



Contents lists available at ScienceDirect

## The Veterinary Journal

journal homepage: [www.elsevier.com/locate/tvj](http://www.elsevier.com/locate/tvj)

## Review

## Equine influenza: A review of an unpredictable virus

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## ARTICLE INFO

## Article history:

Accepted 30 June 2010

Available online xxx

## Keywords:

Equine influenza

Evolution

Interspecies transmission

Pathogenicity

## ABSTRACT

This review discusses some of the challenges still faced in the control of equine influenza virus H3N8 infection. A widespread outbreak of equine influenza in the United Kingdom during 2003 in vaccinated Thoroughbred racehorses challenged the current dogma on vaccine strain selection. Furthermore, several new developments in the first decade of the 21st century, including transmission to and establishment in dogs, a presumed influenza-associated encephalopathy in horses and an outbreak of equine influenza in Australia, serve as a reminder of the unpredictable nature of influenza viruses. The application of newly available techniques described in this review may further elucidate some of the viral factors that underlie recent events and provide the tools to better evaluate when vaccine strains should be updated.

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## Introduction

Equine influenza is a common respiratory infection of horses caused by an orthomyxovirus of the genus influenza A. Influenza A viruses can be divided into sub-types on the basis of the antigenic reactivity of the surface glycoproteins, the haemagglutinin (H1–H16) and neuraminidase (N1–N9) molecules (Fouchier et al., 2005). The viruses currently circulating in horses are of the H3N8 sub-type. Although influenza viruses were first isolated in the 1930s, we still have much to learn about this pathogen. Influenza A viruses have only eight RNA gene segments encoding between them 11 or 12 proteins, yet the existence of two of these proteins was only reported in this decade (Chen et al., 2001; Wise et al., 2009).

Influenza is a classic example of a (re-)emerging infection. Vaccines against influenza have been used in man since the 1940s (Francis et al., 1945), and became available for use in horses 20 years later. However, the existence of a reservoir of virus in aquatic birds and the highly variable nature of the virus means that influenza defies worldwide eradication. This review focuses on some of the obstacles, highlighted by recent events that must still be surmounted if improvements are to be made in the control of equine influenza, while some areas for future research are suggested.

## Vaccination against equine influenza

The main targets of virus-neutralising antibodies are the surface glycoproteins (Askonas et al., 1982): haemagglutinin (HA) is respon-

sible for viral entry into host cells by binding to receptors on the cell surface; neuraminidase (NA) enzymatically releases newly synthesised virus particles from the cell. Vaccine strategies against influenza have traditionally focussed on generating robust antibody responses against the surface glycoproteins, particularly HA.

During the last century a number of improvements were made to methods for standardising inactivated virus vaccines against equine influenza (Wood et al., 1983). Adjuvants and antigen presentation systems were also enhanced to extend the duration of immunity induced by inactivated virus vaccines, although high levels of antibody are required for protection against field infection by these vaccines (Mumford et al., 1983). Alternatively, infection-induced immunity in horses can afford some protection against re-infection even in the absence of high levels of circulating antibody (Hannant et al., 1988; Bryant et al., 2010). Live attenuated or vectored equine influenza vaccines, which may better mimic the immunity generated by influenza infection than inactivated virus vaccines, are now available for use in horses. The various approaches to vaccination against equine influenza are the subject of a recent extensive review (Paillot et al., 2006).

## 21st Century outbreaks of equine influenza

Despite the extensive use of vaccines in some horse populations, outbreaks of equine influenza continue to occur. In 2003, there was a widespread outbreak of equine influenza among regularly vaccinated racehorses in Newmarket, UK (Newton et al., 2006; Barquero et al., 2007). Detailed analyses of factors associated with serologically- and/or virologically-confirmed influenza infection during this outbreak identified multiple significant factors

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and confirmed the complexity of achieving protective immunity at the population level (Barquero et al., 2007).

During 2003, two cases of influenza infection in un-vaccinated horses were seen to develop neurological signs (Daly et al., 2006). One of the horses was euthanased because the prognosis for recovery was poor and post mortem examination of its brain revealed viral-type non-suppurative encephalitis. Influenza virus infection was confirmed by positive immunostaining of sections of nasal mucosa. There was no evidence of equine herpesvirus infection (which can cause neurological disease) in either case. In the absence of an alternative explanation, it was proposed that infection with equine influenza virus had elicited neurological complications (Daly et al., 2006).

Influenza-associated encephalitis/encephalopathy (IAE) has been described during human influenza epidemics, albeit rarely, and is often consistent with serious sequelae or death (Toovey, 2008; Hjalmarsson et al., 2009). The pathogenic mechanisms behind IAE are unknown, but there is increasing evidence that the disease is associated with the innate immune response to infection, with elevated levels of the pro-inflammatory cytokines interleukin (IL)-6 and tumour necrosis factor (TNF)- $\alpha$  in serum and cerebrospinal fluid of affected patients (Togashi et al., 2004; Hidaka et al., 2006).

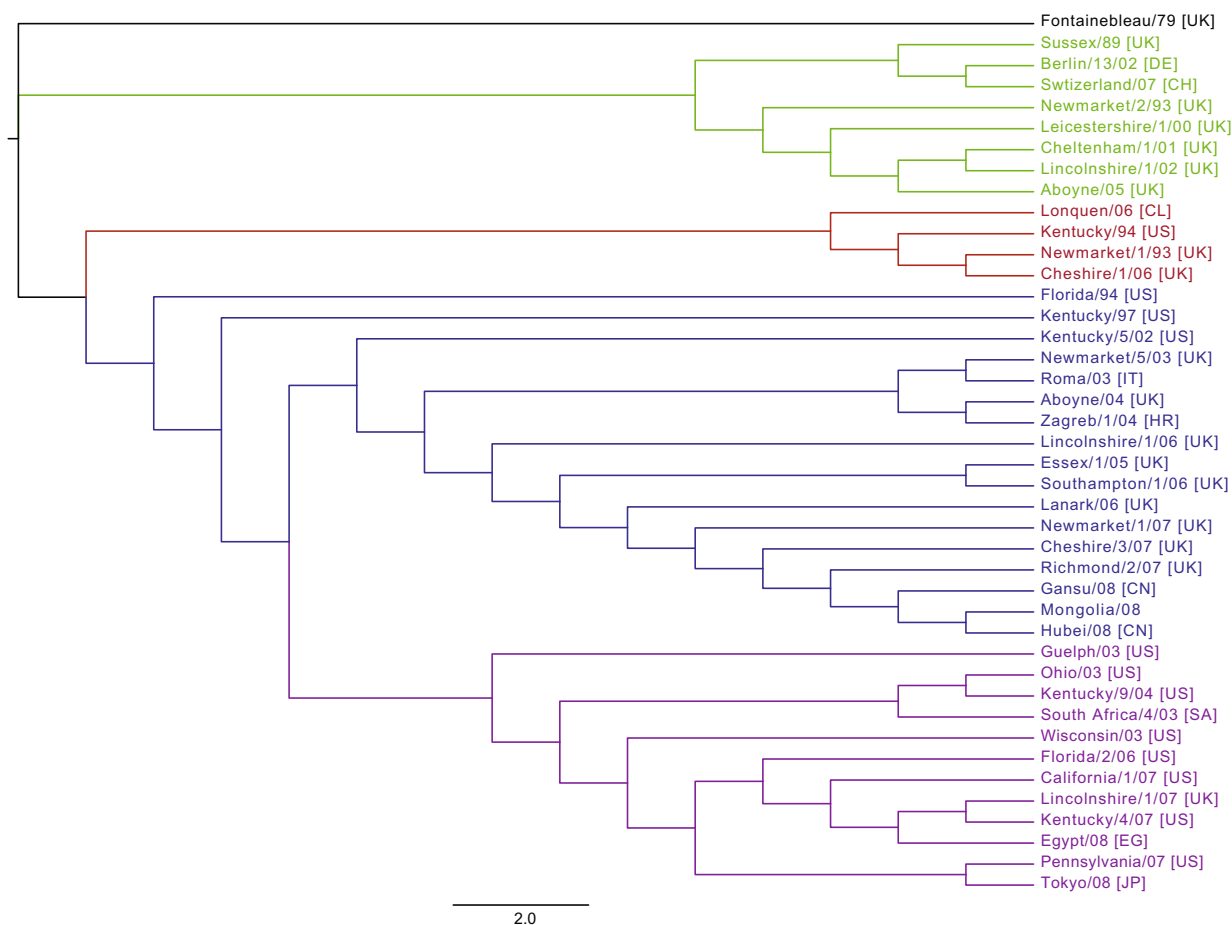
Since 2003, regular outbreaks have been reported in the UK, other European countries and the United States (Damiani et al., 2008; Barbic et al., 2009; Bryant et al., 2009). In addition, other regions that rarely report equine influenza outbreaks have been af-

