]. Neighbor-joining trees [28] with

1,000 replicates were used in the bootstrap analysis with Clustal W [32]. TreeView version 1.6 [25] was used for displaying trees.

Virus isolation: Nasal swabs which were RT-PCR positive were soaked into 1.3 ml Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing antibiotics and vortexed. After centrifuge at 3,000 rpm for 5 min, the supernatants were inoculated into the allantoic cavities of 10–12 day-old embryonated chicken eggs. The eggs were harvested after 3–4 days incubation at 35°C, and the allantoic fluid was tested for the presence of virus by hemagglutination assay with 0.5% chicken red blood cell in physiological salt solution.

Serological test: Hemagglutination-inhibition (HI) tests were performed by the microtiter methods [24]. Kanazawa/07, La Plata/93, and Avesta/93, were propagated in the allantoic cavity of chicken embryos, and the yielded allantoic fluids were used as antigens. To obtain 32 to 64 HA titers, sequential passages in embryonated chicken egg were made. Sera were treated with periodic acid, diluted tenfold, and subjected to HI tests. The HI antibody titer was expressed as the reciprocal of the highest serum dilution that inhibited hemagglutination of the antigen. HI tests were done at least three times.

RESULTS

Clinical examination and virus detection: In the middle of August 2007, an outbreak of equine influenza occurred among vaccinated racehorses of the Kanazawa Racecourse in Ishikawa prefecture after introduction of 13 horses from Miho and Ritto training centers and Fukushima and Niigata Racecourses belonging to Japan Racing Association in August 1–17. All horses had been shot with the Japanese commercial vaccine during the previous three months. Virus detection using rapid diagnostic kits, from nasal swabs of all racehorses in the Kanazawa Racecourse

Table 1. Primers prepared for this study to amplify eight gene segments of H3N8 equine influenza virus

Segments	Forward primers (5' to 3')	Reverse primers (5' to 3')
PB2	466F: GCT AAA GAA GCA CAA GAT G	938R: GCT TTG CAT ATA TCC ACA GC
PB1	275F: GGG TAT GCA CAA ACA GAT TG	1246R: GCA TGT TGA ACA TGC CCA TC
PA	38F: GTG CGA CAA TGC TTC AAT CC	599R: GTC TCT TCG CCT CTC TCG
HA	HA837F: ACA GTA ATG GCA ACT TAG TTG CAC	HA1747R: AGT AGA AAC AAG GGT GTT
NP	NP665F: GTG CAA TAT CCT CAA AGG G	1456R: TTG TCT CCG AAG AAA TAA GA
NA	NA25F: AGC AAA AGC AGG AGT TTA	NA703R: AGT CTC CTT TAA TGC AGG TGC GTG
	NA564F: CGG TGG CAT GGT CAG CAA CAG CAT	NA1444R: AGT AGA AAC AAG GAG TT
M	19F: GTC GAA ACG TAC GTT CTC TC	938R: TGA CCA TCG TCA ACA TCC AC
NS	498F: GGC GAA ATC TCA CCA TTA CC	840R: GAA ACA AGG GTG TTT TTT TAG

Table 2. Comparison of nucleotide sequences of A/equine/Kanazawa/1/2007 (H3N8) with published equine influenza virus sequences using BLAST web-based program

Gene segments (length*)	Determined sequences			Most similar strain(s) (Accession No.)	Similarity (%)
	Size (bp)	position	Accession No.		
PB2 (2,341)	468	499–966	AB370081	California/03 (DQ124182)	99.6
PB1 (2,341)	807	329–1,135	AB370082	Massachusetts/03 (DQ124181)	99.5
				Wisconsin/03 (DQ222919)	
				Florida/03 (DQ124155)	
PA (2,151)	576	18–593	AB370083	Wisconsin/03 (DQ222918)	98.8
HA (1,765)	1,720	24–1,748	AB369862	Wisconsin/03 (DQ222913)	99.1
NP (1,520)	738	699–1,436	AB370084	Wisconsin/03 (DQ222915)	99.3
				Massachusetts/03 (DQ124169)	
				Wisconsin/03 (DQ222914)	
NA (1,460)	1,418	26–1,446	AB369863	Ohio/03 (DQ124188)	98.7
M (1,002)	869	55–923	AB370085	Massachusetts/03 (DQ124162)	99.0
				Florida/03 (DQ124160)	
				Kentucky/02 (DQ124185)	
NS (864)	362	477–838	AB370086	Massachusetts/03 (DQ124170)	99.4
				Florida/03 (DQ124161)	
				Florida/04 (DQ124153)	

