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Review Article

REVIEW ARTICLE

Influenza A virus transmission via respiratory aerosols or droplets as it relates to pandemic potential

Mathilde Richard and Ron A.M. Fouchier*

Department of Viroscience, Postgraduate School Molecular Medicine, Erasmus MC, 3000 CA Rotterdam, the Netherlands

*Corresponding author. Department of Viroscience, Postgraduate School Molecular Medicine, Erasmus MC, Rotterdam, PO Box 2040, 3000 CA Rotterdam, the Netherlands. Tel: +31-10-7043161; Fax: +31-10-7044760. E-mail: r.fouchier@erasmusmc.nl

One sentence summary: This review summarizes recent discoveries on the genetic and phenotypic traits required for airborne transmission of zoonotic influenza viruses of subtypes H5, H7 and H9 and pandemic viruses of subtypes H1, H2 and H3.

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ABSTRACT

Many respiratory viruses of humans originate from animals. For instance, there are now eight paramyxoviruses, four coronaviruses and four orthomxoviruses that cause recurrent epidemics in humans but were once confined to other hosts. In the last decade, several members of the same virus families have jumped the species barrier from animals to humans. Fortunately, these viruses have not become established in humans, because they lacked the ability of sustained transmission between humans. However, these outbreaks highlighted the lack of understanding of what makes a virus transmissible. In part triggered by the relatively high frequency of occurrence of influenza A virus zoonoses and pandemics, the influenza research community has started to investigate the viral genetic and biological traits that drive virus transmission via aerosols or respiratory droplets between mammals. Here we summarize recent discoveries on the genetic and phenotypic traits required for airborne transmission of zoonotic influenza viruses of subtypes H5, H7 and H9 and pandemic viruses of subtypes H1, H2 and H3. Increased understanding of the determinants and mechanisms of respiratory virus transmission is not only key from a basic scientific perspective, but may also aid in assessing the risks posed by zoonotic viruses to human health, and preparedness for such risks.

Keywords: ferret; guinea pig; emerging disease; pandemic; host range

INTRODUCTION

Respiratory tract infections (RTI) are ranked third among the leading causes of death worldwide with around 3.5 million cases each year, or almost 1 in every 15 deaths globally. Viruses are responsible for approximately half of these RTI (WHO 2014b). Although the death toll is much lower in high-income countries than in developing countries, viral RTI are ubiquitous and burdensome nevertheless, accounting for many millions of lost schooldays or workdays and physician visits each year. In certain populations such as neonates, the elderly, immunocompromised individuals and people with underlying disease, viral RTI can have a substantial impact also in the developed world, sometimes with fatal outcome. A wide range of respiratory viruses is known to affect humans, but among these the members of the *Coronaviridae*, *Paramyxoviridae* and *Orthomyxoviridae* virus families are not only notorious to cause recurrent epidemics of RTI in humans associated with severe disease, but are also infamous for their zoonotic potential, i.e. their ability to be transmitted from animals to humans.

Within the *Paramyxoviridae* family, measles virus is one of the most infectious human pathogens known, and has been targeted by the World Health Organisation (WHO) for eradication. Respiratory syncytial virus (RSV), human metapneumovirus,

