Seroepidemiological and molecular evidence for the presence of two H3N8 equine influenza viruses in China in 1993–94

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In May 1993, a severe epidemic of respiratory disease began in horses in Inner Mongolia and spread throughout horses in China. The disease affected mules and donkeys as well as horses but did not spread to other species, including humans. The severity of the disease raised the question of whether the outbreak might have been caused by the new avian-like influenza viruses detected in horses in China in 1989 or by current variants of A/equine/Miami/I/63 (H3N8) (equine-2) or by a reassortant between these viruses. Antigenic and sequence analysis established that all gene segments of the influenza virus causing the epidemic were of recent equine-2 origin and that the virus was not a reassortant. Serological analysis of post-infection horse sera provided evidence for the continued circulation of the A/Equine/Jilin/1/89 (Eq/Jilin) (H3N8) avian-like viruses in horses in Heilongjiang province with original antigenic sin-like responses. It is noteworthy that prior infection with the avian-like Eq/Jilin strain did not afford cross-protection against a current equine-2 strain. Serological evidence for the continued circulation of the avian-like H3N8 influenza virus in horses indicates that this virus has probably established itself in horses in Asia.

Introduction

Epidemics of respiratory disease in horses, due to genetic variants of equine-2 influenza virus (Waddell et al., 1963), continue to occur throughout the world. Major epizootics took place in South Africa in 1986 and in India in 1987 (Mumford & Wood, 1993). More recently, epizootics of equine-2 influenza have arisen in Europe (Livesay et al., 1993) and in North America, including Alaska (Alstad et al., 1993). In 1993, there was an outbreak of equine-2 influenza among vaccinated horses in Hong Kong (Watkins et al., 1993), probably due to a virus introduced from England (Alexander et al., 1994). By contrast, descendants of the A/Equine/Prague/1/57 (H7N7) [equine-1] virus (Sovinova et al., 1958) have not been isolated from horses since 1980, although serological evidence for its circulation in unvaccinated horses is still being found in different parts of the world (Webster, 1993).

Mainland China has been the site of repeated outbreaks in recent years. A severe outbreak struck horses in the Jilin and Heilongjiang provinces of northeastern China in March 1989 (Guo et al., 1990), with morbidity and mortality rates reaching 81% and 20% in some herds. A second outbreak, in April 1990 in Fuyu County, Heilongjiang province was associated with 41% morbidity and no mortality. Both outbreaks were caused by H3N8 influenza viruses of recent avian origin (Guo et al., 1992) and are antigenically and genetically distinct from currently circulating equine-2 strains. From 1990 to 1992 there was a single report of respiratory disease in horses in Xin Jang, in north-western China (Wei Wu ER & Zu Zi Zhi Ou, personal communication). However, beginning in May 1993, a severe outbreak of respiratory disease with high morbidity again affected horses in China. Quite possibly, this widespread epidemic could signal the reappearance of H3N8 influenza viruses of recent avian origin, or it may reflect the introduction of classical equine influenza viruses or possibly genetic reassortment between avian and equine H3N8 viruses. In this report, we characterize the causative agent of the 1993–94 equine influenza epidemic in China as an H3N8 influenza A virus whose gene segments are derived entirely from classical equine-2 influenza viruses and...
provide serological evidence for the continued circulation of the avian-like H3N8 virus in horses in China.

**Methods**

**Viruses and clinical material.** Forty nasal swabs were collected from horses with clinical signs of respiratory disease in August 1993 and in March and May 1994 in the Heilongjiang, Gansu and Sichuan provinces. The samples were inoculated into the allantoic cavity of 9- to 10-day-old embryonated chicken eggs, and the viral isolates were sent to the WHO reference laboratory in Beijing. Each isolate from the Gansu province, designated A/Equine/Gansu/2/94, A/Equine/Gansu/3/94 and A/Equine/Gansu/5/94, was subsequently included in the antigenic analysis, with A/Equine/Gansu/2/94 used for detailed molecular study. Sequence information on other H3N8 influenza viruses was from the repository at St Jude Children's Research Hospital, Memphis, Tenn., USA or from GenBank. The abbreviations listed in Table 1 were used to designate viruses used in the phylogenetic analysis.

All viruses were grown in 11-day-old embryonated chicken eggs and purified by velocity sedimentation through 25–70% sucrose gradients in a Beckman SW28 rotor. Virion RNA was isolated by treating purified virus with proteinase K and SDS, followed by extraction with phenol–chloroform (1:1) as described previously (Bean et al., 1980).