*: Length of nucleotide sequences of A/Puerto Rico/8/34/Mount Sinai [PB2 (AF389115), PB1 (AF389116), PA (AF389117), NP (AF389119), M (AF389121) and NS (AF389122)] and A/duck/Ukuraina/1/1963 [HA (V01087) and NA (L06576)].
A/equine/California/191/03, California/03; A/equine/Massachusetts/213/03, Massachusetts/03; A/equine/Wisconsin/1/03, Wisconsin/03; A/canine/Florida/242/03, Florida/03; A/equine/Kentucky/5/02, Kentucky/02; A/canine/Florida/43/04, Florida/04.

revealed that 117 out of 548 nasal samples (21.3%) were positive. Fifty-five out of 117 (47.0%) horses, which were positive in the rapid diagnostic kits, showed pyrexia (38.2–41.0°C) and nasal discharge with or without coughing, although 62 (53.0%) were mild or subclinical. Subsequently, the positive samples in the antigen detection test were subjected to RT-PCR. One hundred and fifteen out of 117 samples (99.1%) were positive for RT-PCR using the HA1-specific primer-pair. Equine influenza virus, Kanazawa/07, was isolated after third passages in embryonated chicken eggs from RT-PCR positive swab sample of diseased horse.

Comparison of nucleotide sequences: The partial sequences of PB2, PB1, PA, HA, NP, NA, M and NS genes of the Kanazawa/07 were determined. These sequences were compared with published equine influenza virus sequence database using BLAST (<http://blast.ddbj.nig.ac.jp/top-j.html>) web-based program. The nucleotide sequences of the eight genes of the Kanazawa/07 showed high similarity (98.7–99.6%) to those of the Florida sublineage strains, such as A/equine/California/191/2003, A/equine/Massachusetts/213/2003, A/equine/Ohio/1/2003 (Ohio/03), A/equine/Kentucky/5/2002, A/canine/Florida/242/2003 and A/canine/Florida/43/2004 [5] and A/equine/Wisconsin/1/2003 (Wisconsin/03) [26] (Table 2). To define amino acid differences within HA1, multiple alignment of the deduced amino acid sequences of Kanazawa/07 and representative H3N8 strains including vaccine strains was performed (Fig. 1). Although Wisconsin/03 and Ohio/03, which belong to Florida sublineage, were defined as most similar strains to Kanazawa/07 using BLAST, Kanazawa/07 had two amino acid differences in the antigenic sites B1(Pro₁₆₂ to Ser₁₆₂) and B2(Gln₁₈₉ to Lys₁₈₉). Kanazawa/07 had 11 amino acid

differences from vaccine strain La Plata/93 belonging to the American lineage, and four of these changes were involved in antigenic sites [B1 (Asn₁₅₉ to Ser₁₅₉, Pro₁₆₂ to Ser₁₆₂), B2 (Asn₁₈₉ to Lys₁₈₉), and E2 (Val₇₈ to Ala₇₈)]. For vaccine strain Avesta/93 belonging to the European lineage, Kanazawa/07 had six amino acid differences in the antigenic sites B1 (Asn₁₅₉ to Ser₁₅₉, Pro₁₆₂ to Ser₁₆₂, Ile₁₆₃ to Thr₁₆₃), D2 (Glu₂₀₇ to Lys₂₀₇, Val₂₁₃ to Ile₂₁₃) and E2 (Val₇₈ to Ala₇₈).

Phylogenetic analysis: Phylogenetic tree constructed using the nucleotide sequences of the HA1 gene of H3N8 equine influenza viruses is shown in Fig. 2. Kanazawa/07 clustered in the sublineage Florida of American lineage with Wisconsin/03 and Ohio/03, although sublineage Florida was not supported confident bootstrap score (63.8%). The vaccine strains La Plata/93 and Avesta/93 were classified into the sublineage Argentina cluster of American lineage and European lineage cluster supported by 98.2% and 96.0% confident bootstrap values, respectively.

