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# Identification of two antigenically and genetically distinct lineages of H3N8 equine influenza virus in Sweden

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## SUMMARY

Four Swedish strains of equine H3N8 influenza virus isolated from outbreaks during the last 4 years were characterized. Antigenic typing using monoclonal antibodies raised against a variety of H3N8 strains showed that the viruses are heterogeneous, the 1993 isolate being closely related to the 1991 Swedish isolate TAB/91 and the other three isolates from 1994 and 1996 being more closely related to each other. This pattern is reflected in the phylogenetic data calculated from nucleotide sequencing of the haemagglutinin genes. H3N8 equine influenza can be seen to be evolving in two distinct lineages, one European and one American. The 1993 isolate is closely related to the European lineage and is the most recent Swedish strain of this lineage to be isolated. The 1994 and 1996 isolates fit into the American lineage, which contains recent isolates from the United States and also Britain. These results indicate that American-type H3N8 viruses have become endemic in Sweden and, in light of the antigenic differences which can be observed between viruses belonging to the two lineages, we believe that equine influenza virus vaccines should be updated with an American-type virus strain.

## INTRODUCTION

H3N8 equine influenza virus is a common upper respiratory pathogen of equines. The virus causes acute respiratory disease, with subsequent fever and loss of condition. The disease causes significant discomfort to the individual horse, and predisposes to secondary bacterial infection as well as causing significant losses to the equine industry due to cancellation of competitions and interrupted training of horses. Equine influenza has also been proposed as a component in the aetiology of chronic obstructive pulmonary disease (COPD) in equine species [1].

The H3N8 virus has been shown to circulate in many countries, with the exception of Australia and New Zealand. Due to the ubiquitous nature of the virus, vaccination of horses is common practice. Despite extensive vaccination, however, the virus continues to circulate. Earlier studies have indicated that H3N8 equine influenza virus, like its human counterpart H3N2, is subjected to antigenic drift [2–9]. This has been proposed to explain the reduction in vaccine efficacy seen when vaccinated animals are infected with virus strains phylogenetically distant from the vaccine strain [10, 11].

Antigenic drift of influenza virus is attributed to cumulative mutations in the haemagglutinin (HA) surface antigen. This protein is the major target of neutralizing antibodies, and mutation enables escape from neutralizing antibody responses (reviewed in [12]). Because of the continual emergence of new

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antigenic variants, surveillance of new equine influenza virus strains is a priority if vaccines are to be updated [13].

Here we present results of antigenic and genetic characterization of recent strains of equine influenza virus isolated in Sweden. The data indicate that the most recent Swedish strains belong to a distinct phylogenetic branch when compared to previous Swedish epidemic variants.

## MATERIALS AND METHODS

### Viruses and viral RNA

The A/equi 2/Avesta/93, A/equi 2/Söderala/94, A/equi 2/Bollnäs/96 and A/equi 2/Älvdalen/96 viruses characterized in this study were obtained from the repository at the Swedish National Veterinary Institute. The origins and designations of viruses are listed in Table 1. Viruses were grown in 11-day-old embryonated hens' eggs. Virus to be used for serological analysis was stored as allantoic fluid at −70 °C, and virus for RNA extraction was purified by differential sedimentation through sucrose gradients. Virus RNA was obtained by sodium dodecyl sulphate and proteinase K treatment, followed by phenol/chloroform (1:1) extraction [14].

### Preparation of monoclonal antibodies

Monoclonal antibodies were prepared as described elsewhere [15] using the BRE/79, SOL/79 and BRL/91 strains of H3N8 equine influenza as antigens. After screening potentially positive antibodies using the haemagglutination inhibition (HI) assay with homologous virus, stocks from positive clones were prepared from cell culture supernatants by ammonium sulphate precipitation [15]. These stocks were then used for HI assays.

### Serological tests

HI assays were performed in microtitre plates with 1% chick erythrocytes as described by Klingeborn and colleagues [2]. All antibody–virus combinations were assayed five times, and the geometric mean was calculated from these values.