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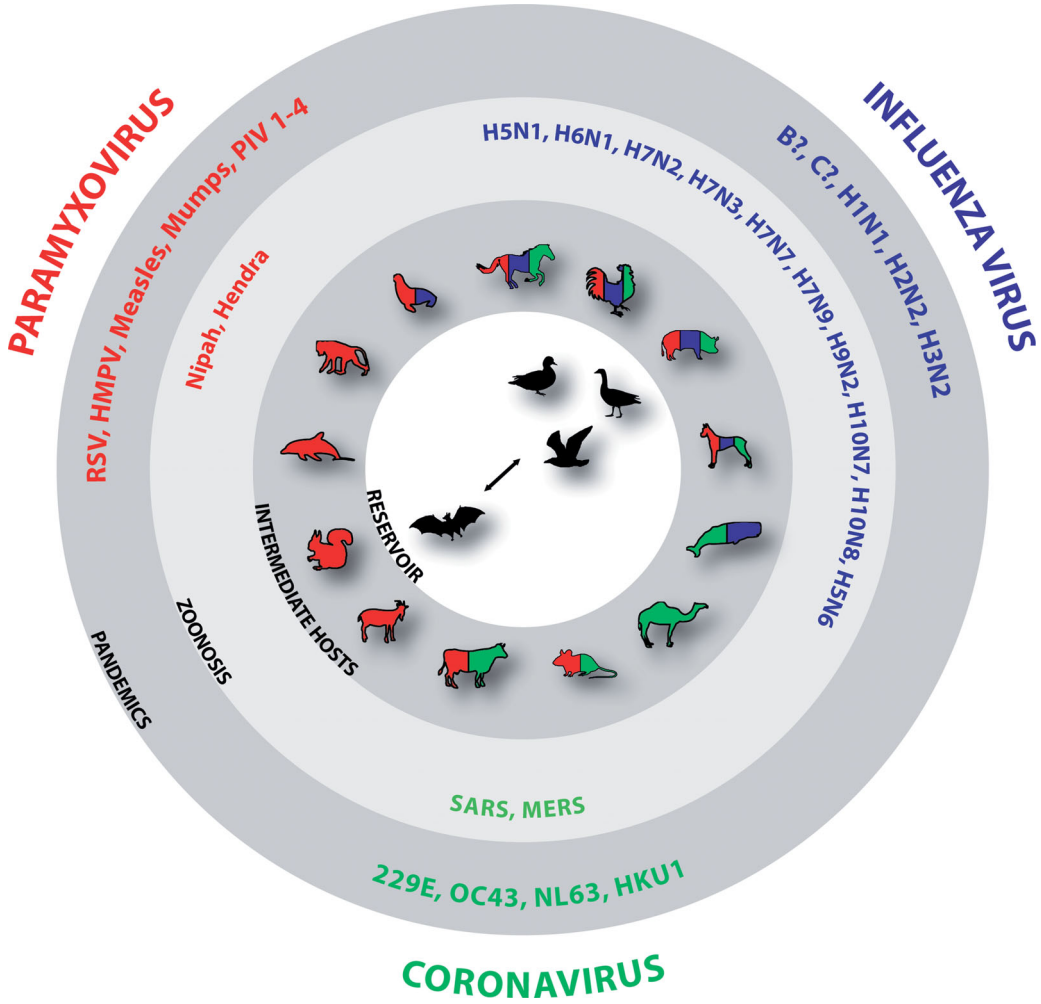

Figure 1. Paramyxovirus, coronavirus and influenza virus hosts. Original reservoirs for influenza viruses (blue), paramyxoviruses (red) and coronaviruses (green) are shown in the center of the figure in black. Recent studies have placed bats as tentative hosts at ancestral nodes to both major Paramyxoviridae subfamilies (Paramyxovirinae and Pneumovirinae) (Drexler *et al.* 2012). Moreover, bats host the broadest diversity of coronaviruses including close relatives of humans coronaviruses, supporting the zoonotic origin of several humans coronaviruses (Vijaykrishna *et al.* 2007; Drexler, Corman and Drosten 2014). Birds have also been suggested to be the gene pool of group 3 coronaviruses (Woo *et al.* 2009).The presence of influenza viruses in both waterfowl and bats suggested that transmission from birds to bats may have occurred in the past (illustrated with the arrow between the two host groups). As humans come rarely into contact with bats and waterfowl, they are more likely to become infected with zoonotic viruses via intermediate hosts, such as domestic birds or mammals. Usually, these zoonotic events are restricted to isolated cases of human infection with no onward transmission between humans subsequently. Rarely, upon mutation or reassortment, zoonotic viruses adapt to become human-to-human transmissible, and may start a pandemic and become endemic in humans. Numerous viruses of animal origin are endemic in humans (outer circle). Four influenza pandemics have occurred in the last century. RSV: respiratory syncytial virus; HMPV : human metapneumovirus; PIV: parainfluenza virus; MERS: Middle-East Respiratory Syndrome; SARS: Severe Acute Respiratory Syndrome.