Serological test: Convalescent sera were collected from 16 horses that recovered from infection on September 11. These 16 horses were positive for the RT-PCR on August 17–21. HI tests were performed using convalescent sera and the causative influenza virus isolate and vaccine strains. Geometric means of HI titers for 16 sera against three viruses are shown in Table 3. High titers, 226–10,240 (geometric mean: 1,457.7) were observed to Kanazawa/07, while titers to La Plata/93 and Avesta/93 were 80–3,620 (388.9) and 57–2,560 (452.6), respectively. Four- to eight-fold differences of HI titers between Kanazawa/07 and the vaccine strains were observed by the sera from horses No.1, 5, 12, 15, and 16.

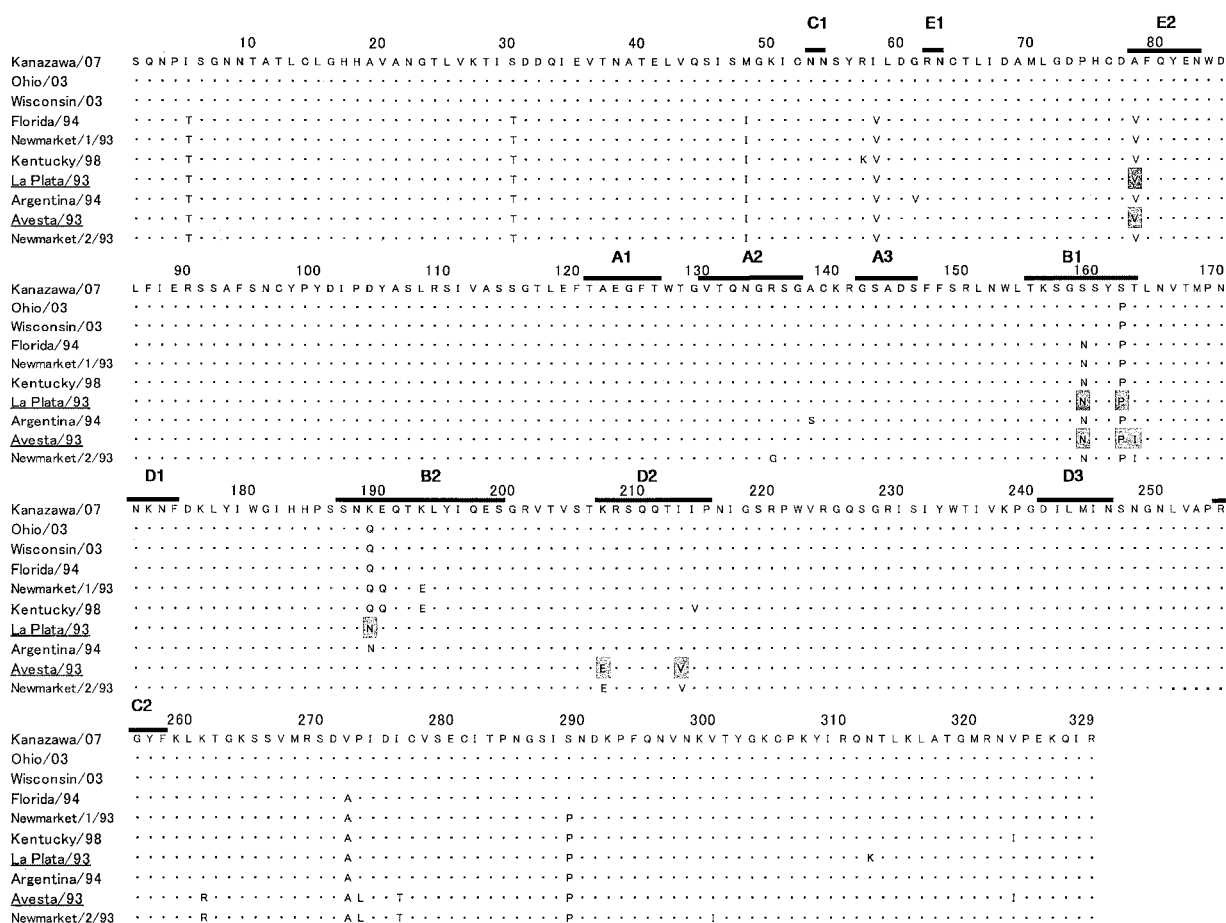


Fig. 1. Alignment of the deduced amino acid sequences from complete HA1 region of A/equine/Kanazawa/1/2007 (Kanazawa/07) with nine strains [A/equine/Ohio/1/2003 (Ohio/03), A/equine/Wisconsin/1/03 (Wisconsin/03), A/equine/Florida/1/94 (Florida/94), A/equine/Newmarket/1/93 (Newmarket/1/93), A/equine/Kentucky/1/98 (Kentucky/98), A/equine/La Plata/1/93 (La Plata/93), A/equine/Argentina/1/94 (Argentina/94), A/equine/Avesta/93 (Avesta/93), A/equine/Newmarket/2/93 (Newmarket/2/93)]. Residues identical to the consensus are indicated by dots. The positions of antigenic sites (A1, A2, A3, B1, B2, C1, C2, D1, D2, D3, E1 and E2) are indicated by line above the amino acid letter. Amino acid differences in the antigenic regions between A/equine/Kanazawa/1/2007 and the vaccine strains are shaded in gray. The vaccine strains are underlined.

DISCUSSION

