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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

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Abstract

In December 2005, equine influenza virus infection was confirmed as the cause of clinical respiratory disease in vaccinated horses in Apulia, Italy. The infected horses had been vaccinated with a vaccine that contained strains representatives from both the European (A/eq/Suffolk/89) and American (A/eq/Newmarket/1/93) H3N8 influenza virus lineages, and the H7N7 strain A/eq/Praga/56. Genetic characterization of the hemagglutinin (HA) and neuraminidase (NA) genes of the virus from the outbreak, indicated that the isolate (A/eq/Bari/2005) was an H3N8 strain closely related to recent representatives (Kentucky/5/02-like) of the American sub-lineage Florida, that was introduced in Italy through movement of infected horses from a large outbreak described in 2003 in United Kingdom. Strain A/eq/Bari/2005 displayed 9 amino acid changes in the HA1 subunit protein with respect to the reference American strain A/eq/Newmarket/1/93 contained in the vaccine. Four changes were localized in the antigenic regions C–D and likely accounted for the vaccine failure.
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1. Introduction

Equine influenza is a severe acute respiratory disease, caused by a type A influenza virus, family Orthomyxoviridae. Symptoms include pyrexia, dyspnoea, anorexia and coughing and frequent sequelae are chronic obstructive pulmonary disease and bacterial

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superinfection (Gerber, 1969; Mumford et al., 1990). The infection in horses is highly diffusive and the control requires preventive vaccination and, to avoid virus spread from influenza outbreaks, interruption of activities inherent equine reproduction and competitions.

Two different subtypes of equine influenza virus, H7N7 and H3N8, have been associated with disease in the horse. H7N7 was first recognized in 1956 in Eastern Europe but it has not been isolated from horses for nearly 25 years and it is presumed not to circulate at the present time (Sovinova et al., 1958; Webster, 1993). H3N8 was first documented in 1963 in Florida (Waddell et al., 1963) and subsequently it was described throughout the world (Burrows and Denyer, 1982; Burrows et al., 1981; Hinshaw et al., 1983; Thomson et al., 1977). Recent studies of the H3N8 subtype of equine influenza viruses have demonstrated that, starting from the early 1990s, these strains have diverged into two distinct evolutionary lineages designated as European (A/eq/Suffolk/89-like) and American (A/eq/Newmarket/1/93-like), while the oldest H3N8 strains, circulating in the 1970 and 1980s (A/eq/Fontainebleau/76-like) and the 1960s (Miami/63-like) apparently went extinct (Borchers et al., 2005; Daly et al., 1996; Lai et al., 2001; Manuguerra et al., 2000; Oxburgh et al., 1994; Oxburgh and Klingeborn, 1999).

Five antigenic sites (A–E) on the three-dimensional structure of the HA protein of A/Aichi/2/68 (H3N2) have been mapped (Wiley et al., 1981; Wilson and Cox, 1990). H3 human and equine strains are thought to be evolutively related and the HA of A/eq/Miami/63 and A/Aichi/68 share more than 85% aa identity (Bean et al., 1992). Analysis of the mutational patterns of H3 humans strain revealed that the antibody-binding sites are under strong positive selection, suggesting that changes in the antigenic regions enhance virus survival (Bush et al., 1999). Representatives of the American and European H3N8 lineages, A/eq/Suffolk/89 and A/eq/Newmarket/1/93, differ in nine residues throughout the HA antigenic regions A–D. A number of studies have investigated the functional significance of this phylogenetic dichotomy and antigenic drift, suggesting that inter-lineages differences may compromise cross-lineage protection after vaccination or infection and representatives of both H3N8 lineages are now included in current vaccines (Crouch et al., 2004; Daly et al., 1996, 2004; Oxburgh and Klingeborn, 1999; Yates and Mumford, 2000; Mumford, 2003). Strains within the American lineage further segregate into at least three distinct sub-lineages, namely Florida, Kentucky and Argentina (Lai et al., 2004).

In December 2005, an outbreak of respiratory disease was reported in horses vaccinated against influenza in Martina Franca, Bari, Italy. Analysis of the strains provided convincing evidence for the circulation in Italy of strains closely related to recent American strains (Kentucky/5/02-like) within the sub-lineage Florida.