**Antisera and specific antibodies.** Antisera to the equine strains were prepared in ferrets as recommended by Kendal & Pereira (1982). Monoclonal antibodies to the haemagglutinin (HA) of equine-2 influenza viruses were prepared according to Kohler & Milstein (1976).

**Serological tests.** HA titrations and haemagglutination-inhibition (HI) tests were performed in microtitre plates with receptor-destroying enzyme-treated sera (Kendal & Pereira, 1982). Neuraminidase (NA) titrations and NA inhibition tests were done by the procedure of Katz et al. (1980). Acute sera were collected from horses at 1–7 days, and convalescent sera at 1–2 months, after the onset of disease.

**RNA extraction, cDNA generation, PCR and nucleotide sequencing.** Methods for extracting influenza RNA from tissue culture or allantoic fluid have been described (Bean et al., 1980). RNA was resuspended in 3.5 µl of water with 1 µl of DNA primer U12 (1 µg/µl, 5'-AGCAAA-AGCAGG 3', corresponding to virion RNA nucleotides 1 to 12) and cDNA was generated with 20 units of reverse transcriptase (Life Sciences), 2 µl 2.5 mm-dNTPs (Pharmacia) and 20 units of RNAsin (Promega) in 0.25 M-Tris-HCl (pH 8.0), 50 mm-MgCl₂, 50 mm-DTT and 0.35 m-KCl. After incubation at 42 °C for 1 h, the reaction was stopped by heating at 100 °C for 5 min.

PCR amplification (Saiki et al., 1988) of the HA1 coding region was performed as previously described (Katz et al., 1990) with use of 5'-TTGCCCTAAAAACTTCCCCG 3' and 5'-CGTCTCCATCCTCCTCCA 3' primers, which are complementary to RNA nucleotides 72 to 91 and 1183 to 1164, respectively. Amplified products were purified by the 'Gene Clean' procedure, as described previously (Saiki et al., 1988; Katz et al., 1990). Purified DNA products were sequenced by the dideoxyxynucleotide chain-termination method (Katz et al., 1990) and the US Biochemical Sequenase reaction. After termination of strand elongation, the reaction products were resolved under reducing conditions (7 M-urea) on 6% polyacrylamide gels containing either a 1.0-3.5 x TBE (90 mM-Tris-borate, pH 8.0, 1 mM-EDTA) gradient or 40% formamide to avoid a compression effect in certain regions of the HA cDNA. Sequence analyses of the HA1 of the cloned reference viruses were performed by fmol DNA sequencing with end-labelled primers (Promega).

**Phylogenetic analysis of the sequence data** was performed with PAUP (Phylogenetic Analysis Using Parsimony), software version 2.4 (David Swoford, Illinois Natural History Survey, Champaign, Ill., USA). The full designation of each virus used in the phylogenetic analysis is provided in Table 1.

**Results**

**Clinical features of respiratory disease in horses and other animals**

The incubation period ranged from 1 to 3 days, with disease onset marked by the development of high fever (≥ 40 °C), dry cough, nasal discharge with purulent conjunctivitis, pharyngitis and pneumonia. Deaths occurred mainly in foals and older horses. Recovery began within 5 to 7 days, and by 2 weeks all signs of disease had disappeared in animals that did not develop pneumonia. Donkeys and mules were affected as severely as horses.

**Epidemiological features of the outbreak**

The epidemic was first detected in horses in Nei Meng Gu, Zhi Zhi Qu, in Inner Mongolia in May 1993, spreading gradually to horses in the Hebei province of northern and north-eastern China in June and July of 1993. The disease was detected in horses in the suburbs of Beijing in January through to March 1994, as well as in horses in the Gansu and Qinghai provinces. Subsequently, in April 1994, the disease spread to horses in the Sichuan province in southern China. By July 1994, the epidemic had largely abated. In the Gansu province of north-western China, approximately 624,897 horses were affected, with 2166 dying out of an estimated horse population of 2452,500; thus, 25% of the horses were affected and 0.09% died. The overall morbidity rate during the epidemic ranged from 25% to 100%, and the mortality rate from 0.5% to 1%. Similar percentages of donkeys and mules were affected in all areas. There were no obvious signs of disease among humans or other

