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Alternate circulation of recent equine-2 influenza viruses (H3N8) from two distinct lineages in the United States

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Abstract

Phylogenetic and antigenic analyses indicate that recent circulating equine-2 influenza viruses in the United States have been alternating between two genetic and antigenic distinct lineages since 1996. The evolution rates for these two lineages, the Kentucky and the Florida lineage, are very similar. For the earlier isolates in the Kentucky lineage, there are multiple and sequential nonsynonymous substitutions at antigenic sites B and D. However, there are no changes at any of these antigenic sites for KY98 and OK00. In the Florida lineage, except for NY99 with one amino acid substitution at antigenic site B, viruses in this lineage do not have nonsynonymous substitutions at any of the antigenic sites. The lack of amino acid substitutions at these antigenic sites suggests a mechanism other than immune selection is responsible for the maintenance of these viral lineages. Serological analysis indicates that these two lineages are antigenic distinct, and the pattern of reactivity of horse sera towards these two lineages alternates in consecutive years, parallel to the "switching" of virus lineage seen in the phylogenetic tree. This alternate circulation may play a role in the maintenance of these two lineages of equine-2 influenza virus.

Keywords: Equine influenza virus (H3N8); Evolution; Alternate circulation; Phylogenetic analysis; Hemagglutinin (HA)

1. Introduction

Equine influenza is a severe acute upper respiratory infection of the horse, and typical symptoms include pyrexia, dyspnoea, anorexia and coughing (Mumford et al., 1990; Wilson, 1993; Chambers et al., 1995a,b). The etiological agent, equine influenza virus, is a type A influenza virus, a member of family Orthomyxoviridae. It is the most common cause of respiratory infection in the horse (Mumford et al., 1998). Spread of equine influenza virus is very rapid and explosive (Powell et al., 1995). Equine influenza causes significant interruption of activities and economic loss for the equine industry. Of the two circulating subtypes, equine-1 influenza virus (H7N7) has not been isolated for more two decades (Webster, 1993); though serological evidence remains in some herds. The highly lethal avian-like equine influenza virus (H3N8) (Guo et al., 1992) was only found in China in 1989–1990, and has not been isolated since. Equine-2 influenza virus (H3N8), however, remains in circulation despite intensive vaccination programs. In most instances, vaccine failure is attributed to the antigenic drift of the virus (Newton et al., 1999).

The hemagglutinin (HA) gene of A/Eq/Miami/63 (H3N8) and A/Aichi/68 (H3N2), prototype viruses for equine-2 influenza virus and human influenza virus, respectively, has more than 85% amino acid identity. Despite the fact that both are of H3 subtype, the rate of amino acid substitutions in the HA gene of equine-2 influenza virus is only one-third to that of human influenza virus (Fitch et al., 1997; Both et al., 1983; Lai et al., 2001). The basis for this slower evolution rate for equine-2 influenza virus is not known. Possible mechanisms include alternate reversions of amino acid substitution, amino acid substitutions at alternate antigenic sites, by "switching" of virus lineages, and by a lower selection pressure.

We have previously reported that there are two distinct Eurasian and American branches of equine-2 influenza virus (Daly et al., 1996), and that for the viruses within the Ameri-

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can branch, they have further diverged into genetic and antigenic distinct lineages (Lai et al., 2001). We are reporting here that since 1996, there have been two "parallel" evolutionary lineages of equine-2 influenza virus circulating in the United States, and each lineage contributes viruses to circulate in alternate years. In addition, the more recent isolates have fewer amino acid substitutions at the antigenic sites. This is in contrast to the earlier isolates, which have multiple nonsynonymous substitutions at antigenic sites B and D. This alternate circulation may play a role in the maintenance of equine-2 influenza virus in a highly vaccinated population.

2. Materials and methods

2.1. Viruses

Equine-2 influenza viruses A/Eq/New York/99 (NY99) and A/Eq/Oklahoma/00 (OK00) were isolated in fertilized eggs, as part of a diagnostic test during outbreaks of respiratory infections in affected horses. The other equine-2 influenza viruses used in the antigenic analysis are also field isolates, with the exception of A/Eq/Miami/63 (MI63) and A/Eq/Prague/56 (PR56). These two viruses were generous gifts from Dr. Robert Webster, St. Jude Children's Hospital. The nucleotide sequences of the HA_1 gene for the viruses as listed in Table 1, except for NY99 and OK00, were obtained from the GenBank with the corresponding accession numbers.