2. Materials and methods

2.1. Outbreak description

In December 2005, an acute respiratory disease appeared in a horse center managed by the Forest Service Rangers in Martina Franca, Apulia, Italy. Thirty-three adult horses (3–6 years old) were housed in the yard. The animals had been vaccinated for equine influenza during the previous 3 months, using a carbopol/aluminum hydroxide-adjuvanted vaccine that contained strains representatives from both the European (A/eq/Suffolk/89) and American (A/eq/Newmarket/1/93) H3N8 influenza virus lineages, and the H7N7 strain A/eq/Praga/56. A total of 24 out of 33 animals presented pyrexia, dyspnoea, anorexia and coughing, but all the animals recovered from the disease over a nearly 2-week-period. One week after the onset of the respiratory signs in the yard, nasal swabs from four animals presenting fever and nasal discharge were collected and sent to our laboratories for bacteriological and virological investigations. The RNA was extracted using the RNeasy Kit (Qiagen, Gmbh, Germany) according to the manufacturer's instructions, and eluted in 20 μl of TE (Tris–EDTA) buffer (pH 8.0). Influenza A RNA (5.15×10^3–2.03×10^5 copies/μl of template) was detected by a real time PCR with primer pair *Flu-M32For*/*Flu-M179Rev* that targets the M gene (Di Trani et al., 2006) in all the nasal swabs.

2.2. Virus isolation

Nasal swab samples were inoculated into the allantoic cavities of 10-day embryonated hens' eggs

after the addition of antibiotics and antimycotics. The eggs were harvested after 3 days, and the allantoic fluid was tested for the presence of virus by hemagglutination assay with 1% chicken erythrocytes in phosphate-buffered saline. Sequential passages in embryonated hen's egg were made until HA titers ranging from 1:128 to 1:256 were obtained.

2.3. Sequence and phylogenetic analysis

The full-length HA and NA genes were reverse transcribed and amplified with primers *Bm-H1*/*Bm-NS 890R* and *Ba-Na1*/*Ba-Na11413R*, respectively. To avoid accumulation of adaptive mutations during cultivation in eggs, the RNA extracted from the nasal swabs was used (Hoffman et al., 2001). To obtain a PCR product for sequencing of the HA and NA genes, 0.5 μl each of primers (50 pmol/μl) was added to a total of 49 μl of the reaction mixture containing 0.2 mM of each dNTP, 1.2 mM $MgSO_4$ and 1 μl of a mix of SuperScript II H—Reverse Transcriptase and Platinum Taq HiFi (Invitrogen—Life Technologies, Milan, Italy). The RNA was reverse transcribed and immediately subjected to PCR amplification in a single-step protocol, using SuperScript One-Step RT-PCR kit (Invitrogen—Life Technologies, Milan, Italy). Reverse transcription was carried out at 48 °C for 60 min, followed by denaturation of the reverse transcriptase at 95 °C for 2 min. Amplification was conducted by a temperature cycling protocol consisting of 35 cycles of 30 s of denaturation at 94 °C, 1 min of primer annealing at 55 °C, and 1 min of extension at 68 °C, followed by 10 min of the final extension phase at 68 °C.

The PCR products were purified with the Wizard PCR Preps DNA Purification System (Promega, Madison, Wis). Then, the DNA was used as template for direct sequencing. The DNA was sequenced by using the conserved primers and specific primers designed according to an overlapping strategy. The sequences were assembled using Bioedit software package version 2.1 (Hall, 1999.) and compared to cognate sequences in the genetic databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and FASTA (http://www.ebi.ac.uk/fasta33) web-based programs. The HA sequence of the Italian strain A/Eq/Bari/2005 is available in GenBank under accession numbers EF117330. Phylogenetic reconstruction was carried out using the MEGA software version 3.0 (Kumar et al., 2004) using the Kimura 2-parameter model as a method of substitution and the UPGMA method to construct the phylogenetic tree. The statistical significance of the phylogenies inferred was estimated by bootstrap analysis with 1000 pseudoreplicate data sets.