ected. In December 2003 through January 2004, South Africa experienced only its second major outbreak of equine influenza. As for the first outbreak in 1986/1987 (Guthrie et al., 1999), a breakdown in biosecurity protocols was at the root of the outbreak (Guthrie, 2006).

In 2008, India similarly reported an outbreak of equine influenza after an interval of around 20 years since the last outbreak (Virmani et al., 2008). Australia, a country previously free of equine influenza, suffered an outbreak in 2007. An inquiry into the events surrounding the Australian outbreak found that it was – again – most likely to have been the result of a failure in biosecurity measures at a quarantine station (Anon, 2008; Callinan, 2008). The virus was apparently brought into Australia with horses imported from Japan (Bryant et al., 2009), despite the fact that Japan itself had not experienced an outbreak of equine influenza for 35 years prior to 2007 (Yamanaka et al., 2008).

Control of the disease among the fully susceptible population of un-vaccinated Australian horses required establishment of strict movement controls in restriction zones established in the two affected states of New South Wales and Queensland. Recently reported spatio-temporal analyses of data from the outbreak have highlighted the critical role played by rapid recognition of the infection and imposition of stringent animal movement restrictions soon afterwards in controlling the outbreak (Cowled et al., 2009).

The canarypox-vectored vaccine (ProteqFlu, Merial) was used to create buffer zones of vaccinated horses in Australia. This vaccine



**Fig. 1.** Phylogenetic tree created from nucleotide sequences of the HA1 portion of the haemagglutinin molecule of 40 equine influenza A H3N8 strains isolated in the 21st century and prototype strains of the different lineages and clades. Pre-divergence shown in black, European lineage in green, US (Kentucky) lineage in red, variant US (Florida) lineage in blue (clade 1) and purple (clade 2). The tree was constructed under the general time reversible substitution model and tree bisection-reconnection branch swapping using PAUP\* 4.0b10 (Swofford, 1999). The country of origin of each strain is denoted by the ISO two-letter code in brackets after the name.

was chosen because it will induce antibodies to only one protein of the equine influenza virus (HA). It would therefore be possible, using a diagnostic enzyme-linked immunosorbent assay (ELISA) that detected the nucleoprotein of the virus (Sergeant et al., 2009), to differentiate infected from vaccinated animals (DIVA) so permitting continued serological surveillance for new cases of infection (Uttenthal et al., 2010). The last confirmed equine influenza case in Australia was diagnosed about 5 months after the start of the outbreak and the restrictions on movement were lifted 2 months later (Anon, 2008). The costs associated with the outbreak were considerable (Callinan, 2008), and the debate continues as to whether routine vaccination should be introduced in case of further introductions of the virus.

### Evolution of equine influenza viruses

Phylogenetic analysis of HA sequences revealed that equine H3N8 viruses, which had been evolving as a single lineage for at least two decades (Kawaoka et al., 1989), diverged into two distinct lineages during the mid-1980s (Daly et al., 1996). Viruses in one lineage were predominantly isolated from horses on the continent of America, while viruses in the other lineage were almost exclusively isolated from horses in Europe and Asia. The most recent phylogenetic tree of equine H3N8 strains is more complex (Fig. 1). The 'Eurasian lineage' strains (shown in green in Fig. 1), represented by Newmarket/2/93, continue to form a single clade, but have rarely been isolated in recent years (Bryant et al., 2009). The 'American lineage' strains currently predominate, but the lineage has evolved into three distinct clades. The original American lineage strains, represented by Newmarket/1/93 and Kentucky/1994 (red in Fig. 1), have not been completely superseded, with isolations of strains from this clade in the UK (Bryant et al., 2009) and Chile (Muller et al., 2009) in 2006.

Within the American lineage is a variant sub-lineage known as the Florida lineage (blue and purple in Fig. 1). The strains that gave rise to the outbreak in Newmarket in 2003 and spread across Europe shortly thereafter belonged to this Florida sub-lineage, as have the majority of strains isolated in Europe since 2003 (Damiani et al., 2008; Bryant et al., 2009; Rozek et al., 2009). However, two clades can now be distinguished in this sub-lineage; Florida Clade 1 viruses have been isolated in North America since 2003 (e.g., Ohio/2003) and are distinct from the Florida Clade 2 strains that spread to Europe (e.g., Newmarket/5/03). Florida Clade 1 viruses caused outbreaks in South Africa at the end of 2003 and subsequently in Japan and Australia in 2007 (Bryant et al., 2009).

Florida Clade 2 viruses were responsible for the major outbreaks in China, Mongolia and India from 2007 to 2009 (Qi et al., 2010; Virmani et al., 2010). The phylogenetic analysis points to sporadic incursions of virus from North America into Europe and other regions, as happened around 1993 and 2003, followed by a period of more localised divergent evolution.