Table 1
Accession numbers for the viruses used in the phylogenetic analysis

Virus	Abbreviation	Accession number	Country of origin
A/Eq/Saskatoon/90	SA90	AF197243	Canada
A/Eq/Suffolk/89	SU90	X68437	United Kingdom
A/Eq/Lambourne/92	LM92	X85087	United Kingdom
A/Eq/Hong Kong/92	HK92	L27597	China
A/Eq/Kentucky/91	KY91	L39918	United States
A/Eq/Kentucky/92	KY92	L39917	United States
A/Eq/Kentucky/94	KY94	L39914	United States
A/Eq/Kentucky/95	KY95	AF197247	United States
A/Eq/Kentucky/96	KY96	AF197248	United States
A/Eq/Kentucky/97	KY97	AF197249	United States
A/Eq/Kentucky/98	KY98	AF197241	United States
A/Eq/Florida/93	FL93	L39916	United States
A/Eq/Florida/94	FL94	AF197242	United States
A/Eq/Argentina/93	AR93	L39913	Argentina
A/Eq/Argentina/94	AR94	AF197245	Argentina
A/Eq/Argentina/95	AR95	AF197244	Argentina
A/Eq/Argentina/96	AR96	AF197246	Argentina
A/Eq/New York/99[a]	NY99	AY273167	United States
A/Eq/Oklahoma/00[a]	OK00	AY273168	United States

[a] The HA_1 sequences determined in this study.

2.2. Sequence determination for the hemagglutinin gene (HA_1)

Viral RNA was extracted from allantoic fluid and RT-PCR was performed to synthesize the templates for sequence determination, using primers uni-12 (AGCAAAAGCAGG), EH3-1 (AGCAAAAGCAGGGGATATTTCTGTC) and EH3-1061- (TGTGATTTGCTTTTCTGGTAC), as described previously (Lai and Chambers, 1995). Some of the sequences were determined by automated DNA sequencing using a Perkin-Elmer (Applied Biosystems, Foster City, California) Model 373 XL DNA sequencing system. Sequencing reactions were performed multiple times, and both forward and reverse sequencing primers were used in determining the sequence. Sequence data was complied and analyzed using GeneTool version 1.1 (BioTools Incorporated, Edmonton, Canada). Phylogenetic analysis was performed by using the deduced HA_1 amino acid sequence and the PHYLIP software package (Felsenstein, 1993). Distance matrix was calculated by using the Protdist program, and an unrooted tree generated by using the Fitch program. Bootstrap value was set at 100, and the consensus values were determined by using the Consensus program. Regression analysis and plotting of the curve was performed by using the distance data from the consensus tree and by using the Sigma Plot program (Jandel Scientific).

2.3. Serological analysis

Serum samples were collected by venipuncture from recovering horses. Hemagglutination-inhibition (HI) assay

Table 2
Hemagglutination-inhibition titers of horse sera to equine-2 influenza viruses

Convalescent sera (year of collection)	Virus antigen								
	MI63	KY91	KY97	KY98	NY99	KY99	OK00	KY01	PR56
1995[a]	–	320	–	80	–	80	–	80	–
1998[b]	40	–	80	640	80	–	320	–	80
1999A[b]	20	–	160	80	320	–	160	–	40
1999B[a]	–	160	–	80	–	320	–	320	–
2001[a]	–	320	–	80	–	160	–	160	–
2002[b]	80	–	20	80	20	–	320	–	20

The titers of the sera towards the homologous virus or viruses in the same phylogenic lineage is highlighted by grey boxes. (–) not done.
[a] Sera collected from convalescence or experimental infected horses in Kentucky.
[b] Sera collected from horses with respiratory infections at the Large Animal Hospital, College of Veterinary Medicine, Oklahoma State University.

was performed as described (Mahy and Kangro, 1996). These horse sera were collected from outbreak investigations or from client horses visiting the Large Animal Hospital, Oklahoma State University, for respiratory infections or other unrelated ailments. The serum samples found to have HI titer towards equine-2 influenza virus were used in the antigenic analysis shown in Table 2.