mumps virus and parainfluenza viruses types 1 through 4 are also endemic, causing recurrent epidemics in humans globally (Lamb and Griffith 2013). The paramyxoviruses switch hosts at a higher rate than numerous of other RNA virus families and infect a wide range of non-human hosts, including primates, horses, dogs, sheep, pigs, cats, mice, rats, dolphins, porpoises, fish, seals, whales, birds, cattle and bats (Kitchen, Shackelton and Holmes 2011). Newly emerging members of the family—Hendra virus and Nipah virus—have caused numerous fatal infections in humans upon zoonoses from horses and pigs, but originating from bats (Fig. 1) (Lamb and Griffith 2013).

Coronaviruses also infect and cause disease in a wide variety of species, including bats, birds, cats, dogs, pigs, mice, horses, whales and camels. Four coronaviruses are currently endemic, generally associated with relatively mild RTI in humans: HCoV-229E, HCoV-OC43, HCoV-HKU1 and HCoV-NL63 (Masters and Perlman 2013). The Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) that emerged in 2002 was rather virulent, causing approximately 800 deaths among 8000 human cases of RTI detected in 30 countries (Peiris *et al.* 2003). First, the isolation of a coronavirus closely related to the SARS-CoV from palm civets pointed at this species as being the natural hosts of SARS-CoV (Wang, Yan and Xu 2005). However, subsequent studies demonstrated that the true original reservoir of the SARS-CoV were bats of the genus *Rhinolophus* (Li *et al.* 2005). Similarly, since the first detection of the Middle East Respiratory Syndrome coronavirus (MERS-CoV) in 2012 (Zaki *et al.* 2012), nearly 1000 laboratory-confirmed cases of RTI have been reported, of which approximately a third with fatal outcome (WHO 2015). The identification of a closely related MERS-like CoV from the feces of South Africa bats of the family *Vespertilionidae* suggests that bats may also be a natural reservoir for the MERS-CoV (Ithete *et al.*

2013). These zoonoses of SARS-CoV and MERS-CoV highlight the ongoing threat of coronaviruses to cross the species barriers and cause outbreaks in humans (Fig. 1).

Influenza A viruses belong to the *Orthomyxoviridae* family and are enzootic in wild migratory birds of aquatic habitats around the world. They occasionally spill over from this bird 'virus reservoir' into other animal hosts, including domestic poultry, pigs, horses, a variety of carnivores and marine mammals. Sporadically, the viruses adapt to their new animal hosts, leading to enzootic virus circulation for years, decades or centuries. Zoonotic influenza A virus infections occur relatively frequently, but often without serious consequences for the public at large. However, the introduction of 'novel' influenza viruses from animals into the human population can result in pandemics, i.e. global epidemics caused by a new subtype of influenza viruses to which the immunity of the population is low or inexistent, as it was the case four times in the last 100 years alone (Fig. 1).

The zoonotic events caused by Hendra virus, Nipah virus, SARS-CoV, MERS-CoV and various animal influenza A viruses highlight the ongoing threat of respiratory viruses to cross the species barriers and cause outbreaks in humans. Luckily, most of these zoonotic viruses have so far displayed a very limited ability of transmission between humans, and hence zoonoses have not lead to global virus spread to cause a pandemic. However, given that some members of the same virus families have caused pandemics in the past repeatedly (as observed in the last century for influenza A viruses, or inferred from virus genome sequences for the other viruses), we remain aware that we might be less lucky with the next series of zoonotic events. Unfortunately, we have very little knowledge of what makes an animal virus transmissible between humans, the key factor determining the difference between sporadic zoonotic infections and—potentially devastating—pandemics. In fact, despite the significant impact of respiratory viruses on global health and economy, surprisingly little is known about (the determinants of) transmission routes. We know that respiratory viruses may spread via small aerosols (generally defined as <5 μm) or larger respiratory droplets upon coughing, sneezing or breathing (hereafter collectively referred to as 'airborne transmission') or by direct person-to-person contact or via contaminated surfaces or fomites. Influenza viruses are well known for their ability to transmit efficiently via the airborne route, while some paramyxoviruses (e.g. RSV) are thought to spread primarily via contact and fomites. What determines these routes and mechanisms of transmission has remained unknown. For several respiratory viruses, we do not even know with certainty what is the most important route of transmission. As a consequence of these knowledge gaps, key questions in public health such as whether particular newly emerging coronaviruses, paramyxoviruses and influenza viruses could acquire the ability of sustained transmission between humans to trigger a pandemic remain unanswered. Because of the relatively high frequency of influenza A virus zoonoses and recurrence of influenza pandemics (~every 30 years in the last centuries), researchers have started to investigate influenza virus transmission between mammals. Here we review the current state of influenza virus transmission research and discuss future challenges.