### Generation of cDNA copies of HA genes

cDNA copies of the HA genes were prepared from virion RNA by two methods. For the SOD/94 isolate, virion RNA was transcribed and cloned as a library using the Pharmacia Timesaver cDNA kit (Pharmacia Biotech). Clones encoding SOD/94 HA sequences were selected by colony hybridization using random-primed probes complementary to cDNA copies of TAB/91 HA [10]. For the AVE/93, BOL/96 and ALV/96 strains, virion RNA was reverse transcribed according to the method described by Huddleston & Brownlee [16]. In both cases, the 13-mer oligonucleotide reported to be complementary to the 3′ ends of all influenza virus segments [17] was used for first-strand synthesis.

### PCR amplification of cDNA from AVE/93, BOL/96 and ALV/96 strains

First-strand cDNA was used as template for PCR using primers complementary to nucleotides 9–19 and 1733–1745. PCR was performed using VentR polymerase (New England Biolabs) and a thermal cycle consisting of denaturation at 94 °C for 45 s, annealing at 60 °C for 1 min and elongation at 72 °C for 1·5 min. The cycle was repeated 32 times. Products were analysed by agarose gel electrophoresis.

### Sequencing of HA genes

Plasmid DNA encoding the SOD/94 HA was sequenced using the Pharmacia T7 sequencing kit and a set of primers specific for the HA gene described previously [8]. PCR products from AVE/93, BOL/96 and ALV/96 were sequenced on an ABI automated sequencer using the primers described previously [8].

### Sequence analysis

Sequence data were analysed using the UWGCG software package [18]. Phylogenetic analysis of amino acid sequence data was performed with the PHYLIP software package [19]. A distance matrix was calculated and an unrooted tree was fitted using the Fitch program.

## RESULTS

### Antigenic analysis

Antigenic analysis of the equine influenza strains characterized in this study was performed using monoclonal antibodies raised against the BRE/79 strain (ISCA2:1, 2ISCA2:2 and 2ISCA2:12), SOL/79 (MS23) and BRL/91 (MB series) in HI assays. The

Table 1. *Strains of equine influenza with which the genomes of four Swedish isolates* (*in bold*) *were compared*