3. Results

3.1. Deduced amino acid sequence of the HA_1

The accession numbers for the deduced HA_1 amino acid sequences of A/Eq/New Yok/99, A/Eq/Oklahoma/00, and other equine-2 influenza viruses used in the phylogenetic analysis are shown in Table 1. Since the receptor for equine-2 influenza virus is "avian-like", it is unlikely that nucleotide or amino acid changes are a result of egg passage. The ratio of synonymous and nonsynonymous nucleotide substitutions is approximately two to one, as expected (data not shown), indicating that there are no constraint on mutations in this viral gene. However, from the deduced amino acid sequence, nonsynonymous substitutions appear to cluster at the putative antigenic sites A–D, as shown in Table 3. We have previously reported that antigenic site B, which locates at the tip of the globular structure of the HA, is a "hot spot" for amino acid substitutions in equine-2 influenza virus (Lai et al., 2001). This is particularly true for the Kentucky lineage. However, since 1998, antigenic site D has become the site of amino acid substitutions for viruses in the Kentucky lineage, with a change of Ile_{216} to Val_{216}. In contrast, viruses in the Florida lineage have amino acid changes at antigenic site B, with a Gln_{191} to Glu_{191} switch from KY97 to NY99 (Table 3). Therefore, nonsynonymous substitution at site B is not restricted for viruses in the Kentucky lineage. Furthermore, there are no changes at all at these antigenic sites for the most recent isolates, KY98 and OK00, of the Kentucky lineage.

3.2. Phylogenetic and regression analysis

Fig. 1 is a phylogenetic tree generated by using the deduced HA_1 amino acid sequence of equine-2 influenza viruses as listed in Table 1. The Eurasian and American branches, as indicated by A and B respectively, had diverged since 1990. For the American branch, it has further diverged into three distinct lineages: Argentina, Kentucky, and Florida. In addition, since 1996, circulating viruses in the United States have been derived from the Kentucky and Florida lineages during alternate years, with KY96, KY98, and OK00 from the Kentucky lineage, and KY97 and NY99 from the Florida lineage.

Fig. 2 is a plot of the distance from the node of the virus isolate (based on the phylogenetic tree in Fig. 1) against the year of isolation. The calculated nonsynonymous substitution rate is similar to that for the earlier viruses as described by Bean et al. (1992). Specifically, the evolution rate for the Eurasian viruses is 0.99 amino acids per year, or 3.00×10^{-3} amino acids substitutions per site per year. For the American viruses, it is 0.88 (2.67×10^{-3} amino acids per site per year) and 0.93 (2.82×10^{-3} amino acids per site per year)

Table 3
Amino acid substitutions at the antigenic sites of recent equine-2 influenza viruses hemagglutinin

Virus	Site A														Site B																		Site C										Site D														
	146*														199																		55				278						174				217						246						
MI63	Q	N	G	G	S	S	A	C	R	R	G	S	A	D	S	T	N	N	E	Q	T	K	L	Y	V	Q	A	S	C	N	N	P	P	I	D	T	C	W	N	D	N	F	K	R	S	Q	Q	T	I	I	P	D	V	L	M	I	N
[2]FL93	R	G	K												S	Q								I	E								S				I	V					K												I					
[2]FL94	R	G	K												S	Q								I	E								S				I	V					K												I					
[1]KY94	R	G	K												S	Q	Q		E					I	E								S				I	V					K												I					
[1]KY95	R	G	K												S	Q	K		E					I	E								S				I	V					K												I					
[1]KY96	R	G	K												S	H	K		E					I	E								S				I	V					K												I					
[2]KY97	R	G	K												S	Q								I	E								S				I	V					K												I					
[1]KY98	R	G	K												S	Q			E					I	E								S				I	V					K												V	I				
[2]NY99	R	G	K												S	Q	E							I	E								S				I	V					K												I					
[1]OK00	R	G	K												S	Q			E					I	E								S				I	V					K												V	I				

(*) The sequence and position of amino acids at the antigenic sites for the prototype equine-2 influenza virus, A/Eq/Miami/63. (1) Kentucky lineage (highlighted in grey box). (2) Florida lineage.