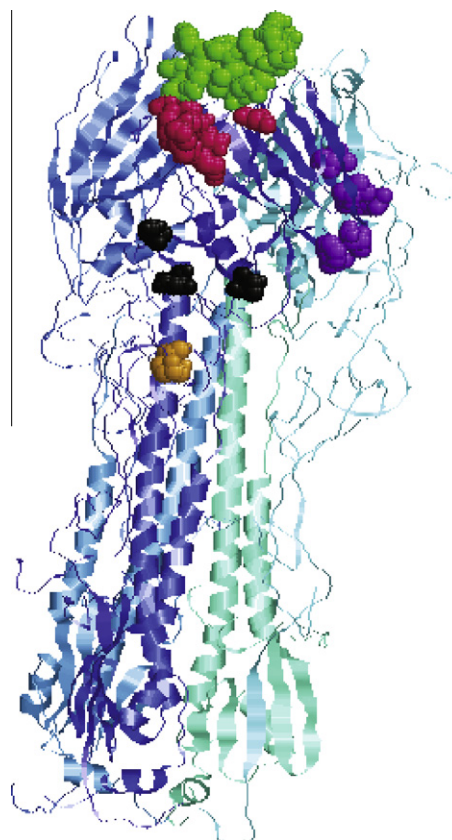
### Assessing the impact of antigenic drift on vaccine efficacy

Antigenic drift involves the accumulation of amino acid changes in the surface glycoproteins. Randomly occurring mutations subtly change the structure of the protein so that, over time, it becomes increasingly unrecognisable by host antibodies raised to an earlier strain. The assays routinely used to measure antibody levels rely on antibody binding. Some of the antibodies raised against one virus may bind to a different virus at conserved sites remote from the receptor-binding site, but these cross-reactive antibodies are believed to be much less efficient at neutralising virus infectivity than strain-specific antibody (Haaheim and Schild, 1980). If suffi-

cient changes occur in antigenic sites (located around the receptor-binding site), the virus may escape neutralisation (Fig. 2).

Human influenza vaccines are re-formulated frequently (often annually) to ensure that they are representative of circulating strains, with the aim of avoiding vaccine failure due to antigenic drift. The importance of antigenic drift for efficacy of equine influenza vaccines has also been demonstrated (Newton et al., 1999, 2000; Daly et al., 2003, 2004). Data from both field observations and vaccine challenge studies, in particular those that quantitatively describe protective antibody thresholds, have been used to set the parameters for mathematical models (Glass et al., 2002; Park et al., 2004; Baguelin et al., 2010). These models have demonstrated how the small changes in the ability of a vaccine to protect against infection by a variant strain observed at the individual pony level in experiments can have large effects on transmission of equine influenza when scaled up to the population level (Park et al., 2004).

Since 1993, a more global approach to equine influenza surveillance has been adopted and a vaccine strain selection system has been implemented. The rate of antigenic drift reported for equine influenza H3N8 strains (Daly et al., 1996) is much slower than that reported for human influenza A viruses (Fitch et al., 1997), vaccine strain updates are therefore not required as frequently. In 1995, it was recommended by the panel of experts appointed by the World Organisation for Animal Health (OIE) and World Health Organization (WHO) that the H3N8 vaccine strains should be updated to in-



**Fig. 2.** Changes to amino acids in antigenic sites on the haemagglutinin (HA) molecule mean that antibodies generated by previous infection or vaccination are no longer able to neutralise virus infectivity by blocking the receptor-binding site. In the diagram, the HA structure of a human influenza H3N2 virus (Protein database [PDB] accession number 2HMG) is visualised using RasMol. Three HA monomers combine to form the glycoprotein spike that projects from the virus surface. Amino acids in putative antigenic sites (Wilson et al., 1981) are indicated as coloured space-fill molecules (site A = pink; B = green; C = orange; D = purple; E = black).

clude Suffolk/1989 or Newmarket/2/93 to represent the Eurasian lineage and Newmarket/1/93 or Kentucky/1994 for the American lineage (OIE, 1996). The most recently published change to these recommendations was made in 2004, when it was recommended that the American lineage strain be updated to South Africa/4/03 or Ohio/03 (OIE, 2006).

The equine influenza strain surveillance programme is modelled on the surveillance program for human influenza, the mainstay of which is analysis of antigenic differences between strains using mono-specific ferret antisera in haemagglutination inhibition (HI) assays. The HI assay is based on the ability of influenza viruses to agglutinate red blood cells and the ability of animal antisera raised against the same or related strains to block this agglutination (Hirst, 1942). The HI assay has provided essential data for human influenza strain selection for over 60 years, but complex tables of HI data are difficult to interpret. Recently, Smith et al. (2004) established a method described as 'antigenic cartography'. Antigenic maps created from HI data provide a simple visual interpretation of the data and increase the resolution with which viruses can be distinguished because they do not rely on pair-wise comparisons alone. Antigenic cartography was applied to provide a detailed retrospective analysis of the evolution of human influenza H3N2 viruses (Fig. 3), and is now being applied to the analysis of equine influenza virus evolution.

The widespread outbreak of equine influenza that occurred in 2003 challenged pre-conceptions about the extent of antigenic drift that will cause reduced protection against infection. During the outbreak in Newmarket, horses became infected despite recent vaccination and high levels of serum antibody (Barquero et al.,

2007), which would suggest that there was a significant mismatch between the vaccine and outbreak strain. However, this was not fully supported by applying the current antigenic criteria for what constitutes a significant mismatch (Newton et al., 2006). In addition to antigenic analysis, the HA1 gene of virus isolates is routinely sequenced to assess the extent of genetic variation underlying phenotypic differences between strains. When comparing the HA1 sequence of isolates from the 2003 outbreak with the Newmarket/1/93 vaccine strain, only five consistent changes were seen, and only two of these were in a putative antigenic site, both in site B, a region of variable amino acids close to the receptor-binding site (Newton et al., 2006).

A tenet from human strain surveillance is that a minimum of 4–5 changes in at least two antigenic sites is required to determine a significant difference (Wilson and Cox, 1990). However, it has recently become apparent that just one or two amino acid substitutions in the human influenza HA molecule may have a significant impact on the antigenicity of a strain, depending on where they are located (Jin et al., 2005). Further mathematical modelling of data from vaccination and challenge studies of equine influenza has looked at the effective reproduction number, which is the number of new cases that an infectious individual gives rise to, on average, in a population with mixed susceptibility, such as a population in which not all individuals are vaccinated. An outbreak will potentially arise if the effective reproduction number begins to exceed 1.0, and the mathematical model suggested that this can happen when antigenic drift resulted in at least two amino acid differences in antigenic sites (Park et al., 2009).

A relatively new laboratory technique is increasingly being used to investigate human influenza viruses, namely, plasmid-based reverse genetics (Neumann et al., 1999). A reverse genetics system for equine influenza virus has also been developed (Quinlivan et al., 2005). In the standard reverse genetics system, each of the eight gene segments of influenza virus is cloned into plasmids under the control of a promoter, which, when the plasmids are transfected into a suitable cell, directs their synthesis into RNA segments. By transfecting all eight plasmids into a single cell together with four plasmids expressing each of the viral proteins required to hijack the cell's machinery and direct synthesis of viral proteins from the RNA segments, the cells can be turned into a virus-synthesising factory (Fig. 4). As each viral segment is carried by a separate plasmid, it is relatively straightforward to swap segments from different virus strains or use the molecular technique of site directed mutagenesis to introduce mutations into a gene of interest. This technique can therefore be used to gain a better understanding of what mutations are likely to have a significant impact on the epidemic potential of an emerging virus strain, which may help inform future decisions about when vaccine strains should be updated.