3. Results

The HA protein of strain A/eq/Bari/2005 displayed the highest identity (99.1%) to American H3N8 strains within the Florida sub-lineage, such as strain A/Eq/Kentucky/2002. There was only one 1 amino acid change, 213-Iso → Met, in region D between strain A/eq/Bari/2005 and A/Eq/Kentucky/2002. There were nine amino acid changes between the HA1 subunit protein of the reference strain A/eq/Newmarket/1/93, contained in the vaccine, and the HA of strain A/eq/Bari/2005. Four changes occurred in the antigenic regions C (48-Iso → Met), B (190-Gln → Glu and 193-Glu → Lys) and D (213-Iso → Met) (Fig. 1). The NA protein of strain A/eq/Bari/2005 displayed the highest identity (95.6%) to the H3N8 strain A/Equine/Tennessee/5/86, while identity to strain A/Eq/Kentucky/2002 was 94.2%.

In the HA phylogenetic tree (Fig. 2), H3N8 strains were clustered in well-defined groups (bootstrap values higher than 75%) according to a clear temporal pattern. The oldest strains, dating back to the 1960s and to the beginning of the 1970s, were included in lineage I, Miami/63-like. All the strains detected through the 1970s and mid-1980s were grouped in lineage II, Fontainebleau/76-like. All the strains detected after 1989–1990 segregated in two separate lineages. Lineage III (Eurasian) included strains circulating mostly in the European continent and the prototypes are strains A/eq/Newmarket2/93 and A/eq/Suffolk/89. Lineage IV (American) included strains detected prevalently in the American continent and the prototype is strain Newmarket1/93. Lineage IV was further distinguished into three discrete sub-lineages, IV-a (Argentina) IV-b (Kentucky), and IV-c (Florida).

4. Discussion

The mechanisms driving evolution of influenza viruses are accumulation of single point mutations,