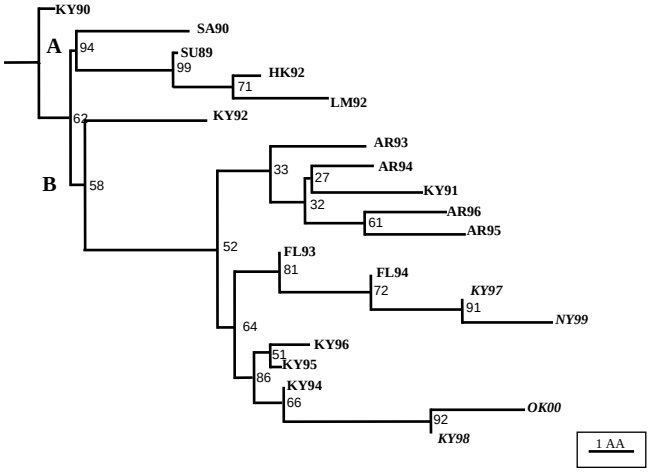

Fig. 1. Phylogenetic tree generated using the HA_1 amino acid sequence of eqine-2 influenza virus. The horizontal distances from the nodes are in scale, which is proportional to the number of nonsynonymous substitutions. Boxed bar = 1 amino acid. (A) Eurasian branch; (B) American branch. Bootstrap values ($n = 100$), is shown at the branch points.

for the Florida and Kentucky lineages, respectively. As indicated in the Fig. 2, these two lineages are almost parallel, offset only by one year. The lower rate of 0.20 (0.61×10^{-3} amino acids per site per year) for the Argentina lineage is probably due to alternate reversion of Ala_{138} to Ser_{138}, as described previously (Lai et al., 2001).

3.3. Antigenic analysis

The result of hemagglutination-inhibition assay for convalescent horse sera against various equine influenza viruses is shown in Table 2. Each row represents the HI titers of a specific serum (collected at the specified year) against various viruses listed in separate columns. The prototype equine-2 influenza virus, A/Eq/Miami/63 (MI63) is shown in the first column, followed by KY91, KY97, KY98, NY99, KY99, OK00, and KY01 for equine-2 influenza virus (H3N8), and PR56 in the last column for equine-1 influenza virus (H7N7). KY97 and NY99 are members in the Florida lineage, whereas KY98 and OK00 belong to the Kentucky lineage.

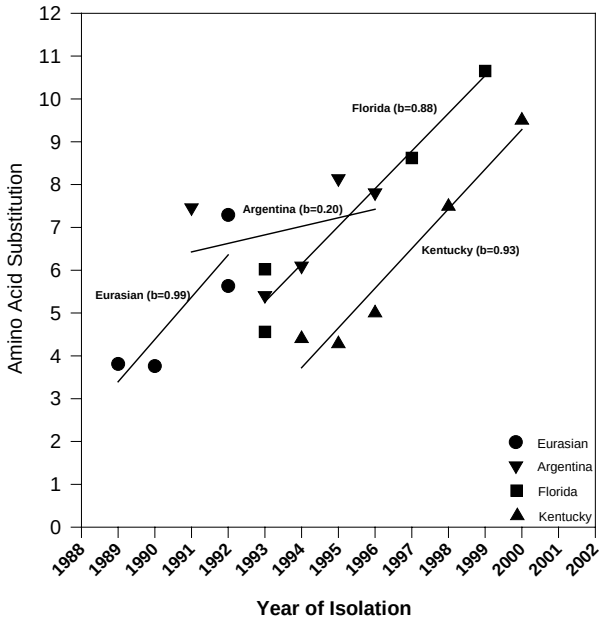

Fig. 2. Regression analysis of recent equine-2 influenza viruses, based on the phylogenetic tree shown in Fig. 1. The distance from a common node is plotted against the year of isolation. b = slope.

The HI titer against KY91 for serum collected in 1995 is 320, while the HI titers for KY98, KY99, and KY01 are 80, 80, and 80, respectively. The higher HI titer against KY91 is probably vaccine-induced, as some commercial vaccines at the time contained an earlier Kentucky isolate such as KY81 or KY87 for one of virus antigens in a trivalent vaccine, in addition to MI63 and PR56. Since KY95 is a member of the "early" Kentucky lineage, the antiserum (serum 1995) it elicited has a lower HI titer of 80 towards the more recent equine-2 influenza viruses, KY98, KY99 and KY01 is not unexpected. Serum 1998 has low HI titers of 40 and 80 towards MI63 and PR56, respectively, suggesting prior vaccination with no recent boosting. It has a high titer of 640 towards the homologous virus, KY98, is expected. A somewhat lower titer of 320 towards OK00, is also expected, as these viruses are from the same phylogenetic lineage. An eight-fold reduction in the HI titer towards KY97 (and NY99) indicates an antigenic distinction from KY98 (and OK00). For serum 1999A, it has a high HI titer of 320 towards the homologous virus NY99, but a two to four-fold reduction towards viruses of the other lineage, OK00 and KY98, respectively. For serum 1999B, the HI titer towards the homologous virus, KY99, and KY01 (same lineage) are both at 320. Similar to serum 1999A, there is a four-fold reduction (HI titer of 80) towards KY98 (different lineage). Serum 2001 has HI titers of 160 towards the homologous virus (KY01) and virus of the same lineage (KY99), but a two-fold reduction of 80 towards KY98. Interestingly, serum 2002 retains a high HI titer of 320 towards OK00 of the same lineage, but has a significant lower HI titer of 20 towards the viruses in the other lineage.