### Pathogenicity of equine influenza viruses

The 2003 outbreak of equine influenza led to a re-examination of the potential impact of limited antigenic drift on vaccine efficacy and raised questions about other factors, such as pathogenicity, which may define the epidemic potential of an equine influenza strain. During the outbreak, there were reports of unusually severe clinical signs, particularly coughing among un-vaccinated animals and even among vaccinated animals (Newton et al., 2006).

The clinical signs exhibited by un-vaccinated control animals during vaccine challenge experiments may vary depending on the strain used in the experimental infection (J. Daly, unpublished data). This observation was supported by collating data from the control animals of 14 studies involving four different prototype equine influenza strains (Table 1). In each of these studies, infec-

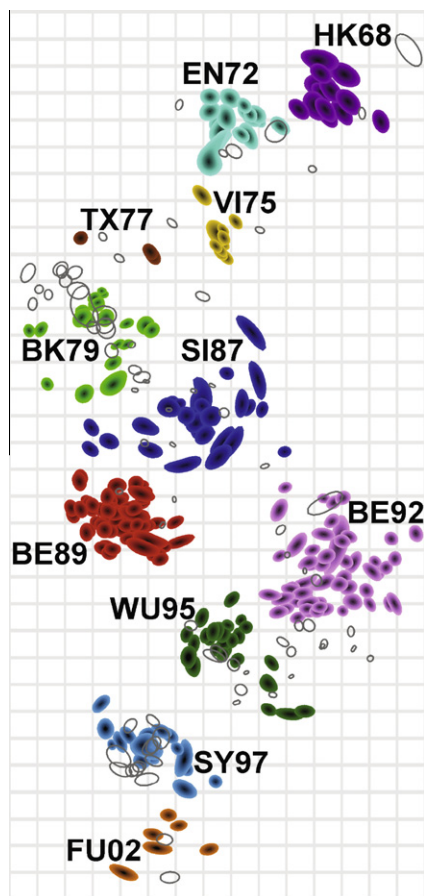
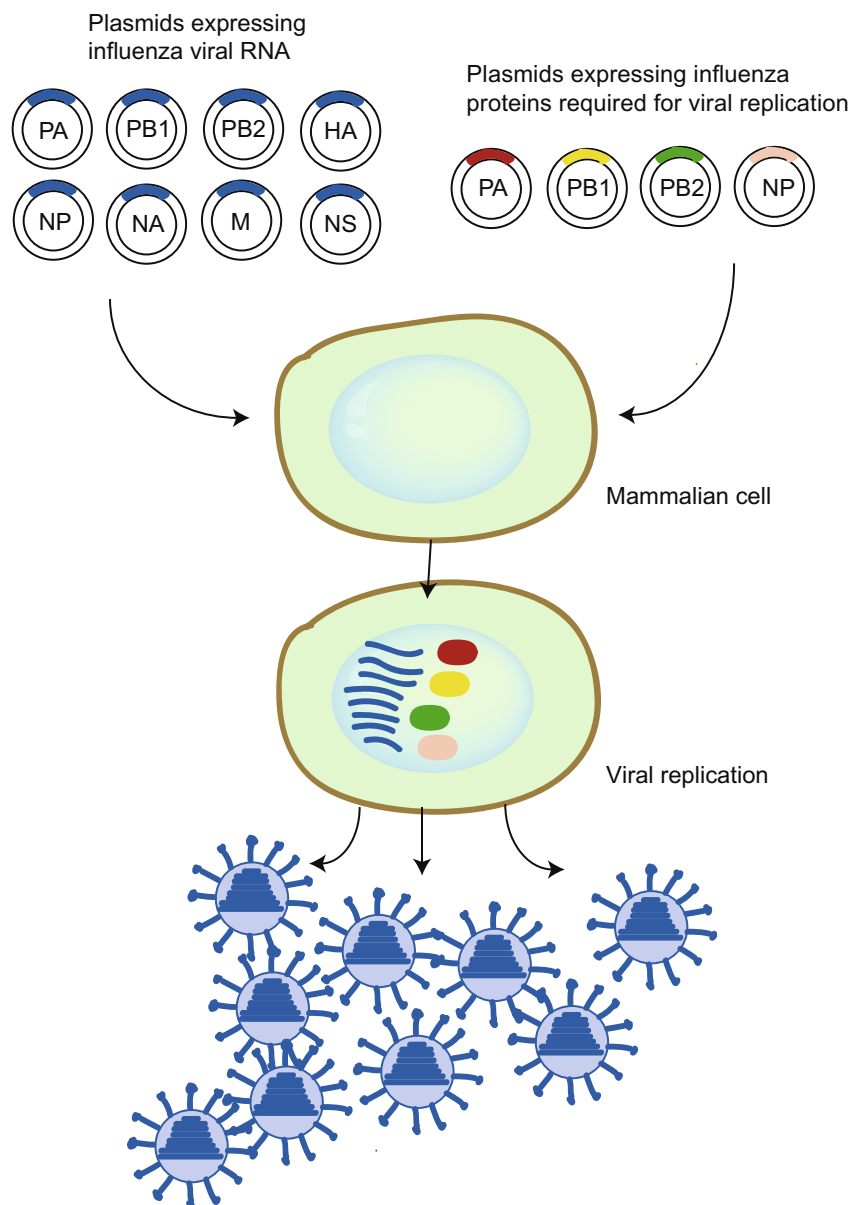


Fig. 3. Antigenic map of human influenza A (H3N2) strains from 1968 to 2003 reproduced with permission from Science (Smith et al., 2004).



**Fig. 4.** Diagram of plasmid-based reverse genetics technique, which can be used to synthesise viruses with specific mutations on a conserved background.

**Table 1**

Duration of virus shedding and clinical signs after experimental infection of ponies with different equine H3N8 influenza virus strains.

Challenge information			Mean duration in days (95% confidence intervals) <sup>a</sup>		
Strain	Dose <sup>b</sup>	No. ponies	Virus shedding	Pyrexia	Coughing
Sussex/1989	8.6	31	5.1 (4.86–5.34)	4.9 (4.03–5.77)	4.9 (3.82–5.98)
Newmarket/1/93	8.9/9.0	21	5.3 (4.86–5.74)	2.1 (1.22–2.98)	2.6 (1.38–3.82)
Newmarket/2/93	10.5	22	4.8 (4.03–5.57)	1.9 (1.01–2.79)	2.9 (1.36–4.44)
Newmarket/5/03	8.9	17	5.2 (4.61–5.79)	3.6 (2.05–5.15)	10.4 (9.22–11.58)

<sup>a</sup> Non-overlapping 95% confidence intervals indicate statistically significant difference in parameter between strains.