INFLUENZA A VIRUS ZOONOSES

Zoonotic transmission of influenza A viruses directly from wild aquatic birds to humans has been rarely reported, presumably because of limited human exposure to excreta from wild birds. Apart from a few cases where close contact with wild birds has been associated with zoonotic events, e.g. during hunting, or defeathering (Kurtz, Manvell and Banks 1996; Gilsdorf *et al.* 2006), humans have been primarily infected with zoonotic influenza viruses via intermediate species to which human exposure is more frequent, such as pigs and poultry (Fig. 2). The vast majority of human cases of zoonotic virus infections was sporadic, without evidence of sustained transmission between humans. Infections resulted from activities in close contact with animals, such as visiting live markets or fairs, care giving, meat processing, culling of infected animals or performing necropsies of infected animals.

Upon transmission of influenza A viruses from birds or humans to pigs, numerous influenza virus lineages have become enzootic in pig populations across the world (Vincent *et al.* 2014). Three subtypes, H1N1, H1N2 and H3N2, as well as reassortants between these enzootic swine influenza swine lineages, were proven to be able to cause human infections (Freidl *et al.* 2014). One swine influenza virus noteworthy for its zoonotic ability is the H3N2v virus, a reassortant between the North American swine influenza virus lineage and the 2009 pandemic H1N1 virus (pH1N1). Since the first documented cases in the fall of 2011, over 300 human infections with swine H3N2v virus have been reported (Freidl *et al.* 2014).

Initially, pigs were considered a necessary intermediate host to allow the adaptation of avian influenza viruses to mammals, as the respiratory tract of pigs contains receptors for both human and avian influenza viruses. However, occasional transmission of avian H5N1 influenza viruses directly from terrestrial poultry to humans since 1997 resulted in a revision of the paradigm that only pigs can act as bridge species for influenza virus zoonoses (Subbarao and Katz 2000). The H5N1 virus zoonotic events pointed at terrestrial poultry as an alternative potential intermediate host for transmission of influenza viruses from wild waterfowl to humans. Since these first human cases of avian influenza H5N1 virus infection, reports of zoonotic events arising directly from poultry have increased, presumably in part as a consequence of increased awareness and increased surveillance and pandemic preparedness. Avian influenza viruses of subtypes H5, H6, H7, H9 and H10 have indeed demonstrated the ability to infect humans (Richard, de Graaf and Herfst 2014). Transmission of avian influenza viruses of subtypes H5, H7 or H9 to humans was associated with frequent outbreaks caused by these viruses in poultry or their establishment in terrestrial birds, such as H9N2 viruses in Asia, H7N2 viruses in the USA and H5N1 viruses in a number of Asian, European and African countries (Alexander 2007). Although H6 viruses also have been widespread in poultry (Cheung *et al.* 2007), only one human case of avian H6N1 virus infection has been reported so far (Yuan *et al.* 2013). On the other hand, while outbreaks of H10 influenza viruses in poultry appear to be relatively rare, human infections with influenza viruses of the H10N7 and H10N8 subtypes have been reported (ProMED 2004; Arzey *et al.* 2012; Zhang *et al.* 2014). The discordance between the number of human cases of infection and the extent of virus circulation in poultry populations suggests that differences exist in the zoonotic potential of different influenza viruses or virus subtypes.