| Abbreviation | Full strain name | Country | Reference | Acc. no.* |
|---|---|---|---|---|
| MIA/63 | Miami/1/63 | USA | (5) | M24719 |
| URG/63 | Uruguay/1/63 | Uruguay | (5) | M24718 |
| FRA/67 | France/67 | France | (7) | |
| TKY/2/71 | Tokyo/2/71 | Japan | (5) | M24720 |
| TKY/3/71 | Tokyo/3/71 | Japan | (7) | |
| ALG/72 | Algiers/72 | Algeria | (5) | M24721 |
| NEW/76 | Newmarket/76 | UK | (5) | M24722 |
| FRA/76 | France/76 | France | (25) | M73773 |
| FON/79 | Fontainebleau/79 | France | (5) | M24723 |
| SOL/79 | Solvalla/79 | Sweden | (8) | Y14054 |
| BRE/79 | Brentwood/79 | UK | | |
| ROM/80 | Romania/80 | Romania | (5) | M24724 |
| KEN/81 | Kentucky/1/81 | US | (7) | |
| ABY/84 | Åby/84 | Sweden | (8) | Y14055 |
| SAN/85 | Santiago/85 | Chile | (5) | M24725 |
| TEN/86 | Tennessee/5/86 | USA | (5) | M24726 |
| KEN/86 | Kentucky/2/86 | USA | (5) | M24727 |
| KEN/87 | Kentucky/1/87 | USA | (5) | M24728 |
| BHI/87 | Bhiwani/87 | India | (26) | |
| LUD/87 | Ludhiana/87 | India | (26) | |
| BRA/87 | Brazil/87 | Brazil | (7) | |
| LPL/88 | La Plata/88 | Argentina | (7) | |
| SKA/88 | Skara/88 | Sweden | (8) | Y14053 |
| ELL/89 | Ella/89 | Holland | (9) | X85086 |
| SUS/89 | Sussex/89 | UK | (9) | X85090 |
| SUF/89 | Suffolk/89 | UK | (11) | X68437 |
| YVE/89 | Yvelines/2136/89 | France | (21) | D30679 |
| JIL/89 | Jilin/1/89 | China | (23) | M65018 |
| VIS/90 | Visingsö/90 | Sweden | (8) | Y14056 |
| KEN/90 | Kentucky/90 | USA | (27) | L39915 |
| ARU/91 | Arundel/91 | UK | (9) | X85085 |
| IBA/6/91 | Ibadan/6/91 | Nigeria | (22) | X95637 |
| IBA/9/91 | Ibadan/9/91 | Nigeria | (22) | X95638 |
| ROME/91 | Rome/5/91 | Italy | (21) | D30684 |
| BRL/91 | Borlänge/91 | Sweden | (10) | |
| TAB/91 | Täby/91 | Sweden | (10) | S64310 |
| KEN/91 | Kentucky/91 | USA | (27) | L39918 |
| ALA/91 | Alaska/1/91 | USA | (21) | D30680 |
| HON/92 | Hong Kong/J/92 | Hong Kong | (28) | L27597 |
| LAM/92 | Lambourn/92 | UK | (9) | X85087 |
| KEN/92 | Kentucky/92 | USA | (27) | L39917 |
| **AVE/93** | **Avesta/93** | **Sweden** | **This paper** | **Y14057** |
| NEW/1/93 | Newmarket/1/93 | UK | (9) | X85088 |
| NEW/2/93 | Newmarket/2/93 | UK | (9) | X85089 |
| ARG/93 | Argentina/93 | Argentina | (9) | L39913 |
| LPL/93 | La Plata/93 | Argentina | (21) | D30686 |
| FLO/93 | Florida/93 | USA | (27) | L39916 |
| KEN/94 | Kentucky/94 | USA | (9) | L39914 |
| **SOD/94** | **Söderala/94** | **Sweden** | **This paper** | **Y14058** |
| **BOL/96** | **Bollnäs/96** | **Sweden** | **This paper** | **Y14060** |
| **ALV/96** | **Alvdalen/96** | **Sweden** | **This paper** | **Y14059** |

* EMBL database accession numbers.

Table 2. *Haemagglutination-inhibition titres of monoclonal antibodies*

| | Monoclonal antibody | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Virus | ISCA2:1* | 2ISCA2:2* | 2ISCA2:12* | MS23† | MB6‡ | MB19‡ | MB21‡ | MB22‡ |
| MIA/63 | 438§ | 69 | 27 | 594 | < 4 | < 4 | < 4 | < 4 |
| SOL/79 | 174 | 174 | 276 | 594 | < 4 | < 4 | < 4 | < 4 |
| ABY/84 | < 4 | 109 | 22 | 374 | < 4 | < 4 | < 4 | < 4 |
| SKA/88 | 438 | 174 | 69 | 551 | < 4 | < 4 | < 4 | < 4 |
| VIS/90 | < 4 | 138 | < 4 | 74 | 187 | 34 | 9 | 297 |
| TAB/91 | < 4 | 138 | < 4 | 59 | 187 | 187 | 472 | 297 |
| AVE/93 | < 4 | 138 | < 4 | 37 | 297 | 236 | 187 | 276 |
| SOD/94 | 438 | 138 | < 4 | 695 | < 4 | 8 | < 4 | < 4 |
| BOL/96 | 438 | 219 | < 4 | 437 | < 4 | < 4 | < 4 | < 4 |
| ALV/96 | 297 | 174 | < 4 | 374 | < 4 | < 4 | < 4 | < 4 |

* Made against virus strain BRE/79.
† Made against virus strain SOL/79.
‡ Made against virus strain BRL/91.
§ Figure is reciprocal of geometric mean titre calculated from five assays.