<sup>b</sup> Log<sub>10</sub> 50% egg infectious doses in aerosolised inoculum.

tion was generated by exposure to an aerosol of virus using a DeVilbiss nebuliser. Nasopharyngeal swabs for detection of virus shedding (determined by virus isolation in embryonated hens' eggs) were taken daily for 10 days and clinical signs, including coughing and rectal temperature, were monitored daily for 14 days after challenge. Although there are limitations to comparing data from studies that were conducted at different times and using different

doses of virus, there were no significant differences in duration of virus shedding between the strains. In contrast, the period of pyrexia induced by the two 1993 isolates was shorter than for the 1989 and 2003 epidemic strains. Furthermore, a field observation during the 2003 outbreak reported that coughing was a particular feature of infection (Newton et al., 2006), which was confirmed by the significantly longer duration of coughing observed after exper-

imental infection with the Newmarket/5/03 strain compared with the three older isolates (Table 1).

Wattrang et al. (2003) compared the production of cytokines associated with a pro-inflammatory innate immune response in two groups of 10 ponies challenged with either Sussex/1989 or Newmarket/2/93. As expected from earlier observations, ponies infected with Sussex/1989 exhibited the most pronounced clinical signs (Wattrang et al., 2003). Type I interferon activity was detected in nasal secretions of all ponies infected with Sussex/1989 but only in two ponies infected with Newmarket/2/93. IL-6 activity was detected in nasal secretions of all of the ponies from day 2 onwards, but markedly higher IL-6 responses were observed in ponies infected with Sussex/1989 (Wattrang et al., 2003). This study provided evidence that, as with influenza virus in other species (van Reeth, 2000), the clinical signs of infection are related to the duration and level of cytokine responses, rather than directly to the extent of virus replication.

Experimental infection of five ponies with Newmarket/5/03 induced type I interferon activity in a significantly greater proportion of the ponies ( $P < 0.001$ ) than infection with Sussex/1989 or Newmarket/2/93, and for a significantly longer period ( $P < 0.001$ ) than Newmarket/2/93 (E. Wattrang, personal communication).

Both strains isolated in Newmarket in 1993, despite being from different lineages according to phylogenetic analysis of the HA gene, were from minor outbreaks that only affected horses in one or two yards. In contrast, the two strains that caused more severe clinical disease (Sussex/1989 and Newmarket/5/03) were representative of strains that caused widespread epidemics across Europe, but also belonged to different HA lineages. This suggested that although characterisation of the HA of a virus can indicate whether vaccine breakdown may occur, it is not necessarily indicative of the potential for a virus to cause severe disease and/or become widespread. Pathogenicity of influenza viruses is known to be a multigenic trait (Wright et al., 2007), therefore sequencing of other genes, in addition to that encoding HA, is now being increasingly performed in the surveillance of human and equine influenza.

### Interspecies transmission of equine influenza viruses

Influenza A viruses of aquatic birds have been proposed as the ancestors of all influenza virus sub-types existing in other animals, including humans, pigs and horses. Only a restricted number of sub-type combinations have become established in mammalian species (e.g., H7N7 and H3N8 in horses; H1N1, H3N2 and H2N2 in humans). Studies have shown that the H3N8 sub-type was introduced into horses a long time ago and the lack of exchange of virus genes between the equine viruses and viruses from other species (Gorman et al., 1991) led to the suggestion that horses may be a 'dead-end' host. However, an outbreak of respiratory disease among racing greyhounds in Florida (US) at the beginning of 2004 resulted in several greyhounds dying from haemorrhagic pneumonia. An equine influenza virus was identified as the cause of the outbreak by isolation of virus from one case and the detection of specific antibodies in other cases (Crawford et al., 2005). Genetic sequence analysis and phylogenetic comparisons determined that the canine isolates were closely related to contemporary strains of equine influenza H3N8 virus (Crawford et al., 2005).

The interspecies transmission of equine influenza to dogs was subsequently shown not to be unique to the USA. Equine influenza was retrospectively shown to be responsible for an outbreak of respiratory disease in English foxhounds that occurred in the UK in 2002 (Daly et al., 2008). An outbreak in a second pack of foxhounds was subsequently diagnosed retrospectively on the basis of serology (Newton et al., 2007). Furthermore,

transmission of equine influenza virus to dogs during the recent outbreak in Australia has also been reported (Kirkland et al., 2010).

The ability of the equine influenza virus to become established in the American racing greyhound population was perhaps unsurprising as the dogs travel widely and intermingle at events, providing opportunities for the virus to spread to further susceptible individuals. The virus has also since become established in other canine populations in the US (Crawford et al., 2005), probably via rescue shelters, and a vaccine against canine influenza is now commercially available in the US (Deshpande et al., 2009). There is no evidence that canine influenza is causing similarly widespread problems in the UK or elsewhere in Europe, but the veterinary research community is mindful of this risk and surveillance for equine influenza is ongoing in Europe (R. Newton, D. Elton, unpublished observations).

Interspecies transmission of influenza A viruses depends upon several factors. For influenza viruses to enter host cells, the HA protein must bind to sialic acid (SA) receptors on the cell surface (Gotschalk et al., 1972). Sialic acid is a nine-carbon sugar that terminates glycans found on glycoproteins and glycolipids. It is attached via the second carbon to a variety of glycans in the underlying sugar chain. Viruses isolated from wild aquatic birds bind strongly to SA in a 2,3 linkage (SA 2,3) (Connor et al., 1994). The same linkage is recognised by equine influenza virus and is the predominant linkage found on cells lining the equine upper respiratory tract (Suzuki et al., 2000). In contrast, human-adapted influenza viruses recognise and bind SA 2,6 receptors, and these are the receptors that predominate in the human respiratory tract (Couceiro et al., 1993).

Using two plant lectins that bind to either SA 2,3 or SA 2,6 linkages, the predominance of the SA 2,3 linkage preferred by equine influenza viruses on canine tracheal epithelial cells has been demonstrated (Daly et al., 2008). The availability of relevant receptors for equine influenza virus on the respiratory tract of dogs poses the question of why infection of dogs had not been reported prior to 2005. Mild infections may have been overlooked as part of the 'kennel cough' syndrome (which is associated with infection with pathogens including, but not limited to, parainfluenza-3 virus and *Bordetella bronchiseptica*). However, the multiple independent transmissions of equine influenza virus to dogs in recent years (Crawford et al., 2005; Newton et al., 2007; Daly et al., 2008; Kirkland et al., 2010) suggested that the viruses recently circulating in horses are particularly transmissible.

The initial transmission of virus between two species requires some form of interaction between them and the manner of transmission may depend on the nature of this interaction. Although influenza viruses are usually transmitted between individuals of a mammalian species (e.g., man-to-man or horse-to-horse) by the aerosol route, transmission can also occur by contact. The predominant mode of transmission may be influenced by factors such as climate (Lowen and Palese, 2009). The UK foxhounds that were affected in 2002 were fed horse meat shortly before the outbreak, leading to speculation that they may have inhaled virus during consumption of raw lung material (Daly et al., 2008). This route of transmission to carnivores has been demonstrated for highly pathogenic avian influenza of the H5N1 sub-type (Keawcharoen et al., 2004; Songserm et al., 2006).