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**Table 1. Influenza A viruses used in these studies**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Abbreviation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duck/Hokkaido/21/82 (H3N8)</td>
<td>DKH2182</td>
</tr>
<tr>
<td>Duck/Hokkaido/8/80 (H3N8)</td>
<td>DKB880</td>
</tr>
<tr>
<td>Duck/Ukraine/1/63 (H3N8)</td>
<td>DUK63</td>
</tr>
<tr>
<td>Aichi/2/68 (H3N2)</td>
<td>AICH1</td>
</tr>
<tr>
<td>Memphis/102/72 (H3N2)</td>
<td>MEM72</td>
</tr>
<tr>
<td>Bangkok/1/79 (H3N2)</td>
<td>BANGKOK79</td>
</tr>
<tr>
<td>Equine/Jilin/1/89 (H3N8)</td>
<td>EQJL89</td>
</tr>
<tr>
<td>Equine/Miami/1/63 (H3N8)</td>
<td>EOMIA63</td>
</tr>
<tr>
<td>Equine/New Market/79 (H3N8)</td>
<td>EQNM79</td>
</tr>
<tr>
<td>Equine/Tennessee/5/86 (H3N8)</td>
<td>EQTN86</td>
</tr>
<tr>
<td>Equine/Taby/91 (H3N8)</td>
<td>EQBY91</td>
</tr>
<tr>
<td>Equine/Gansu/2/94 (H3N8)</td>
<td>EQGAN94</td>
</tr>
</tbody>
</table>

* Used in the phylogenetic analysis (see Fig. 1).
animals including domestic poultry that had been in contact with infected horses.

**Antigenic characterization of infectious agents**

Results of HI assays with isolates obtained from three horses in the Gansu province yielded identical results; hence, the data reported in Table 2 represent a single isolate, Eq/Gansu/94. The highest titres were obtained with H3N8 equine influenza virus isolates from Kentucky and Italy (A/Eq/Italy/1062/91 and A/Eq/Ken/1/92), and the lowest with Eq/Jilin (H3N8). These findings indicate that the HA of the Gansu isolates is antigenically most closely related to H3N8 equine-2 influenza viruses currently circulating in horses in other parts of the world.

To confirm the subtype and relatively recent derivation of the HA glycoprotein of the Gansu viruses, we performed HI assays using a panel of monoclonal antibodies raised against the HA of equine-2 viruses isolated before 1989 (Table 3). Each antibody reacted to produce high HI titres with pre-1989 viruses, including A/Eq/Ken/698/88 (H3N2), but showed markedly lower titres with the Gansu isolates (<100–200). Thus, the Gansu viruses differ antigenically from equine-2 viruses at the epitopes represented by this group of monoclonal antibodies. Analysis of the NA in serological assays showed that it was most closely related to current equine-2 influenza viruses and only marginally to the Eq/Jilin (H3N8) isolate (results not shown). We conclude that both of the surface glycoproteins of viruses isolated from horses in the Gansu province were of the H3N8 subtype and were most closely related to the equine-2 influenza viruses currently circulating in horses in other parts of the world.

**Seroepidemiological survey**

To test whether the Gansu H3N8 viruses were responsible for the outbreaks of respiratory disease in horses in China in 1993, we conducted a seroepidemiological study of acute and convalescent sera collected in August 1993 from Heilongjiang province and in April–May 1994 from Gansu and Sichuan provinces (Table 4). Serological analysis of the acute sera from horses in Heilongjiang province showed that four of the 50 sera had HI titres of >160 to Eq/Jilin (H3N8) virus. Unfortunately, acute phase sera could not be collected in Sichuan. From 82.0% to 95.3% of the convalescent sera from each region had detectable HI antibodies to the Eq/Gansu/94 virus, compared with 11.8% to 13.7% of the acute sera. The highest antibody levels were detected in sera from the Gansu and Sichuan provinces [geometric mean titre (GMT) values of 130 and 181, respectively] with lower levels found in samples from the Heilongjiang
Table 4. Seroepidemiologic survey of horses in China

<table>
<thead>
<tr>
<th>Province</th>
<th>Serum*</th>
<th>No. of sera examined</th>
<th>Anti-A/Eq/Gansu/2/94 (H3N8)</th>
<th>Anti-A/Eq/Jilin/1/89 (H3N8)</th>
<th>Anti-A/Eq/Prague/1/56 (H7N7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive (%)</td>
<td>GMT†</td>
<td>Positive (%)</td>
<td>GMT</td>
</tr>
<tr>
<td>Gansu</td>
<td>A</td>
<td>34</td>
<td>11.8</td>
<td>15</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>36</td>
<td>91.7</td>
<td>130</td>
<td>11.1</td>
</tr>
<tr>
<td>Heilongjiang</td>
<td>A</td>
<td>51</td>
<td>13.7</td>
<td>15</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>50</td>
<td>82.0</td>
<td>35</td>
<td>44.0</td>
</tr>
<tr>
<td>Sichuan</td>
<td>C</td>
<td>43</td>
<td>95.3</td>
<td>181</td>
<td>16.3</td>
</tr>
</tbody>
</table>