Although zoonoses with every subtype of influenza virus may be of concern, viruses of the H5, H9 and H7 subtypes are considered the greatest threat, because of the frequency of zoonoses and/or the severity of disease. The H5N1 virus was first detected in humans in Hong Kong in 1997, when 18 persons were found to be infected, of whom 6 died (de Jong *et al.* 1997). This

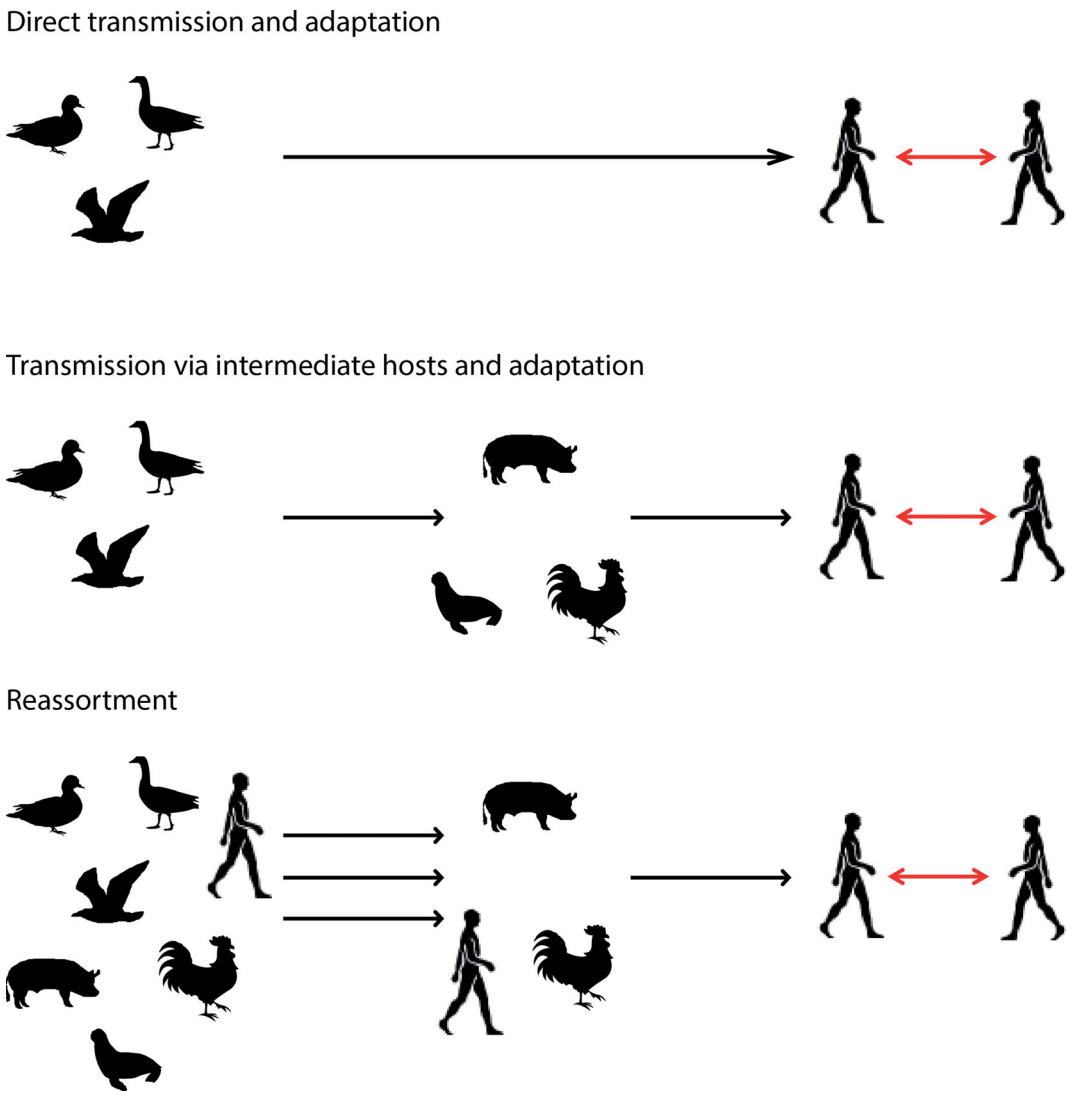

Figure 2. Potential routes of transmission and adaptation of influenza viruses. Many scenarios could support the transmission of influenza viruses from their original reservoir to humans and subsequent adaption to transmit via the airborne route: (i) direct transmission from waterfowl to humans and subsequent adaptation in humans; (ii) transmission from waterfowl to intermediate hosts, adaptation in these hosts and subsequent transmission to humans; and (iii) reassortment in intermediate hosts of influenza viruses originating from diverse animals species and transmission to humans.

virus was eradicated from Hong Kong by culling the local poultry population. In 2001 and 2002, poultry outbreaks of H5N1 viruses were reported in Hong Kong again, but without any human casualties (Sims *et al.* 2003). Between 2000 and 2002, H5N1 viruses were isolated from healthy domestic ducks in Southern China (Chen *et al.* 2004) and two H5N1 virus outbreaks were recorded in wild birds in 2002 (Ellis *et al.* 2004). In 2003 and 2004, H5N1 viruses spread from East Asia to Southeast Asia, including Vietnam, Thailand, Cambodia, Laos, Malaysia and Indonesia, and sporadic human infections were recorded along with outbreaks in terrestrial and aquatic poultry (Li *et al.* 2004; The World Health Organization Global Influenza Program Surveillance Network 2005; Smith *et al.* 2006). A second wave of virus spreading was recorded after the Qinghai Lake outbreak in waterfowl in 2005 in China (Chen *et al.* 2005; Liu *et al.* 2005), with H5N1 viruses spreading westward to India (Chakrabarti *et al.* 2009), to Europe (Lipatov *et al.