Enhanced pathogenicity of a virus will increase the risk that a horse will die or be euthanased while infected, which, in turn, will increase the likelihood of a dog being fed meat, including lung tissue, with a high viral load. The more usual route of aerosol transmission must also be considered, with the pronounced coughing seen during the Newmarket/5/03 infection potentially increasing the effectiveness of transmission by this route. It has been demonstrated that equine influenza can be transmitted from an experi-

mentally infected horse to a dog housed in the same stable (Yamanaka et al., 2009).

The foxhounds infected in 2002 and 2003 in the UK were housed alongside horses, and in the second report of infection it was speculated that transport of foxhounds in the same vehicle as horses may have provided the opportunity for transmission by aerosol (Newton et al., 2007). Kirkland et al. (2010) reported that in Australia, some dogs that became infected with equine influenza virus were kept near to (but not in direct contact with) infected horses, although there was no evidence for transmission (e.g., by fomites) to dogs kept further afield.

Further evidence that the horse may not be a dead-end host has arisen from the characterisation of two viruses isolated from pigs showing respiratory disease in 2005 and 2006 (Tu et al., 2009). The HA sequences of the two isolates were found to be most closely related to European lineage equine influenza viruses isolated in the early 1990s (Tu et al., 2009).

It is equally possible that a new influenza sub-type could emerge in horses from the avian reservoir. Although it did not replace the current equine H3N8 virus that has been circulating in horses for many years (Gorman et al., 1991), cross-species transmission of avian H3N8 influenza virus into horses occurred in Jilin Province in China during 1989, causing mortality of up to 20% in some herds (Guo et al., 1991). This transient re-emergence of the H3N8 sub-type rather than any other may reflect the fact that this sub-type is commonly isolated from the avian reservoir (see, for example, Sharp et al., 1997). Various combinations of the 16 different HA and 9 different NA sub-types have been isolated from aquatic birds, but we currently have no means of predicting which, if any, of these could emerge (or re-emerge) in the equine population in the future.

## Conclusions

Equine influenza A H3N8 viruses continue to cause widespread problems in horses despite control measures including quarantine and vaccination, and international spread of the virus occurs as horses travel for racing and breeding purposes. The first decade of this century has highlighted the unpredictable nature of equine influenza viruses and provided salient reminders that there is no room for complacency in the arena of influenza surveillance and research.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

## Acknowledgments

Much of the data presented in this paper has been generated through the collaborative efforts of several research teams. The authors would particularly like to thank past and present members of the equine influenza group at the Animal Health Trust, which was originally established by Dr. Jenny Mumford. We are indebted to the combined efforts of all the laboratories involved in equine influenza surveillance and to veterinary practitioners who submit nasal swab samples for diagnostic testing. The authors greatly appreciate the continued financial support of the Horserace Betting Levy Board for the Animal Health Trust Equine Influenza Programme. Additional funding came from the British Biological Research Council, Cambridge Infectious Diseases Consortium (Defra/HEFCE), equine influenza vaccine manufacturers, and other UK animal charities.

## References

- Anon, 2008. Summary of the Australian equine influenza outbreak. *Veterinary Record* 163, 378.
- Askonas, B.A., McMichael, A.J., Webster, R.G., 1982. The antibody response to influenza virus. In: Beare, A.S. (Ed.), *Basic and Applied Influenza Research*. CRC Press, Boca Raton, Florida, pp. 164–181.
- Baguelin, M., Newth, J.R., Demiris, N., Daly, J., Mumford, J.A., Wood, J.L., 2010. Control of equine influenza: scenario testing using a realistic metapopulation model of spread. *Journal of the Royal Society, Interface* 7, 67–79.
- Barbic, L., Madic, J., Turk, N., Daly, J., 2009. Vaccine failure caused an outbreak of equine influenza in Croatia. *Veterinary Microbiology* 133, 164–171.
- Barquero, N., Daly, J.M., Newton, J.R., 2007. Risk factors for influenza infection in vaccinated racehorses: lessons from an outbreak in Newmarket, UK in 2003. *Vaccine* 25, 7520–7529.
- Bryant, N.A., Rash, A.S., Russell, C.A., Ross, J., Cooke, A., Bowman, S., MacRae, S., Lewis, N.S., Paillot, R., Zanoni, R., Meier, H., Griffiths, L.A., Daly, J.M., Tiwari, A., Chambers, T.M., Newton, J.R., Elton, D.M., 2009. Antigenic and genetic variations in European and North American equine influenza virus strains (H3N8) isolated from 2006 to 2007. *Veterinary Microbiology* 138, 41–52.
- Bryant, N.A., Paillot, R., Rash, A.S., Medcalf, E., Montesso, F., Ross, J., Watson, J., Jeggo, M., Lewis, N.S., Newton, J.R., Elton, D.M., 2010. Comparison of two modern vaccines and previous influenza infection against challenge with an equine influenza virus from the Australian 2007 outbreak. *Veterinary Research* 41, 19.
- Callinan, I., 2008. Equine Influenza: The August 2007 Outbreak in Australia. Report of the Equine Influenza Inquiry. The Commonwealth of Australia. <<http://www.equineinfluenzaenquiry.gov.au/eiexhibits/REP.0001.001.0001.pdf>> (accessed 26.02.10).
- Chen, W., Calvo, P.A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J.R., Yewdell, J.W., 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nature Medicine* 7, 1306–1312.
- Connor, R.J., Kawaoka, Y., Webster, R.G., Paulson, J.C., 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* 205, 17–23.
- Couceiro, J.N., Paulson, J.C., Baum, L.G., 1993. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Research* 29, 155–165.
- Cowled, B., Ward, M.P., Hamilton, S., Garner, G., 2009. The equine influenza epidemic in Australia: spatial and temporal descriptive analyses of a large propagating epidemic. *Preventive Veterinary Medicine* 92, 60–70.
- 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., Donis, R.O., 2005. Transmission of equine influenza virus to dogs. *Science* 310, 482–485.
- Daly, J.M., Lai, A.C.K., Binns, M.M., Chambers, T.M., Barrandeguy, M., Mumford, J.A., 1996. Antigenic and genetic evolution of equine H3N8 influenza A viruses. *Journal of General Virology* 77, 661–671.
- Daly, J.M., Yates, P.J., Browne, G., Swann, Z., Newton, J.R., Jessett, D., Davis-Poynter, N., Mumford, J.A., 2003. Comparison of hamster and pony challenge models for evaluation of effect of antigenic drift on cross-protection afforded by equine influenza vaccines. *Equine Veterinary Journal* 35, 458–462.
- Daly, J.M., Yates, P.J., Newton, J.R., Park, A., Henley, W., Wood, J.L.N., Davis-Poynter, N., Mumford, J.A., 2004. Evidence supporting the inclusion of strains from each of the two co-circulating lineages of H3N8 equine influenza virus in vaccines. *Vaccine* 22, 4101–4109.
- Daly, J.M., Whitwell, K.E., Miller, J., Dowd, G., Cardwell, J.M., Smith, K.C., 2006. Investigation of equine influenza cases exhibiting neurological disease: incidence or association? *Journal of Comparative Pathology* 134, 231–235.
- Daly, J.M., Blunden, A.S., Macrae, S., Miller, J., Bowman, S.J., Kolodziejek, J., Nowotny, N., Smith, K.C., 2008. Transmission of equine influenza virus to English foxhounds. *Emerging Infectious Diseases* 14, 461–464.
- Damiani, A.M., Scicluna, M.T., Ciabatti, I., Cardeti, G., Sala, M., Vulcano, G., Cordioli, P., Martella, V., Amaddeo, D., Autorino, G.L., 2008. Genetic characterization of equine influenza viruses isolated in Italy between 1999 and 2005. *Virus Research* 131, 100–105.
- Deshpande, M.S., Jirjis, F.F., Tubbs, A.L., Jayappa, H., Sweeney, D., Spencer, S.J., Lakshmanan, N., Wasmoen, T.L., 2009. Evaluation of the efficacy of a canine influenza virus (H3N8) vaccine in dogs following experimental challenge. *Veterinary Therapeutics: Research in Applied Veterinary Medicine* 10, 103–112.
- 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. *Proceedings of the National Academy of Science of the United States of America* 94, 7712–7718.
- Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B., Osterhaus, A.D., 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of Virology* 79, 2814–2822.
- Francis, T., Salk, J.E., Pearson, H.E., Brown, P.N., 1945. Protective effect of vaccination against induced influenza A. *Journal of Clinical Investigation* 24, 536–546.
- Glass, K., Wood, J.L., Mumford, J.A., Jessett, D., Grenfell, B.T., 2002. Modelling equine influenza 1: a stochastic model of within-yard epidemics. *Epidemiology and Infection* 128, 491–502.
- Gorman, O.T., Bean, W.J., Kawaoka, Y., Donatelli, I., Guo, Y., Webster, R.G., 1991. Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. *Journal of Virology* 65, 3704–3714.