* A, acute phase; C, convalescent phase.
† Geometric mean titre.

province (GMT of 35). A small percentage of convalescent horses in the Gansu and Sichuan provinces were positive for the Eq/Jilin (H3N8) influenza viruses (11.1% and 16.3%, respectively), as compared to 44% of horses in the Heilongjiang province. This suggests that the horses in Heilongjiang province were previously infected with the Eq/Jilin (H3N8) virus and infection with the Gansu (H3N8) virus elicited an antibody response to the earlier virus. None of the sera tested showed detectable antibodies to equine-1 influenza virus [A/Equine/Prague/1/56 (H7N7)]. These results demonstrate that the H3N8 viruses originally detected in the Gansu province were responsible for the influenza outbreaks that affected other regions of China in 1993 and that horses in the Heilongjiang province had serological evidence of infection with the avian-like H3N8 viruses.

Table 5. Genetic characterization of equine/Gansu/2/94 (H3N8)

<table>
<thead>
<tr>
<th>Gene segment</th>
<th>Regions of gene sequenced</th>
<th>Total base pairs sequenced (% of gene)</th>
<th>Virus with the highest identity</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>48-275, 370-582</td>
<td>441 (19.7)</td>
<td>A/Ep/Fla/91 (H3N8)</td>
<td>96.8</td>
</tr>
<tr>
<td>PB1</td>
<td>2036-2231</td>
<td>188 (80)</td>
<td>A/Ep/Fla/91 (H3N8)</td>
<td>99.5</td>
</tr>
<tr>
<td>PB2</td>
<td>990-1244, 1309-1598</td>
<td>545 (23.2)</td>
<td>A/Ep/Ky/2/86 (H3N8)</td>
<td>97.2</td>
</tr>
<tr>
<td>NP</td>
<td>1084-1331</td>
<td>248 (15.8)</td>
<td>A/Ep/Tn/5/86 (H3N8)</td>
<td>95.6</td>
</tr>
<tr>
<td>M</td>
<td>52-439, 545-884</td>
<td>705 (68.6)</td>
<td>A/Ep/Tn/5/86 (H3N8)</td>
<td>99.3</td>
</tr>
<tr>
<td>NS</td>
<td>43-165, 722-854</td>
<td>255 (28.7)</td>
<td>A/Ep/Tn/5/86 (H3N8)</td>
<td>99.2</td>
</tr>
<tr>
<td>HA</td>
<td>305-691</td>
<td>364 (21.8)</td>
<td>A/Ep/Taby/91</td>
<td>98.4</td>
</tr>
</tbody>
</table>

**Genetic characterization of the equine Gansu H3N8 isolates**

Isolation of an avian-like H3N8 influenza virus (Eq/Jilin) from horses in north-western China in 1989 (Guo et al., 1992) suggested that the more recent outbreak of respiratory disease may have been caused by a reassortant virus possessing gene segments from the two different H3N8 influenza viruses. We therefore undertook sequencing of each of the eight gene segments of Eq/Gansu/2/94 and compared the results with listings in GenBank (Table 5). Each portion of the gene segments analysed had the greatest identity with an equine-2 influenza virus. The PB1, M and NS genes were > 99% identical with recent equine-2 influenza viruses; the most divergent segment was the nucleoprotein (NP), which showed 95.6% identity with A/Ep/Tn/5/86 (H3N2). None of the gene segments were closely related to Eq/Jilin (H3N8), indicating that they were all of recent equine-2 origin and did not arise from genetic reassortment.