An outbreak of equine influenza in the Kanazawa Racecourse on August 2007 persisted for about three weeks, and a significant economic loss was caused by the postponement of four race days and movement of horses for competition purpose. All horses had been shot with a Japanese commercial vaccine [23] during the previous three months, and booster vaccination was given at least once a year. The object of this study was to characterize the virus that caused outbreak among vaccinated horses in the Kanazawa Racecourse genetically and antigenically.

The present phylogenetic analysis and comparison of deduced amino acid sequences of the HA, and comparison of nucleotide sequences of HA, NA and six internal proteins using BLAST web-based program revealed that Kanazawa/07, causative virus of the outbreak, belongs to the sublin-

age Florida of the American lineage. On the other hand, the vaccine strain La Plata/93 belonging to the American lineage was clustered into Argentina sublineage. Since influenza epidemics are thought to be caused by the viruses that have escaped from the defense mechanisms of acquired immunity of the host by altering the antigenicity of the HA protein [19], amino acid sequence analysis of the HA protein is important for epidemiological investigation. From analyses of natural occurring and laboratory-selected antigenic variations, five antigenic sites on the HA protein have been identified with H3 influenza viruses, and antigenic sites were further subdivided into two to three regions (A1, A2, A3, B1, B2, C1, C2, D1, D2, D3, E1 and E2) [4, 21, 30, 33, 36, 38]. For human influenza A viruses, commonly at least four amino acid changes in two separate antigenic sites of the HA are required for significant antigenic drift [37]. Kanazawa/07 revealed four and six amino acid differences