BRE/79 has been demonstrated to be closely antigenically related to SOL/79 (data not shown). The BRL/91 strain has previously been shown to be antigenically closed related to the TAB/91 strain [10]. Four basic patterns of reactivity were found (Table 2). 2ISCA2:2 and MS23 react with all strains used in the study. 2ISCA2:12 reacts only with strains isolated before 1990. MB6, MB19, MB21 and MB22 react with strains isolated between 1990 and 1993. ISCA2:1 reacts with strains isolated before 1990 (with the exception of ABY/84) and strains isolated after 1993.

The existence of a pre-1988 epitope, absent from later strains, indicates that the cross-reactivity of pre-1990 and post-1993 strains with ISCA2:1 is not due to re-emergence of an early virus, but rather to reversion of an epitope. The four anti-BRL/91 antisera recognize an epitope unique to the 1990–3 strains. This epitope could be linked to the epitope recognized by MS23, because a reduction in binding of this antibody is seen in the 1990–3 strains.

### Nucleotide sequences of recent Swedish H3N8 isolates

The first 1060 nucleotides of the HAs of AVE/93, SOD/94, BOL/96 and ALV/96 were determined, and the nucleotide sequences have been submitted to the EMBL database (accession numbers listed in Table 1).

### Amino acid sequences

Figure 1 shows an alignment of deduced amino acid sequences of HA proteins of strains sequenced in this study and in previous studies by other authors (Table 1). BRA/87 and ALV/96 have not been included in the alignment because their deduced amino acid sequences are identical to LPL/88 and BOL/96 respectively.

All HA1 sequences are of the same length with the exception of JIL/89, which contains one extra amino acid at position number four. Since this amino acid is present in this strain only, it has not been included in the numbering (noted as a question mark in the MIA/63 sequence). Apart from the JIL/89 virus, all strains shown in the alignment display cumulative changes in amino acid sequence indicative of antigenic drift. The glycosylation pattern of the different strains is variable at four positions; amino acids 3, 53, 133 and 285. Two of these positions are located at presumptive antigenic sites (see below).

### Positions of antigenic regions and amino acids involved in the receptor activity of the HA1

The position of antigenic regions on the HA1 has been extrapolated from studies performed on human H3N2 influenza virus (reviewed in [20]). The position of these antigenic regions, as well as the exact location of amino acids within these is shown in Figure 1.

Amino acid residues which are important in forming the receptor-binding structure located at the tip of the HA1 molecule are also shown in Figure 1. These positions are also extrapolated from studies performed on human H3N2 influenza virus (reviewed in [20]).