**Phylogenetic relationships of the HA of Eq/Gansu/2/94**

Results of a maximum parsimony analysis of the nucleotide sequence of the partial HA gene sequence of Eq/Gansu/2/94 (Table 5) established that this molecule belongs to the equine-2 lineage of H3, of which Eq/Mia/63 (H3N8) is the prototype strain (Fig. 1). The Eq/Gansu/2/94 HA is most closely related to Eq/Taby/91, an H3N8 equine influenza virus isolated in Sweden in 1991 (Oxburgh et al., 1993). The Eq/Jilin (H3N8) virus represents a separate lineage related to avian influenza viruses.
Discussion

Three major epidemics of equine influenza have occurred in China over the past two decades. The first was associated with an equine-1 virus (H7N7) that appeared in northern China in 1974 (Deng et al., 1980). Fifteen years later an avian H3N8 influenza virus infected horse populations (Guo et al., 1992). The most recent epidemic, which began in Inner Mongolia in May 1993, was caused by an equine-2 (H3N8) influenza virus and is the subject of this report. Equine-2 influenza viruses appear to have previously infected horses in China since low levels of HI antibody to Eq/Miami/63 (H3N8) were detected in acute-phase sera collected in 1989 (Guo et al., 1992). The high morbidity rate in our survey (70–100%) indicates that the majority of horses were susceptible to infection, while the low death rate (0.5–1.0%) suggests some degree of immunological protection due to previous exposure to classical equine-2 (H3N8) influenza viruses. In contrast, horses infected with Eq/Jilin (H3N8) in Heilongjiang province in 1989 had a similar morbidity (81%), but the mortality was as high as 20% (Guo et al., 1990). Hence, we postulate that long-term immunological memory in the absence of detectable antibody modulated the severity of disease in the Chinese epidemic of 1993–94.

Serological and monoclonal antibody studies of the Chinese epidemic virus demonstrated its antigenic similarity to H3N8 viruses isolated since 1988. The actual source of this equine-2 H3N8 influenza virus found in horses in China in 1993 cannot be defined with any certainty; the first clinical cases were detected in horses in Inner Mongolia in May 1993. Whether the virus originated from the outbreak in horses in Hong Kong in 1992 or had come overland from Europe can only be speculated on. The closest phylogenetic relationship, based on partial analysis of the HA gene, was to an H3N8 virus isolated from horses in Sweden in 1991.

The antibody reactivity of convalescent horses in the Heilongjiang province differed appreciably from that of horses in the Gansu or Sichuan province. That is, 82% of the horses were positive for the Eq/Gansu/94 virus (>20 HI) with a GMT of only 35, whereas in the other provinces, 91.7–95.3% of horses had GMTs of 130–181. Similarly, in Heilongjiang, 44% of convalescent horses were positive for Eq/Jilin (H3N8) but had a GMT of 29. A possible explanation is that the Heilongjiang horses had been infected with Eq/Jilin (H3N8) before the Eq/Gansu/2/94 virus appeared, so that infection with the second virus resulted in an antibody recall to the previously circulating virus. Similar responses have been noted in humans and ferrets (Francis, 1953; Fazekas de St Groth & Webster, 1966; Webster, 1966) and are collectively termed ‘original antigenic sin’. This explanation would account for the low antibody levels (GMT of 35) to Eq/Gansu/94 in horses in Heilongjiang.
Heilongjiang in northeastern China showed that four of the 50 acute serum samples had HI titres of $\geq 160$ to Eq/Jilin (H3N8). Since antibodies to equine influenza viruses in horses are short-lived (Bryans, 1981; Mumford et al., 1983), it is not possible to explain the presence of antibody titres of $\geq 160$ in acute sera as residual effects from the 1989–90 outbreaks of infection with the Eq/Jilin (H3N8) virus. This raises the possibility that descendants of the avian-like H3N8 are still circulating in the Heilongjiang province, but actual virus isolations have not been achieved since 1990. If the Eq/Jilin (H3N8) virus is still circulating, it would be available for genetic reassortment with classical equine-2 influenza virus.

Although specific details are not available, the field experience of veterinarians in the Heilongjiang province indicates that horses that had recovered from infection with the avian-like Eq/Jilin (H3N8) viruses were susceptible to infection with the Eq/Gansu/94 (H3N8) virus. This observation may reflect the relatively long elapsed time, 5 years, between infection with Eq/Jilin and the 1993–94 epidemic. Whatever the explanation, the boost in immune responsiveness provided by Eq/Gansu/94 infection does establish that these animals had immunological memory to Eq/Jilin although it was not adequate to protect against infection with the current H3N8 strain.

We find it interesting that no antibodies to equine-1 influenza viruses were detected in this study. Since equine influenza vaccines are not used in China, this lack of reactivity suggests that reports of equine-1 influenza virus antibodies detected in other countries may reflect continued use of vaccines containing this antigen.

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References


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