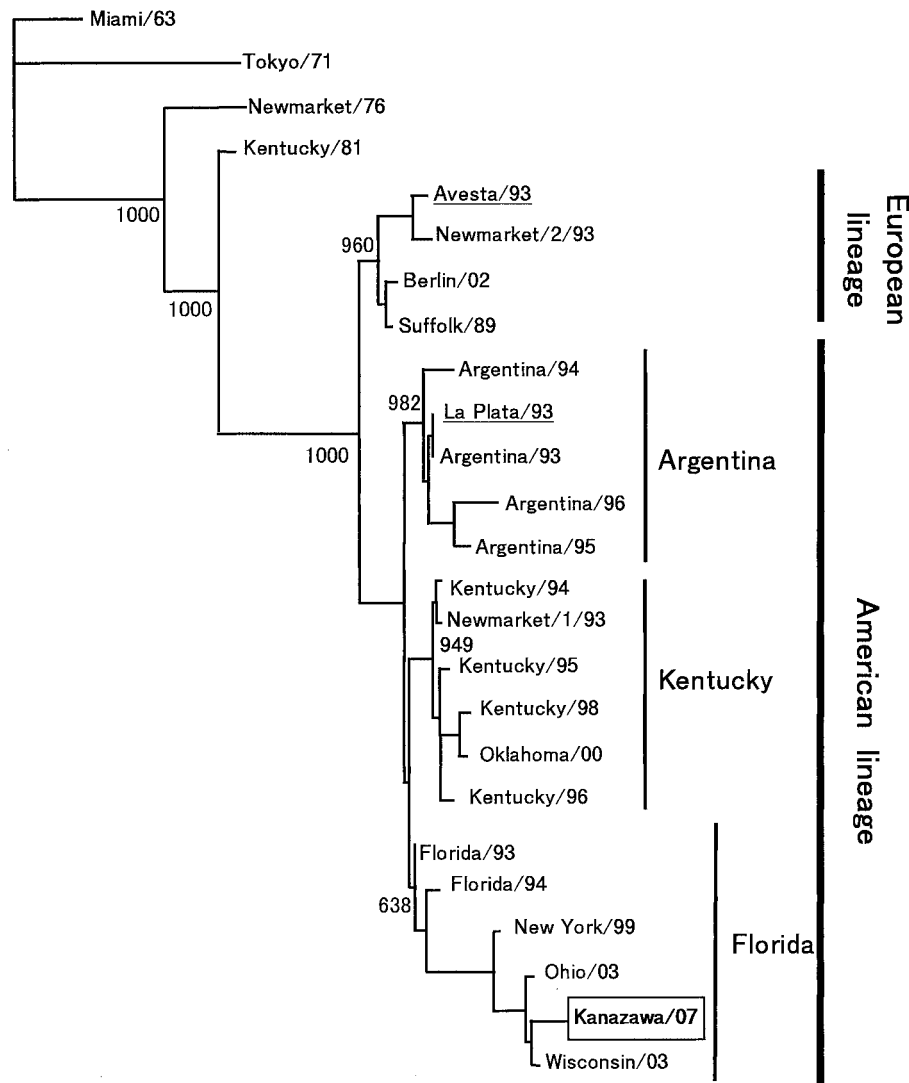


Fig. 2. Phylogenetic analysis of the partial nucleotide sequences of HA1 gene (1,032 bp) of A/equine/Kanazawa/1/2007 with 24 sequences obtained from the DDBJ/EMBL/GenBank databases. Evolutionary distance was estimated by the three-parameter method. The phylogenetic tree was constructed by the neighbor-joining method and bootstrap testing ($n=1,000$). The accession numbers of sequences are as follows: A/equine/Miami/1/1963 (Miami/63), M24719; A/equine/Tokyo/1971 (Tokyo/71), M24720; A/equine/Newmarket/1976 (Newmarket/76), M24722; A/equine/Kentucky/1/81 (Kentucky/81), U58195; A/equine/Avesta/93 (Avesta/93), Y14057; A/equine/Newmarket/2/93 (Newmarket/2/93), X85089; A/equine/Berlin/13/02 (Berlin/02), EF541429; A/equine/Suffolk/89 (Suffolk/89), X68437; A/equine/Argentina/2/94 (Argentina/94), AF197245; A/equine/La Plata/1/93 (La Plata/93), D30686; A/equine/Argentina/1/93 (Argentina/93), L39913; A/equine/Argentina/1/96 (Argentina/96), AF197246; A/equine/Argentina/1/95 (Argentina/95), AF197244; A/equine/Kentucky/1/94 (Kentucky/94), L39914; A/equine/Newmarket/1/93 (Newmarket/1/93), X85088; A/equine/Kentucky/9/95 (Kentucky/95), AF197247; A/equine/Kentucky/1/98 (Kentucky/98), AF197241; A/equine/Oklahoma/2000 (Oklahoma/00), AY273168; A/equine/Kentucky/1/96 (Kentucky/96), AF197248; A/equine/Florida/1/93 (Florida/93), L39916; A/equine/Florida/1/94 (Florida/94), AF197242; A/equine/New York/99 (New York/99), AY273167; A/equine/Ohio/1/2003 (Ohio/03), DQ124192; A/equine/Wisconsin/1/03 (Wisconsin/03), DQ222913. The vaccine strains are underlined.