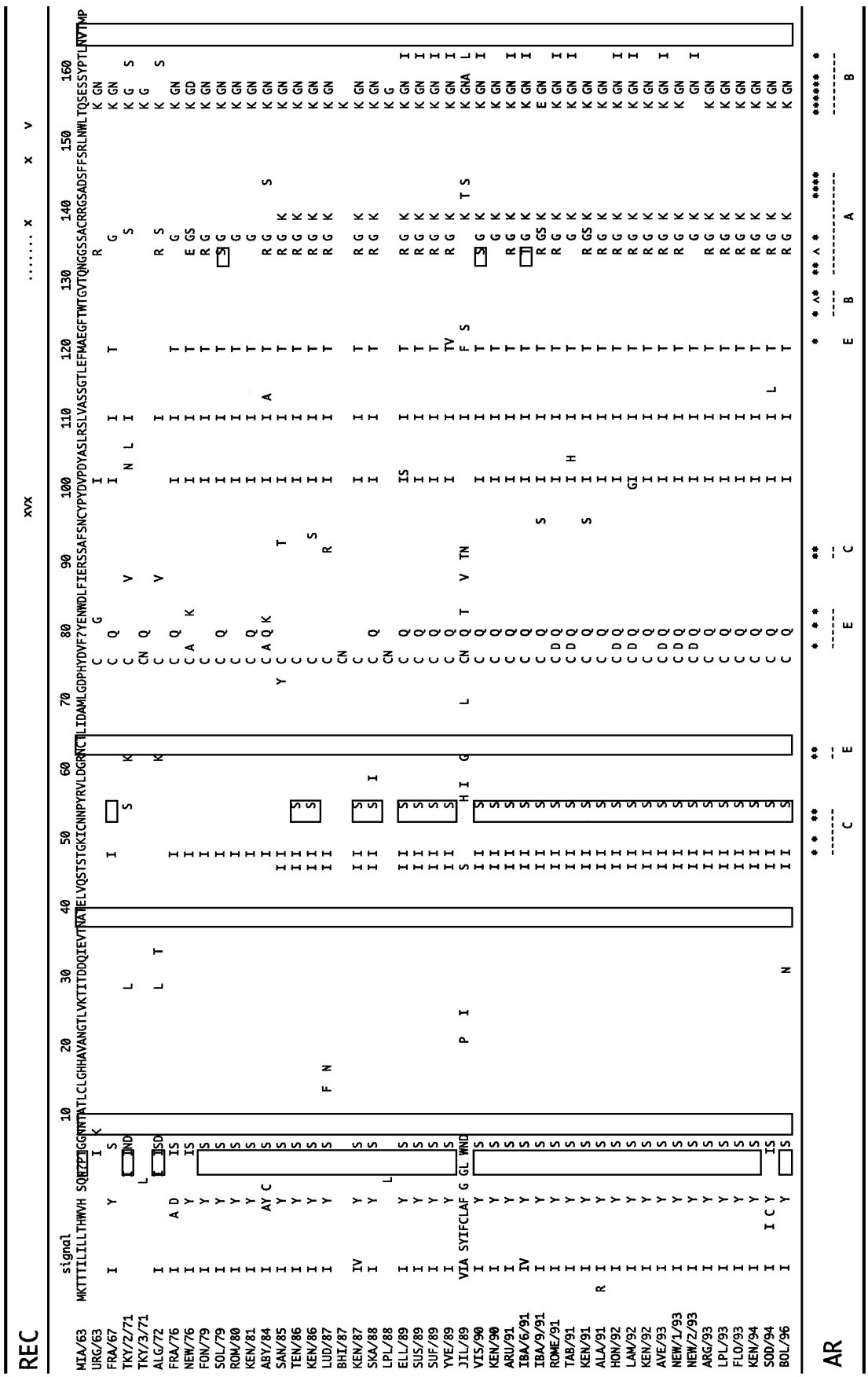


**Fig. 1.** For legend see page 66.

(*b*)