- Gottschalk, A., Belyavin, G., Biddle, F., 1972. Glycoproteins as influenza virus haemagglutinin inhibitors and cellular receptors. In: Gottschalk, A. (Ed.), *The Glycoproteins: Their Composition, Structure and Function*. Elsevier, Amsterdam, pp. 1082–1096.
- Guo, Y., Wang, M., Zheng, S., Wang, P., Ji, W., Chen, Q., 1991. Aetiological study on an influenza-like epidemic in horses in China. *Acta Virologica* 35, 190–195.
- Guthrie, A.J., 2006. Equine influenza in South Africa, 2003 outbreak. In: *Proceedings of the 9th International Congress of World Equine Veterinary Association*. International Veterinary Information Service.
- Guthrie, A.J., Stevens, K.B., Bosman, P.P., 1999. The circumstances surrounding the outbreak and spread of equine influenza in South Africa. *Scientific and Technical Review of the Office International des Epizooties* 18, 179–185.
- Haaheim, L.R., Schild, G.C., 1980. Antibodies to the strain-specific and cross-reactive determinants of the haemagglutinin of influenza H3N2 viruses. Antiviral activities of the antibodies in biological systems. *Acta Pathologica et Microbiologica Scandinavica*. Section B, *Microbiology* 88, 335–340.
- Hannant, D., Mumford, J.A., Jessett, D.M., 1988. Duration of circulating antibody and immunity following infection with equine influenza virus. *Veterinary Record* 122, 125–128.
- Hidaka, F., Matsuo, S., Muta, T., Takeshige, K., Mizukami, T., Nunoi, H., 2006. A missense mutation of the Toll-like receptor 3 gene in a patient with influenza-associated encephalopathy. *Clinical Immunology* 119, 188–194.
- Hirst, G.K., 1942. The quantitative determination of influenza virus and antibodies by means of red cell agglutination. *Journal of Experimental Medicine* 75, 49–64.
- Hjalmarsson, A., Blomqvist, P., Brytting, M., Linde, A., Skoldenberg, B., 2009. Encephalitis after influenza in Sweden 1987–1998: a rare complication of a common infection. *European Neurology* 61, 289–294.
- OIE, 2006. Conclusions and recommendations from the expert surveillance panel on equine influenza vaccines. *Bulletin of the Office International des Epizooties* 2, 35–36.
- Jin, H., Zhou, H., Liu, H., Chan, W.H., Adhikary, L., Mahmood, K., Lee, M.-S., Kemble, G., 2005. Two residues in the hemagglutinin of A/Fujian/411/02-like influenza viruses are responsible for antigenic drift from A/Panama/2007/99. *Virology* 336, 113–119.
- Kawaoka, Y., Bean, W.J., Webster, R.G., 1989. Evolution of the hemagglutinin of equine H3 influenza viruses. *Virology* 169, 283–292.
- Keawcharoen, J., Oraveerakul, K., Kuiken, T., Fouchier, R.A., Amonsin, A., Payungporn, S., Noppornpanth, S., Wattanodorn, S., Theamboonlers, A., Tantilertcharoen, R., Pattanarangsarn, R., Arya, N., Ratanakorn, P., Osterhaus, D.M., Poovorawan, Y., 2004. Avian influenza H5N1 in tigers and leopards. *Emerging Infectious Diseases* 10, 2189–2191.
- Kirkland, P.D., Finlaison, D.S., Crispe, E., Hunt, A.C., 2010. Influenza virus transmission from horses to dogs, Australia. *Emerging Infectious Diseases* 16, 699–702.
- Lowen, A., Palese, P., 2009. Transmission of Influenza Virus in Temperate Zones is Predominantly by Aerosol, in the Tropics by Contact: A Hypothesis. *The Public Library of Science Currents, Influenza* (RRN1002).
- Muller, I., Pinto, E., Santibanez, M.C., Celedon, M.O., Valenzuela, P.D., 2009. Isolation and characterization of the equine influenza virus causing the 2006 outbreak in Chile. *Veterinary Microbiology* 137, 172–177.
- Mumford, J., Wood, J.M., Scott, A.M., Folkers, C., Schild, G.C., 1983. Studies with inactivated equine influenza vaccine: 2. Protection against experimental infection with influenza virus A/equine/Newmarket/79 (H3N8). *Journal of Hygiene (Cambridge)* 90, 385–395.
- Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D.R., Donis, R., Hoffmann, E., Hobom, G., Kawaoka, Y., 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proceedings of the National Academy of Science of the United States of America* 96, 9345–9350.
- Newton, J.R., Verheyen, K., Wood, J.L.N., Yates, P.J., Mumford, J.A., 1999. Equine influenza in the United Kingdom in 1998. *Veterinary Record* 145, 449–452.
- Newton, J.R., Lakhani, K.H., Wood, J.L.N., Baker, D.J., 2000. Risk factors for equine influenza serum antibody titres in young Thoroughbred racehorses given an inactivated vaccine. *Preventive Veterinary Medicine* 46, 129–141.
- Newton, J.R., Daly, J.M., Spencer, L., Mumford, J.A., 2006. Description of the equine influenza (H3N8) outbreak in the United Kingdom during 2003, during which recent vaccination failed to prevent disease in racehorses in Newmarket. *Veterinary Record* 158, 185–192.
- Newton, R., Cooke, A., Elton, D., Bryant, N., Rash, A., Bowman, S., Blunden, T., Miller, J., Hammond, T.A., Camm, I., Day, M., 2007. Canine influenza virus: cross-species transmission from horses. *Veterinary Record* 161, 142–143.
- OIE, 1996. Conclusions and recommendations from the consultation meeting of OIE and WHO experts on equine influenza, Newmarket, United Kingdom, September 18–19, 1995. *Bulletin of the Office International des Epizooties* 108, 482–484.
- Paillot, R., Hannant, D., Kydd, J.H., Daly, J.M., 2006. Vaccination against equine influenza: quid novi? *Vaccine* 24, 4047–4061.