It is interestingly to note that the sera collected from Kentucky represent sera from "highly vaccinated" horses, as indicated by their high HI titer towards KY91, whereas, sera collected from Oklahoma represent "less vaccinated" horses, as shown by their lower HI titer towards MI63 and PR56. Nonetheless, these sera distinguish the antigenic differences between these two viral lineages.

4. Discussions

The existence of two parallel Kentucky and Florida viral lineages described here is not a result of geographic separation. NY99 was isolated from a horse being transported from Oklahoma to New York via Kentucky and Maryland.

It is likely that this virus represents the predominant virus circulating in the United States during that year. However, whether these two lineages are mutually exclusive is unknown. Simultaneous circulation of viruses from both the Kentucky and the Florida lineage is possible. Viruses from both Eurasian and American lineages had been isolated during a single outbreak (Daly et al., 1996), and that these two viral lineages now co-circulate in European countries (Oxburgh and Klingeborn, 1999). Co-circulation of genetic and antigenic distinct equine-2 influenza viruses has also been described in Nigeria (Adeyefa et al., 1996). However, there is no evidence for co-circulation of Eurasian and American viruses in North America. The only Eurasian virus isolated in North America was A/Eq/Saskatoon/90 (Lai et al., 2001), which was isolated in Canada. Other isolates from the same epizootic were identical to the index virus, also of the Eurasian branch (unpublished results). Furthermore, epizootics of equine influenza are explosive and widespread, and hence likely caused by a single predominant virus.

From Table 2, it is clear that these two phylogenetic lineages, the "Kentucky" and "Florida", are antigenic distinct. Horse sera react differentially to viruses of one lineage than the other, with a higher reactivity towards the homologous virus and viruses in the homologous lineage, but a lower reactivity towards viruses of the other lineage. Furthermore, these horse sera, collected in consecutive years, have alternating pattern of reactivity towards the viruses from these two lineages, parallel to the alternating pattern as shown in the phylogenetic tree.

There are at least two nonsynonymous substitutions, one at each of the two antigenic sites (B and D), between the latest viruses of the two viral lineages. However, the difference in the HI titers, as recognized by different sera, ranges from two- to eight-fold. This limited antigenic difference may be a result of multiple prior infections in the donor horses, particularly for the horse sera collected in Oklahoma. Those horses were client horses visiting the Veterinary Hospital and their full history is unknown. The omission of horse serum for 2000 is due to the fact that a "match" serum was not found. Nonetheless, these horse sera can differentiate the two lineages, and the "alternating" pattern of reactivity supports the result of genetic analysis.

This alternating circulation of viruses from distinct viral lineage raises a fundamental question. Since equine influenza is an acute infection, how and where the non-circulating viral lineage is maintained? One possibility is that these two viral lineages are not mutually exclusive. When one is the predominant virus, the other is evading detection, or circulating in other horse populations. However, when multiple viral isolates are available for a given year, the viruses are antigenically, and sometimes genetically, identical (unpublished data). Unlike human influenza virus for which a well-established surveillance program is in place, isolation of equine influenza virus is somewhat biased towards horses with veterinary care. The identity of the virus circulating in inaccessible horse populations cannot be determined. Another possible mechanism for the maintenance of the non-prevalent viral lineage is that, after being the predominant virus during the previous year, this virus produces sub-clinical infection in immune hosts (due to previous infection) in the subsequent year. With additional antigenic changes and a supply of new susceptible hosts, the non-prevalent virus once again becomes the prevalent virus in the following year. It has been shown that maternal antibodies interfere with the immune response to vaccination against equine influenza virus (Wilson et al., 2001; van Maanen et al., 1992). Presumably, maternal antibodies offer protection to the homologous virus—the predominant virus when the foal is born. Yearlings are the most affected horse population during epizootics of equine influenza. These 1-year-old horses, while being protected by maternal antibodies during the previous year as foals, become susceptible to the virus of the "re-emerging" lineage. Therefore, this annual "switching" of viral lineage is possibly a result of the changes in the host. It would be difficult to test this hypothesis though. Detection of viruses in a "sub-clinical host" is impractical, as sub-clinically infected hosts are usually identified retrospectively by serology. However, detecting the circulation of "non-predominant virus" may be achievable, if a serology test that can differentiate the two viral lineages is available.