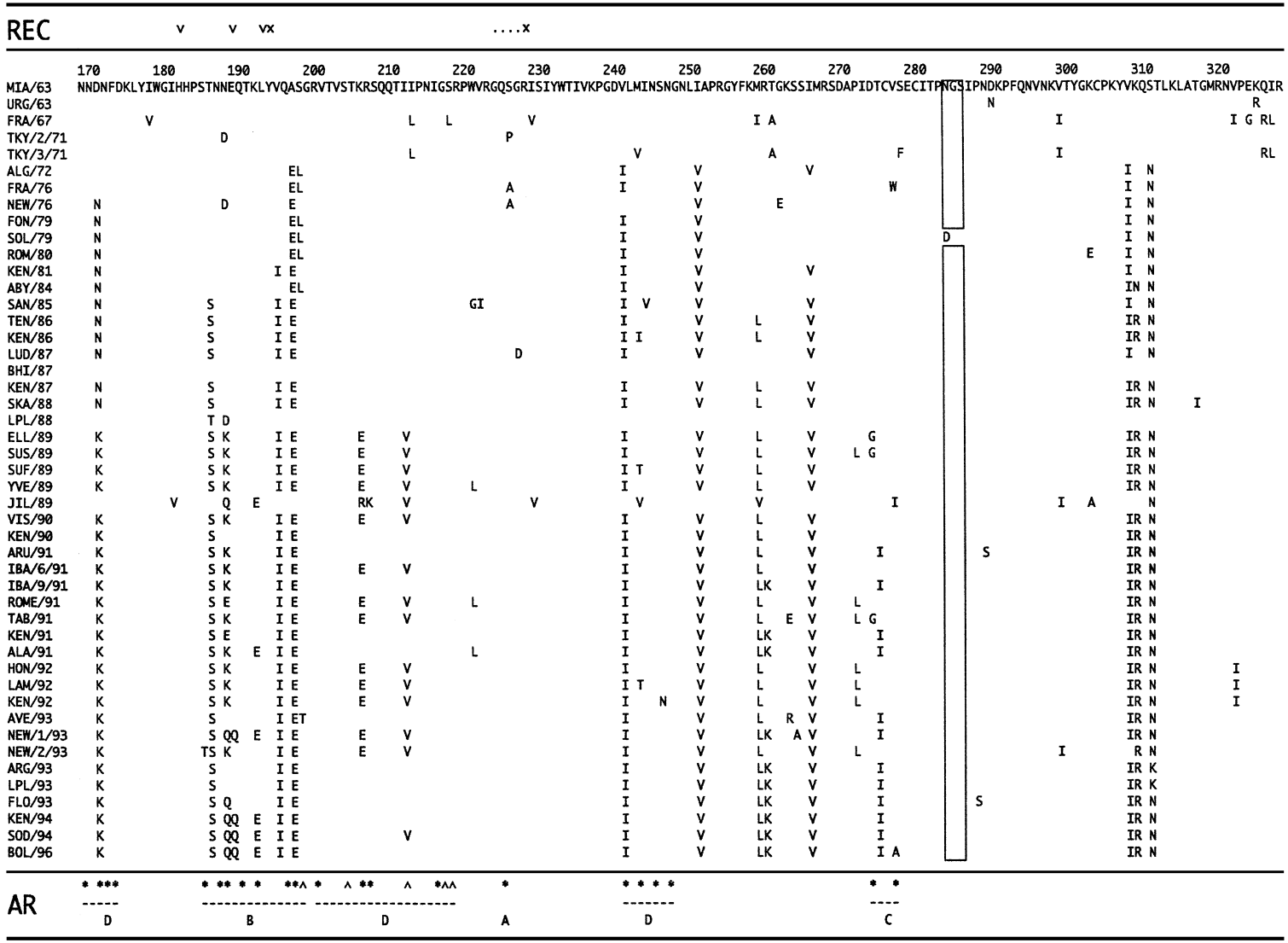


**Fig. 1.** Comparison of deduced amino acid sequences listed in Table 1. Where present, amino acid substitutions are written below the MIA/63 sequence. Possible N-linked glycosylation sites are boxed. The positions of amino acids involved in the receptor activity of HA are shown above the sequence alignment in the row named REC. v, amino acids which make up the receptor binding pocket structure; x, amino acids involved in stabilizing the pocket; ... amino acids which can interact with the receptor. The positions of amino acids which have been identified as components of antigenic regions, and their corresponding antigenic regions, are shown below the sequence alignment on the row designated AR. * amino acids found to vary in epidemic strains of human H3N2 influenza virus; ^ amino acids found to vary in laboratory-selected escape mutants.