Table 3. Hemagglutination-inhibition titers of convalescent sera to A/equine/Kanazawa/1/2007 (H3N8) and vaccine strains

Horse No. of convalescent serum	Virus antigens		
	Kanazawa/07	La Plata/93	Avesta/93
1	453	113	113
2	640	226	80
3	226	80	57
4	453	160	226
5	10240	1810	1810
6	5120	3620	1810
7	640	160	226
8	1280	320	640
9	1280	453	640
10	905	226	320
11	2560	640	1280
12	10240	1810	2560
13	1280	453	905
14	1280	453	905
15	3620	453	453
16	1810	226	226
Geometric means	1457.7	388.9	452.6

in the antigenic regions B and E, and B, D and E in comparison with the vaccine strains La Plata/93 and Avesta/93, respectively. These amino acid changes are within this criteria.

Antibody to the virus HA, that neutralizes virus infectivity by preventing attachment to cell receptors [9], or interfering with fusion of viral envelope with the endosomal membrane [11], is of prime importance in immunity [10]. Antigenic difference of equine influenza viruses within the American sublineages, the Kentucky and the Florida, was demonstrated using convalescent horse sera [16]. The horse sera reacted differently to viruses of one sublineage than the other, with a higher reactivity towards the viruses in the homologous sublineage, and a lower reactivity towards the viruses of the other sublineage [16]. However, antigenic difference between the Argentina sublineage and the Florida sublineage was not mentioned in this report. We, therefore, to evaluate antigenic difference between the causative virus and the vaccine strains, performed HI tests for the sera collected from convalescent 16 horses on September 11. Unfortunately, we could not collect acute phase sera due to too busy for prevention of epidemic, such as disinfection practice. Although the antibody titers of acute phase sera could not be determined, it seems unlikely that the antibody titers of vaccinated horses against the causative virus were higher than against the vaccine strains. Thus, in this study, antigenic difference between isolate and vaccine strains was considered by the antibody titers of convalescent sera. The geometric mean of HI titers against Kanazawa/07 was only 3.2- to 3.7-fold higher than vaccine strains. In addition, for 5 out of 16 sera, HI titers against Kanazawa/07 were four- to eight-fold higher than the vaccine strains. Thus, it is suggested that Kanazawa/07 may be antigenically distinct from the vaccine strains, La Plata/93 and Avesta/93. Further investigations, such as cross-HI and cross-neutralization

tests with antisera against Kanazawa/07 and the vaccine strains are needed. Antigenic site B on the HA molecule is the site for sequential amino acid substitutions found on the HAs of viruses in the Kentucky sublineage of the American lineage and thought to be a 'hot spot' for amino acid changes [15]. There were two amino acid differences in the antigenic sites B1 and B2 between Kanazawa/07 and Wisconsin/03 and Ohio/03 which were most similar strains belonging to the Florida sublineage. Sato *et al.* [29] suggested that an amino acid change of human H3 influenza virus in site B1 might act as a trigger for the emergence of a drift strain. Antigenic drift resulting from the accumulation of amino acid changes that abrogate antibody binding and, consequently, reduces immune level to subsequent infection by antigenic variants [10, 20]. Continuous surveillance of equine influenza virus in Japan to monitor the antigenic drift strains or novel strains is needed.

In previous outbreak of influenza caused by the European lineage strain among vaccinated horses in Hong Kong in 1992, although vaccination did not prevent 75% of the population from becoming infected, the clinical signs of about half of the infected horses were mild [27]. In the outbreaks of influenza caused by the Florida sublineage strain among both vaccinated and unvaccinated horses in UK, the clinical signs were milder among the vaccinated horses than among immunologically naive horses [22]. In the present outbreak in Kanazawa Racecourse, since about half of the infected horses did not show apparent clinical signs, vaccination with Japanese commercial inactivated vaccine was thought to contribute to reducing the morbidity rate and duration of the clinical signs. In these cases, though commendable efficiency of the vaccination was confirmed, the outbreaks of influenza caused by viruses antigenically different from vaccine strains could not prevent by the commercial vaccines. Since the antigenic disparity between the epidemic virus and vaccine strains is a significant factor for insufficient effect of inactivated vaccine, it has been suggested that equine influenza vaccines should be regularly updated [18]. However, due to the occurrence of antigenic variants and recirculation of old strain-like virus, so-called 'frozen evolution' virus [3, 7], it is important to caution in the selection of new vaccine strains and improve the vaccine efficacy or develop new vaccine strategies.