This diverged evolution of equine-2 influenza virus is similar to that observed for influenza B virus (Yamashita et al., 1988; Lindstrom et al., 1999), and this is in contrast to the evolution of human influenza virus (H3N2) with one single dominant lineage (Fitch et al., 1997). Interestingly, the average duration for the circulation of side lineages of human influenza virus (H3N2) is about 1.6 years (Fitch et al., 1991). The extinction of the side branches is a "paradoxical dilemma" for maximizing the genetic and antigenic diversity in the evolution of influenza virus (Ferguson et al., 2003). Some of the earlier lineages described for equine-2 influenza virus have probably become extinct, as viruses from the lineage of A/Eq/Tokyo/71 and A/Eq/Algeria/72 (Endo et al., 1992; Kawaoka et al., 1989) have not been isolated since (unpublished data). Is this diverged evolution of recent equine-2 influenza virus a "transient" extension for the life span of the side branches, or is this pattern a permanent phenomenon and that equine-2 influenza virus is becoming "influenza B virus-like" are the questions remains to be addressed.

Although both equine-2 influenza virus and human influenza virus are of H3 subtype, this alternate circulation may explain the slower evolution rate for the HA gene of equine-2 influenza virus (H3N8) as compared to human influenza virus (H3N2). The presence of genetic and antigenic distinct lineages of equine-2 influenza virus, similar to that of influenza B virus (Lindstrom et al., 1999), may provide a mechanism to reduce the selection pressure—by re-cycling of different lineages—and hence a lower evolution rate. However, only the HA_1 gene was examined in this study, the possibility of the presence of reassortants or

recombinants between these two distinct lineages cannot be ruled out.

In contrast to the annual updating of human influenza virus vaccines, updating of equine vaccines is at best sporadic, and manufacturer-dependent. Some manufacturers update the vaccine more frequently compared to the others. The antigenic disparity between the circulating virus and the vaccine strain is a significant factor for vaccine failure, though vaccination shortens the duration of the disease (Morley et al., 1999). Constant surveillance and updating of vaccine is the key to "pre-empt" epizootics of equine influenza (Mumford et al., 2003).

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References

Adeyefa, C.A., James, M.L., McCauley, J.W., 1996. Antigenic and genetic analysis of equine influenza viruses from tropical Africa in 1991. Epidemiol. Infect. 117 (2), 367–374.

Bean, W.J., Schell, M., Katz, J., Kawaoka, Y., Naeve, C., Gorman, O., Webster, R.G., 1992. Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. J. Virol. 66 (2), 1129–1138.

Both, G.W., Sleigh, M.J., Cox, N.J., 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 (1), 52–60.

Chambers, T.M., Holland, R.E., Lai, A.C.K., 1995a. Equine influenza: current veterinary perspectives. Part I. Equine Practice 17, 19–23.

Chambers, T.M., Holland, R.E., Lai, A.C.K., 1995b. Equine influenza: current veterinary perspectives. Part II. Equine Practice 17, 26–30.

Daly, J.M., Lai, A.C., Binns, M.M., Chambers, T.M., Barrandeguy, M., Mumford, J.A., 1996. Antigenic and genetic evolution of equine H3N8 influenza A viruses. J. Gen. Virol. 77 (Pt. 4), 661–671.

Endo, A., Pecoraro, R., Sugita, S., Nerome, K., 1992. Evolutionary pattern of the H3 haemagglutinin of equine influenza viruses: multiple evolutionary lineages and frozen replication. Arch. Virol. 123 (1–2), 73–87.

Felsenstein, J., 1993. Phylogeny reference package (PHYLIP), version 3.4., Department of Genetics, University of Washington, Seattle (distributed by the author).

Ferguson, N.M., Galvani, A.P., Bush, R.M., 2003. Ecological and immunological determinants of influenza evolution. Nature 422 (6930), 428–433.