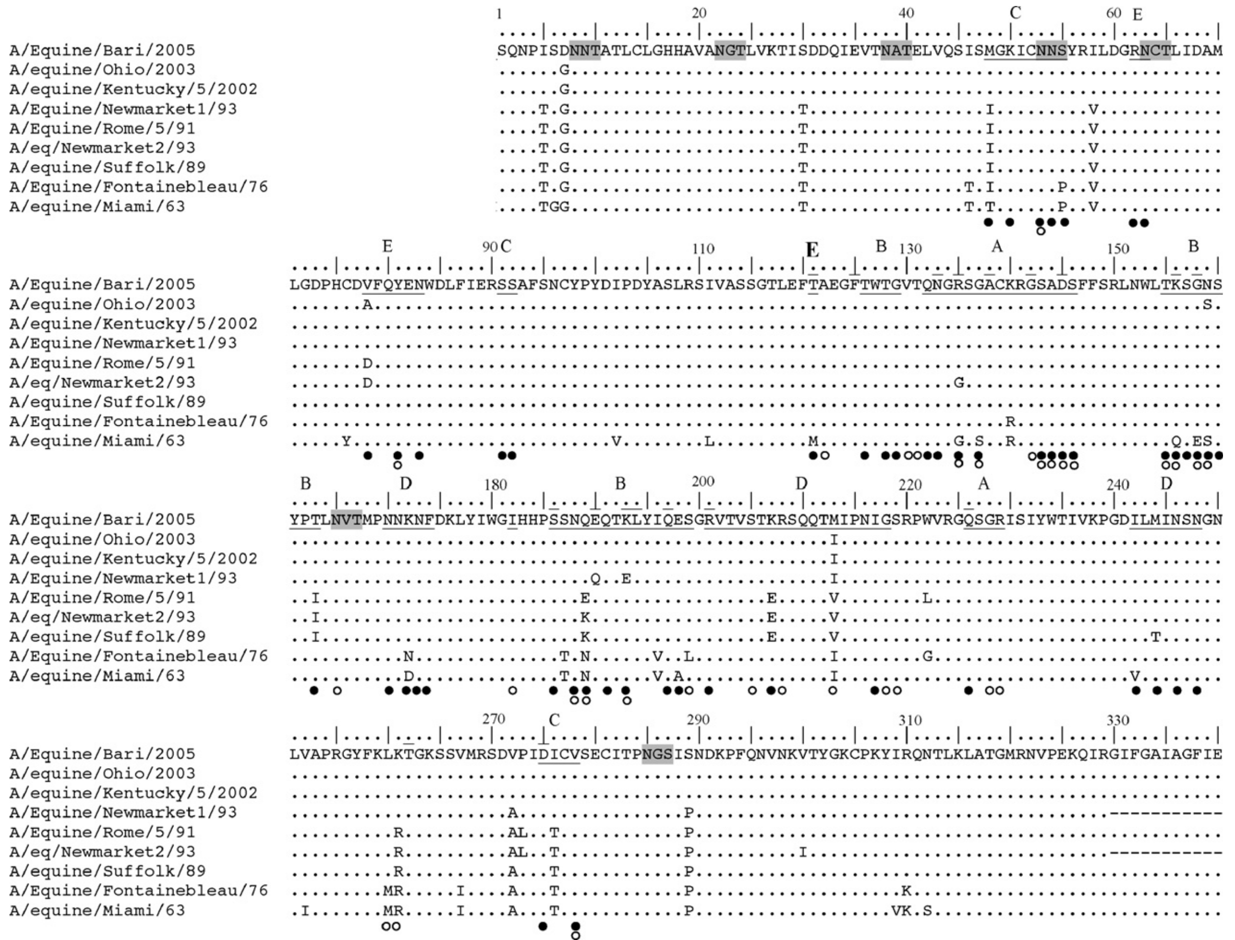

Fig. 1. Amino acid sequence alignment of strain A/eq/Bari/2005 with the HA1 of representative of the various H3N8 lineages and sub-lineages. Possible N-linked glycosylation sites are shaded in gray. (●) denotes an amino acid found to vary in epidemic strains of human influenza virus subtype H3N2, and (○) denotes an amino acid found to vary in laboratory-selected escape mutants (Oxburgh et al., 1994; Webster, 2002; Wiley et al., 1981; Mumford et al., 1990; Jin et al., 2005; Kida et al., 1987; Nakajima et al., 2005; Thomson et al., 1977). The positions of amino acids, which have been identified as components of antigenic regions, and their corresponding antigenic regions, are indicated (line under the amino acid letter). Residues under positive selection in human influenza virus subtype H3N2 are indicated (line above the amino acid letter) (Bush et al., 1999).

reassortment and interspecies transmission (Webster, 2002). Accumulation of single point mutations in the surface proteins steadily produces, over time, heavy structural and antigenic changes, affecting to various extent vaccine efficacy (Nakajima et al., 2005). A commonly held tenet for human influenza A viruses is that at least four amino acid changes in two separate antigenic sites of the HA are required for significant antigenic drift (Wilson and Cox, 1990), although only two amino acid changes may also alter virus antigenicity significantly (Jin et al., 2005), and a model based on the number of amino acid changes in the antigenic regions proved to be a good proxy for prediction of antigenic variation (Lee and Chen, 2004). Ten changes in vaccine composition have been recommended between 1986 and 1998 to account for new antigenic variants of human H3N2 influenza virus A (Hay et al., 2001).

In our and in other phylogenetic reconstructions based on the HA (Lai et al., 2004), H3N8 strains within the American lineage were found to diverge into at least three distinct sub-lineages, namely