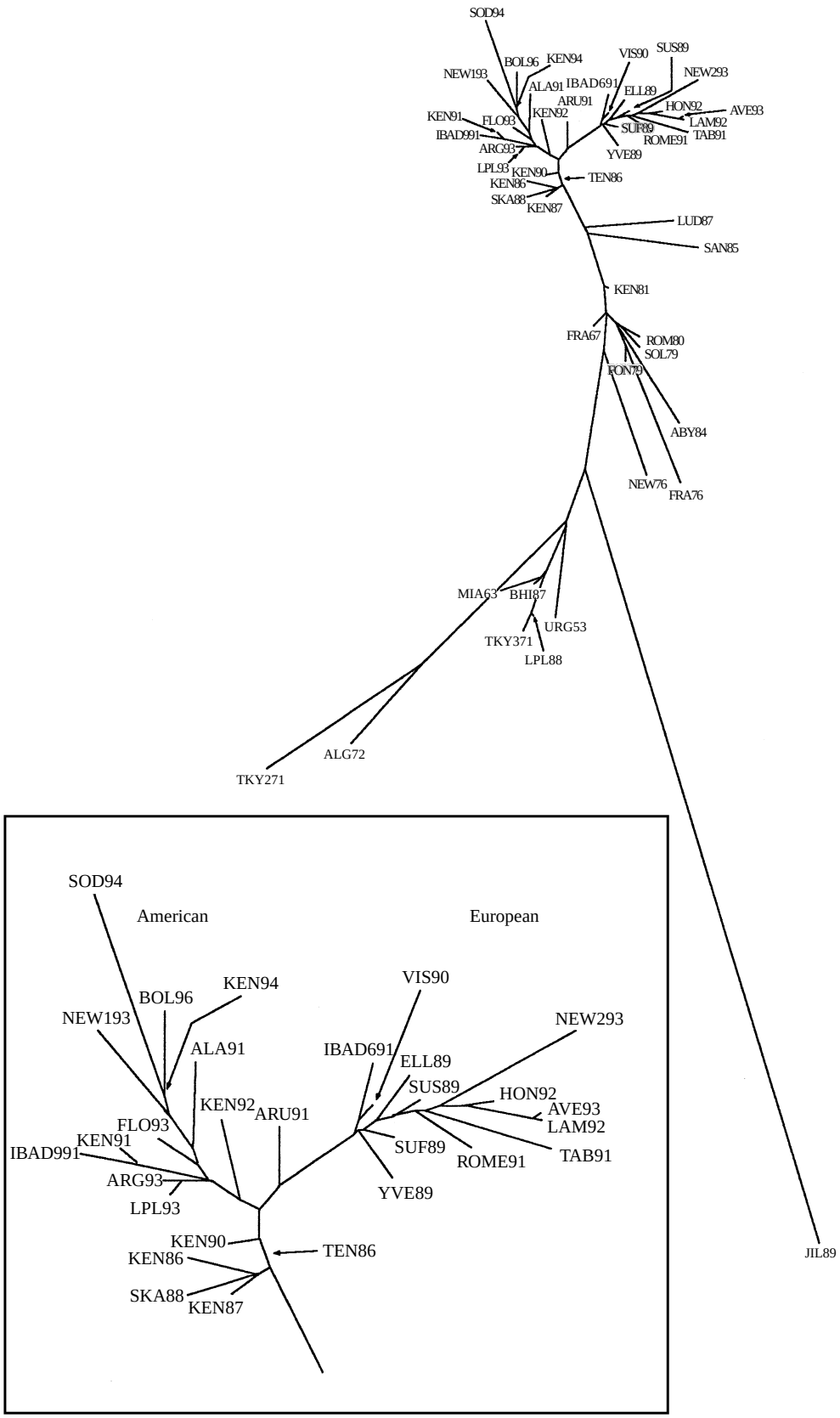


Fig. 2. Phylogenetic tree for all equine H3N8 influenza virus strains available in the EMBL database. Inset is an enlargement of the head of the tree showing the division of recent strains into two separate branches.

### Phylogeny of HA1s of strains isolated between 1963 and 1996

The amino acid sequences shown in Figure 1 were used to construct a phylogenetic tree depicting the evolution of equine influenza from 1963 to 1996. The phylogenetic tree, calculated using the Fitch algorithm, is shown in Figure 2. All H3N8 equine influenza sequences available from the EMBL database were used in this study.

The phylogenetic tree shows a pattern of relatively linear evolution up to 1988, after which the main lineage divides into two branches, one branch containing mainly American isolates and the other containing mainly European isolates.

## DISCUSSION

The results of this study indicate that recent equine influenza viruses isolated in Sweden can be divided into two separate evolutionary lineages. This division can be made both at the serological and sequence levels.