Fitch, W.M., Bush, R.M., Bender, C.A., Cox, N.J., 1997. Long term trends in the evolution of H(3) HA1 human influenza type A. Proc. Natl. Acad. Sci. U.S.A. 94 (15), 7712–7718.

Fitch, W.M., Leiter, J.M., Li, X.Q., Palese, P., 1991. Positive Darwinian evolution in human influenza A viruses. Proc. Natl. Acad. Sci. U.S.A. 88 (10), 4270–4274.

Guo, Y., Wang, M., Kawaoka, Y., Gorman, O., Ito, T., Saito, T., Webster, R.G., 1992. Characterization of a new avian-like influenza A virus from horses in China. Virology 188 (1), 245–255.

Kawaoka, Y., Bean, W.J., Webster, R.G., 1989. Evolution of the hemagglutinin of equine H3 influenza viruses. Virology 169 (2), 283–292.

Lai, A., Chambers, T.M., 1995. Rapid protocol for sequencing RNA virus using ΔTaq version 2.0 DNA polymerase. BioTechniques 19, 704–706.

Lai, A.C., Chambers, T.M., Holland Jr., R.E., Morley, P.S., Haines, D.M., Townsend, H.G., Barrandeguy, M., 2001. Diverged evolution of recent equine-2 influenza (H3N8) viruses in the Western Hemisphere. Arch. Virol. 146 (6), 1063–1074.

Lindstrom, S.E., Hiromoto, Y., Nishimura, H., Saito, T., Nerome, R., Nerome, K., 1999. Comparative analysis of evolutionary mechanisms of the hemagglutinin and three internal protein genes of influenza B virus: multiple cocirculating lineages and frequent reassortment of the NP, M, and NS genes. J. Virol. 73 (5), 4413–4426.

Mahy, B., Kangro, H.O., 1996. Virological Methods Manual. Academy Press.

Morley, P.S., Townsend, H.G., Bogdan, J.R., Haines, D.M., 1999. Efficacy of a commercial vaccine for preventing disease caused by influenza virus infection in horses. J. Am. Vet. Med. Assoc. 215 (1), 61–66.

Mumford, E.L., Traub-Dargatz, J.L., Salman, M.D., Collins, J.K., Getzy, D.M., Carman, J., 1998. Monitoring and detection of acute viral respiratory tract disease in horses. J. Am. Vet. Med. Assoc. 213 (3), 385–390.

Mumford, J., Cardwell, J., Daly, J., Newton, R., 2003. Efforts to pre-empt an equine influenza epidemic. Vet. Rec. 152 (13), 405–406.

Mumford, J.A., Hannant, D., Jessett, D.M., 1990. Experimental infection of ponies with equine influenza (H3N8) viruses by intranasal inoculation or exposure to aerosols. Equine Vet. J. 22 (2), 93–98.

Newton, J.R., Verheyen, K., Wood, J.L., Yates, P.J., Mumford, J.A., 1999. Equine influenza in the United Kingdom in 1998. Vet. Rec. 145 (16), 449–452.

Oxburgh, L., Klingeborn, B., 1999. Cocirculation of two distinct lineages of equine influenza virus subtype H3N8. J. Clin. Microbiol. 37 (9), 3005–3009.

Powell, D.G., Watkins, K.L., Li, P.H., Shortridge, K.F., 1995. Outbreak of equine influenza among horses in Hong Kong during 1992. Vet. Rec. 136 (21), 531–536.

van Maanen, C., Bruin, G., de Boer-Luijtze, E., Smolders, G., de Boer, G.F., 1992. Interference of maternal antibodies with the immune response of foals after vaccination against equine influenza. Vet. Q. 14 (1), 13–17.

Webster, R.G., 1993. Are equine-1 influenza viruses still present in horses? Equine Vet. J. 25 (6), 537–538.

Wilson, W.D., 1993. Equine influenza. Vet. Clin. North Am. Equine Pract. 9 (2), 257–282.

Wilson, W.D., Mihalyi, J.E., Hussey, S., Lunn, D.P., 2001. Passive transfer of maternal immunoglobulin isotype antibodies against tetanus and influenza and their effect on the response of foals to vaccination. Equine Vet. J. 33 (7), 644–650.

Yamashita, M., Krystal, M., Fitch, W.M., Palese, P., 1988. Influenza B virus evolution: co-circulating lineages and comparison of evolutionary pattern with those of influenza A and C viruses. Virology 163 (1), 112–122.