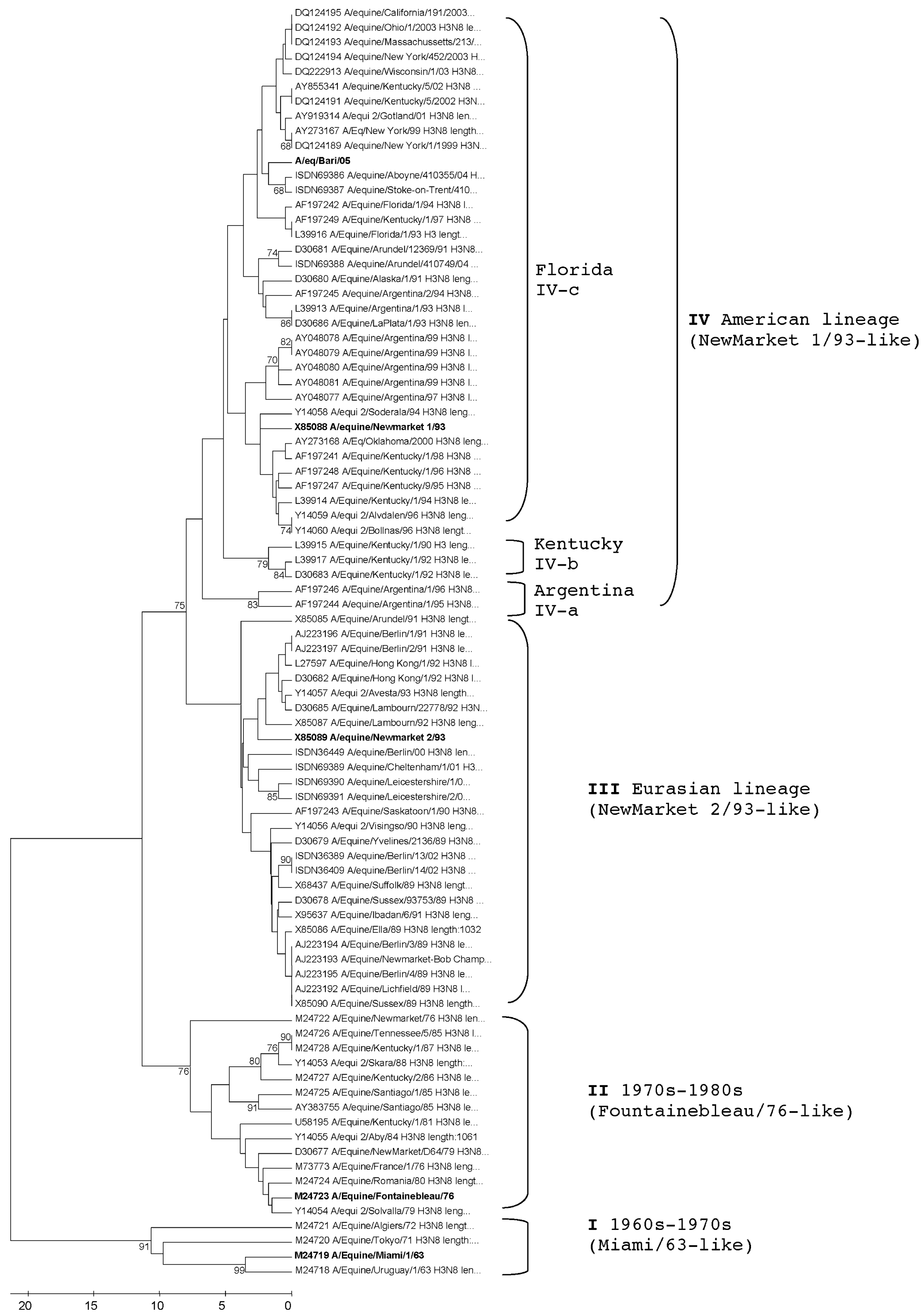

Fig. 2. Phylogenetic analysis of HA1 nucleotide sequences of the various H3N8 equine influenza virus strains. H3N8 prototypes are in boldface. Lineages were designated after Lai et al. (2001).

Argentina (IV-a), Kentucky (IV-b) and Florida (IV-c). Viruses of two such sub-lineages, IV-b and c, appear to circulate in an alternate fashion in the Unites States and to be antigenically distinct. Two amino acid changes in region B and D of HA within representatives of two sub-lineages correlate with up to 1:16 decrease in inhibition of hemagglutination (HI) titers between homologous and heterologous convalescent horse sera (Lai et al., 2004). This pattern of alternate circulation has been interpreted as a mechanism enabling virus persistence by decreasing the host immune pressure (Lai et al., 2004).