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REFERENCES

1. Akiyama, Y., Kumanomido, T., Hirasawa, K., Okuda, Y. and Tabuchi, E. 1972. Studies on outbreak of equine influenza in Japan in 1972—conditions of outbreak and investigation of hemagglutination inhibiting antibody. *Exp. Rep. Equine Health Lab.* 9: 10–28.
2. Bai, G. R., Sakoda, Y., Mweene, A. S., Fujii, N., Minakawa, H. and Kida, H. 2006. Improvement of a rapid diagnosis kit to detect either influenza A or B virus infections. *J. Vet. Med. Sci.* 68: 35–40.

3. Borchers, K., Daly, J., Stiens, G., Kreling, K., Kreling, I. and Ludwig, H. 2005. Characterisation of three equine influenza A H3N8 viruses from Germany (2000 and 2002): evidence for frozen evolution. *Vet. Microbiol.* **107**: 13–21.
4. Both, G. W., Sleight, M. J., Cox, N. J. and Kendal, A. P. 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. *J. Virol.* **48**: 52–60.
5. Crawford, P. C., Dubovi, E. J., Castleman, W. L., Stephenson, I., Gibbs, E. P., Chen, L., Smith, C., Hill, R. C., Ferro, P., Pompey, J., Bright, R. A., Medina, M. J., Johnson, C. M., Olsen, C. W., Cox, N. J., Klimov, A. I., Katz, J. M. and Donis, R. O. 2005. Transmission of equine influenza virus to dogs. *Science* **310**: 482–485.
6. Daly, J. M., Lai, A. C., Binns, M. M., Chambers, T. M., Barandeguy, M. and Mumford, J. A. 1996. Antigenic and genetic evolution of equine H3N8 influenza A viruses. *J. Gen. Virol.* **77**: 661–671.
7. Endo, A., Pecoraro, R., Sugita, S. and Nerome, K. 1992. Evolutionary pattern of the H3 haemagglutinin of equine influenza viruses: multiple evolutionary lineages and frozen replication. *Arch. Virol.* **123**: 73–87.
8. Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.
9. Fleury, D., Barrere, B., Bizebard, T., Daniels, R. S., Skehel, J. J. and Knossow, M. 1999. A complex of influenza hemagglutinin with a neutralizing antibody that binds outside the virus receptor binding site. *Nat. Struct. Biol.* **6**: 530–534.
10. Hay, A. J., Gregory, V., Douglas, A. R. and Lin, Y. P. 2001. The evolution of human influenza viruses. *Phil. Trans. R. Soc. Lond. B* **356**: 1861–1870.
11. Imai, M., Sugimoto, K., Okazaki, K. and Kida, H. 1998. Fusion of influenza virus with the endosomal membrane is inhibited by monoclonal antibodies to defined epitopes on the hemagglutinin. *Virus Res.* **53**: 129–139.
12. Kawaoka, Y., Bean, W. J. and Webster, R. G. 1989. Evolution of the hemagglutinin of equine H3 influenza viruses. *Virology* **169**: 283–292.
13. Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111–120.
14. Kono, Y., Ishikawa, K., Fukunaga, Y. and Fujino, M. 1972. The first outbreak of equine influenza in Japan. *Natl. Inst. Anim. Health Q.* **12**: 183–187.
15. Lai, A. C., Chambers, T. M., Holland, R. E. Jr., Morley, P. S., Haines, D. M., Townsend, H. G. and Barandeguy, M. 2001. Diverged evolution of recent equine-2 influenza (H3N8) viruses in the Western Hemisphere. *Arch. Virol.* **146**: 1063–1074.
16. Lai, A. C., Rogers, K. M., Glaser, A., Tudor, L. and Chambers, T. 2004. Alternate circulation of recent equine-2 influenza viruses (H3N8) from two distinct lineages in the United States. *Virus Res.* **100**: 159–164.
17. Martella, V., Elia, G., Decaro, N., Di Trani, L., Lorusso, E., Campolo, M., Desario, C., Parisi, A., Cavaliere, N. and Buonavoglia, C. 2007. An outbreak of equine influenza virus in vaccinated horses in Italy is due to an H3N8 strain closely related to recent North American representatives of the Florida sub-lineage. *Vet. Microbiol.* **121**: 56–63.
18. Mumford, J. A. and Wood, J. 1993. Conference report on WHO/OIE meeting: consultation on newly emerging strains of equine influenza. *Vaccine* **11**: 1172–1175.