The main conclusions which can be drawn from the antigenic analysis presented in this paper are: (1) that there are epitopes which are conserved among the different strains; (2) that there is an epitope which mutated after the 1988 isolate; (3) that there is an epitope which is shared by the 1990, 1991 and 1993 isolates which has subsequently been lost; and (4) that there is an epitope which is shared by three of the early strains (MIA/63, SOL/79 and SKA/88) and the three most recent strains (SOD/94, BOL/96 and ALV/96). These results indicate that the H3N8 equine influenza virus has split into two discrete serological lineages after the 1988 isolation, the 1990–3 strains belonging to one branch, and the 1994 and 1996 strains belonging to the other. Data using polyclonal antisera raised against equine influenza also support this finding. Daly and colleagues [9] found that antisera raised against a European lineage virus (NEW2/93) had several-fold reduced HI titre against American lineage virus (NEW1/93) as compared to titres against European lineage viruses. This finding emphasizes the significance of the American/European lineage dichotomy.

Phylogenetic analysis of the primary amino acid sequences of equine H3 HA1s demonstrates the division of post-1988 strains into two lineages: a European lineage, to which the VIS/90, TAB/91 and AVE/93 strains belong, and an American lineage, to which the SOD/94, BOL/96 and ALV/96 strains belong. This observation correlates well with findings by Lindstrom and colleagues [21] and Daly and colleagues [9], who previously noted this phylogenetic division. Interestingly, co-circulation of strains belonging to both lineages was shown by Daly and colleagues [9], as well as by Adeyefa and colleagues [22]. The question of co-circulation cannot be addressed in our study, because no virus belonging to the European lineage has been isolated after 1993.

The JIL/89 strain is evolutionarily distant from all other equine H3N8 strains used in this study. This strain has been proposed to be an avian virus which infects equines, without genetic re-assortment [23], and is therefore distantly related to the equine H3N8 viruses.

The question of the location of antigenic sites on equine H3 has been taken up in much previous work, and generally the sites of human H3N2 influenza virus have been extrapolated directly [5, 8–11]. Judging from the correlation seen between sites of amino acid variation found in epidemic equine H3N8 strains, and the positions of antigenic regions seen in human strains, this approach seems valid. There are, however, areas outside the extrapolated antigenic regions on the equine H3 HA in which similar variability can be seen. The area from amino acids 252–267 is the most obvious, with cumulative changes occurring at four positions. Other positions include amino acids 102, 111, and 309–312.

Amino acid substitutions can be seen at all antigenic regions, but region B appears to be under intense selective pressure. This region is placed at the tip of the HA molecule, and variability in this region has been recorded among human H3 strains.

As has been observed for the human H3 HA1, sequential changes of amino acid at a single site are unusual [24]. In the majority of cases, amino acids are conserved following the mutation event.

Taken together, the existence of regions of variability, which are not mapped as antigenic regions on human H3N2 influenza, and the fact that few amino acid changes in H3 equine HAs occur exactly at the same amino acid position as they do in human H3 influenza, suggest either that the equine immune system differs in its recognition of the H3 protein or that structural differences between the two H3s (amino acid identity approximately 80%) lead to differences in accessibility of amino acids to antibody.

Amino acids involved in receptor binding of the HA can be divided into three groups: amino acids

which make up the receptor binding pocket, amino acids which are involved in stabilizing the pocket and amino acids which potentially interact with the receptor (see Fig. 1). Amino acids of the former two groups can be seen to be well conserved, with the exception of site 190 at which a glutamic acid is replaced by glutamine. The consequence of this change is at the moment obscure, since the viruses containing the mutation grow well in embryonated hens' eggs, and no difference in infectivity for avian cells can be detected. Amino acids belonging to the latter group are less well conserved. However, no variability can be seen at position 226, which has been implicated in the preferential affinity of equine and avian H3 viruses for NeuAc$\alpha$2-3Gal and human H3 for NeuAc$\alpha$2-6Gal.

The results presented in this study show that American-type equine influenza strains are circulating in Sweden. In view of the antigenic difference which is seen between representatives of this lineage and the European lineage, we believe that equine influenza vaccines for use in this country should include a representative of the newly introduced virus lineage.

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