In Europe circulation of recent IV-c strains, Kentucky/5/02-like, was first documented in United Kingdom in 2003, where outbreaks of influenza were described regardless of the immunization status of the animal (Newton et al., 2006). Epidemiological tracing revealed that, in May 2003, Kentucky/5/02-like IV-c strains spread outside United Kingdom as a result of transport of horses from Newmarket to a racetrack in Rome, Italy, where an outbreak subsequently occurred (Newton et al., 2006). In our phylogenetic analysis (Fig. 2), a tight homology was found between strain A/eq/Bari/2005 and recent H3N8 strains from United Kingdom (A/eq/Stock-on-Trent/410956/04 and A/eq/Aboyne/410355/04). The findings of the present note provides evidence that Kentucky/5/02-like IV-c strains circulating at present in Italy are tightly related to the viruses from the 2003 outbreaks described in United Kingdom and Rome.

The Italian Kentucky/5/02-like strain A/eq/Bari/2005 differed in four changes throughout the antigenic regions C–D from the vaccine strain A/eq/Newmarket/1/93, that falls within the same genetic cluster IV-c. Whether those changes are responsible for the vaccine failure may be conjectured but it is not clear. The Italian strains appear to have originated from A/Eq/Kentucky/2002-like strains detected in United Kingdom in 2003 from which differ only in the change 213-Met → Iso in region D. Cross-evaluation by HI with ferret antisera failed to reveal substantial antigenic differences between the A/Eq/Kentucky/2002-like strains detected in United Kingdom in 2003 and the vaccine strain A/eq/Newmarket/1/93 (Newton et al., 2006), although those conclusions were apparently inconsistent with the numerous vaccine breakdowns observed in the field (Newton et al., 2006). Intriguingly, anecdotal evidence and preliminary experiments suggest that the 2003 English isolates are more pathogenic than older English strains (Newton et al., 2006). Accordingly, the increased virus pathogenicity, coupled with sub-optimal vaccine protection, could account for the occurrence of symptomatic infections in vaccinated horses.

Despite the intensive use of vaccines, equine influenza outbreaks still continue to occur and therefore it is pivotal to improve the vaccine efficacy or develop new vaccine strategies (Paillot et al., 2006). Recent IV-c American H3N8 equine strains appear to differ in 3–5 residues in the HA antigenic regions from the reference American strain A/eq/Newmarket/1/93, within the same sub-lineage, and inclusion in the vaccines of one such recent strains, A/eq/Ohio/03, has been recommended by OIEs guidelines. However, strain A/Eq/Bari2005 revealed three amino acid changes in the antigenic regions E (78-Ala → Val), B (159-Ser → Asn) and D (213-Met → Iso) with respect to the reference strain A/eq/Ohio/03. Residue 213 appears to be critical for maintenance of a neutralizing epitope, as evidenced by analysis of monoclonal antibody-selected escape mutants (Oxburgh and Klingeborn, 1999) and it is regarded as diagnostic to distinguish between American (IV) and Eurasian strains (III) (Daly et al., 1996). Such residue is Iso in the oldest strains (lineages I and II), while it is Val in lineage III viruses and it is retained as Iso in lineage IV strains, exhibiting a lineage-specific pattern that is suggestive of selective pressure. Accordingly, there is evidence that after their introduction in the European continent, recent IV-c H3N8 strains, Kentucky/5/02-like, have started diverging independently from the analogous strains spreading in Northern America and this should be kept into account for the regional update of the vaccine formulations.

In conclusion, in this study we reported a vaccine breakdown occurred in horses in December 2005 in Italy, due to the spread of a recent American H3N8 strain, of the Florida sub-lineage. Sequence analysis provided evidence for changes in the antigenic regions of the HA between the breakthrough strain and the homologous reference strain contained in the vaccine, that likely accounted for the vaccine failure. The findings of the present study reinforce the proposition that increased international movement of horses for breeding and competition purpose constitutes an

important factor in the spread of equine influenza throughout the world. Continual surveillance is necessary to monitor the onset of novel strains and to adequate the vaccines.

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