19. Nakajima, S., Nobusawa, E. and Nakajima, K. 2000. Variation in response among individuals to antigenic sites on the HA protein of human influenza virus may be responsible for the emergence of drift strains in the human population. *Virology* **274**: 220–231.
20. Nakajima, K., Nobusawa, E., Nagy, A. and Nakajima, S. 2005. Accumulation of amino acid substitutions promotes irreversible structural changes in the hemagglutinin of human influenza HA3 virus during evolution. *J. Virol.* **79**: 6472–6477.
21. Nakajima, K., Nobusawa, E., Tonegawa, K. and Nakajima, S. 2003. Restriction of amino acid change in influenza A virus H3HA: comparison of amino acid changes observed in nature and *in vitro*. *J. Virol.* **77**: 10088–10098.
22. Newton, J. R., Daly, J. M., Spencer, L. and Mumford, J. A. 2006. Description of the outbreak of equine influenza (H3N8) in the United Kingdom in 2003, during which recently vaccinated horses in Newmarket developed respiratory disease. *Vet. Rec.* **158**: 185–192.
23. Ohta, M., Yamanaka, T., Yoshinari, M. and Matsumura, T. 2007. Antibody responses against American and European lineage strains of equine-2 influenza virus among racehorses inoculated with the new vaccine. *J. Equine Sci.* **18**: 117–120.
24. Ozaki, H., Shimizu-Nei, A., Sugita, S., Sugiura, T., Imagawa, H. and Kida, H. 2001. Antigenic variation among equine H3N8 influenza virus hemagglutinins. *Jpn. J. Vet. Res.* **48**: 177–186.
25. Page, R. D. 1996. TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**: 357–358.
26. Peek, S. F., Landolt, G., Karasin, A. I., Slack, J. A., Steinberg, H., Semrad, S. D. and Olsen, C. W. 2004. Acute respiratory distress syndrome and fatal interstitial pneumonia associated with equine influenza in a neonatal foal. *J. Vet. Intern. Med.* **18**: 132–134.
27. Powell, D. G., Watkins, K. L., Li, P. H. and Shortridge, K. F. 1995. Outbreak of equine influenza among horses in Hong Kong during 1992. *Vet. Rec.* **136**: 531–536.
28. Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
29. Sato, K., Morishita, T., Nobusawa, E., Tonegawa, K., Sakae, K., Nakajima, S. and Nakajima, K. 2004. Amino-acid change on the antigenic region B1 of H3 haemagglutinin may be a trigger for the emergence of drift strain of influenza A virus. *Epidemiol. Infect.* **132**: 399–406.
30. Smith, D. J., Lapedes, A. S., de Jong, J. C., Bestebroer, T. M., Rimmelzwaan, G. F., Osterhaus, A. D. and Fouchier, R. A. 2004. Mapping the antigenic and genetic evolution of influenza virus. *Science* **305**: 371–376.
31. Sovinova, O., Tumova, B., Pousky, F. and Nemec, J. 1958. Isolation of a virus causing respiratory diseases in horses. *Acta. Virol.* **1**: 52–61.
32. Thompson, J. D., Higgins, D. S. and Gibson, T. J. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680.
33. Underwood, P. A. 1982. Mapping of antigenic changes in the haemagglutinin of Hong Kong influenza (H3N8) strains using a large panel of monoclonal antibodies. *J. Gen. Virol.* **62**: 153–169.
34. Waddel, G. H., Teighland, M. B. and Siegel, M. M. 1963. A new influenza virus associated with equine respiratory disease. *J. Am. Vet. Med. Assoc.* **143**: 587–590.
35. Webster, R. G. 1993. Are equine 1 influenza viruses still

- present in horses? *Equine Vet. J.* **25**: 537–538.
36. 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.
37. Wilson, I. A. and Cox, N. J. 1990. Structural basis of immune recognition of influenza virus hemagglutinin. *Annu. Rev. Immunol.* **8**: 737–771.
38. Wilson, I. A., Skehel, J. J. and Wiley, D. C. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* **289**: 366–373.
39. Wilson, W. D. 1993. Equine influenza. *Vet. Clin. North Am. Equine Pract.* **9**: 257–282.