- Park, A.W., Wood, J.L.N., Daly, J.M., Newton, J.R., Glass, K., Henley, W., Mumford, J.A., Grenfell, B.T., 2004. The effects of strain heterogeneity on the epidemiology of equine influenza in a vaccinated population. *Proceedings of the Royal Society of London, Series B* 271, 1547–1555.
- Park, A.W., Daly, J.M., Lewis, N.S., Smith, D.J., Wood, J.L.N., Grenfell, B.T., 2009. Quantifying the impact of immune escape on transmission dynamics of influenza. *Science* 326, 726–728.
- Qi, T., Guo, W., Huang, W., Dai, L., Zhao, L., Li, H., Li, X., Zhang, X., Wang, Y., Yan, Y., He, N., Xiang, W., 2010. Isolation and genetic characterization of H3N8 equine influenza virus from donkeys in China. *Veterinary Microbiology*. doi:10.1016/j.vetmic.2010.01.006.
- Quinlivan, M., Zamarin, D., Garcia-Sastre, A., Cullinane, A., Chambers, T., Palese, P., 2005. Attenuation of equine influenza viruses through truncations of the NS1 protein. *Journal of Virology* 79, 8431–8439.
- Rozek, W., Purzycka, M., Polak, M.P., Gradzki, Z., Zmudzinski, J.F., 2009. Genetic typing of equine influenza virus isolated in Poland in 2005 and 2006. *Virus Research* 145, 121–126.
- Sergeant, E.S.G., Kirkland, P.D., Cowled, B.D., 2009. Field evaluation of an equine influenza ELISA used in New South Wales during the 2007 Australian outbreak response. *Preventive Veterinary Medicine* 92, 382–385.
- Sharp, G.B., Kawaoka, Y., Jones, D.J., Bean, W.J., Pryor, S.P., Hinshaw, V., Webster, R.G., 1997. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. *Journal of Virology* 71, 6128–6135.
- Smith, D.J., Lapedes, A.S., de Jong, J.C., Bestebroer, T.M., Rimmelzwaan, G.F., Osterhaus, A.D.M.E., Fouchier, R.A.M., 2004. Mapping the antigenic and genetic evolution of influenza virus. *Science* 305, 371–376.
- Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A., Chutinimitkul, S., Thanawongnuwech, R., Poovorawan, Y., 2006. Fatal avian influenza A H5N1 in a dog. *Emerging Infectious Diseases* 12, 1744–1747.
- Suzuki, Y., Ito, T., Suzuki, T., Holland Jr., R.E., Chambers, T.M., Kiso, M., Ishida, H., Kawaoka, Y., 2000. Sialic acid species as a determinant of the host range of influenza A viruses. *Journal of Virology* 74, 11825–11831.
- Swofford, D.L., 1999. PAUP 4.0: Phylogenetic Analysis using Parsimony (and other methods). In: Sinauer Associates.
- Togashi, T., Matsuzono, Y., Narita, M., Morishima, T., 2004. Influenza-associated acute encephalopathy in Japanese children in 1994–2002. *Virus Research* 103, 75–78.
- Toovey, S., 2008. Influenza-associated central nervous system dysfunction: a literature review. *Travel Medicine and Infectious Disease* 6, 114–124.
- Tu, J., Zhou, H., Jiang, T., Li, C., Zhang, A., Guo, X., Zou, W., Chen, H., Jin, M., 2009. Isolation and molecular characterization of equine H3N8 influenza viruses from pigs in China. *Archives of Virology* 154, 887–890.
- Utenthal, A., Parida, S., Rasmussen, T.B., Paton, D.J., Haas, B., Dundon, W.G., 2010. Strategies for differentiating infection in vaccinated animals (DIVA) for foot-and-mouth disease, classical swine fever and avian influenza. *Expert Review of Vaccines* 9, 73–87.
- Van Reeth, K., 2000. Cytokines in the pathogenesis of influenza. *Veterinary Microbiology* 74, 109–116.
- Virmani, N., Singh, B.K., Gulati, B.R., Kumar, S., 2008. Equine influenza outbreak in India. *Veterinary Record* 163, 607–608.
- Virmani, N., Bera, B.C., Singh, B.K., Shanmugasundaram, K., Gulati, B.R., Barua, S., Vaid, R.K., Gupta, A.K., Singh, R.K., 2010. Equine influenza outbreak in India (2008–09): virus isolation, sero-epidemiology and phylogenetic analysis of HA gene. *Veterinary Microbiology*. doi:10.1016/j.vetmic.2009.12.007.
- Wattrang, E., Jessett, D.M., Yates, P., Fuxler, L., Hannant, D., 2003. Experimental infection of ponies with equine influenza A2 (H3N8) virus strains of different pathogenicity elicits varying interferon and interleukin-6 responses. *Viral Immunology* 16, 57–67.
- Wilson, I.A., Cox, N.J., 1990. Structural basis of immune recognition of influenza virus hemagglutinin. *Annual Review of Immunology* 8, 737–771.
- Wilson, I.A., Skehel, J.J., Wiley, D.C., 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 289, 366–373.
- Wise, H.M., Foeglein, A., Sun, J., Dalton, R.M., Patel, S., Howard, W., Anderson, E.C., Barclay, W.S., Digard, P., 2009. A complicated message: identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *Journal of Virology* 83, 8021–8031.
- Wood, J.M., Schild, G.C., Folkers, C., Mumford, J., Newman, R.W., 1983. The standardization of inactivated equine influenza vaccines by single-radial immunodiffusion. *Journal of Biological Standardization* 11, 133–136.
- Wright, P.F., Neumann, G., Kawaoka, Y., 2007. Orthomyxoviruses. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A. (Eds.), *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia, pp. 1691–1740.
- Yamanaka, T., Niwa, H., Tsujimura, K., Kondo, T., Matsumura, T., 2008. Epidemic of equine influenza among vaccinated racehorses in Japan in 2007. *The Journal of Veterinary Medical Science* 70, 623–625.
- Yamanaka, T., Nemoto, M., Tsujimura, K., Kondo, T., Matsumura, T., 2009. Interspecies transmission of equine influenza virus (H3N8) to dogs by close contact with experimentally infected horses. *Veterinary Microbiology* 139, 351–355.