* 2007; Starick *et al.* 2008; Artois *et al.* 2009) and Northern and Central Africa (Ducatez *et al.* 2007), where they were detected in poultry and many species of wild birds. Wild bird migration and poultry movement have been implicated in the geographic expansion of Qinghai-like H5N1 viruses (Kilpatrick *et al.* 2006; Keawcharoen *et al.* 2008). Human cases have continued to be reported along with poultry outbreaks. Most of the recent reports on H5N1 virus outbreaks have come from Egypt and southeastern parts of Asia (OIE 2014). Recently, zoonotic infections have been reported with H5N6 influenza viruses, caused by reassortants between the H5N1 virus and other influenza virus subtypes (WHO 2014a).

H5N1 viruses are unique in many ways. First, they are the only highly pathogenic avian influenza (HPAI) viruses that are enzootic in poultry in many parts of the world for over a decade. Moreover, as a result of this continued circulation, they diversified in different genetic clades, by accumulation of point mutations leading to distinct phenotypes, including antigenic differences. H5N1 viruses are also unique in the broad host range of species they infect, including poultry, wild birds and various mammals, the high incidence of zoonotic events and the reported severity of human infections. No sustained

transmission in any mammalian species has occurred, but due to continued circulation in both terrestrial and aquatic poultry in many countries across Eurasia and Africa and potentially in wild birds, H5N1 viruses pose continuing animal and human health threats.

H9N2 viruses have become highly prevalent in terrestrial and aquatic poultry in many countries of Eurasia since the middle of the 1990s (Lee *et al.* 2000; Perk *et al.* 2006; Xu *et al.* 2007; Sun *et al.* 2010; Shanmuganatham *et al.* 2013). H9N2 viruses have also been detected in pigs (Peiris *et al.* 2001; Cong *et al.* 2007). Human infections with H9N2 viruses have been reported upon contact with poultry since 1999 (Peiris *et al.* 1999; Butt *et al.* 2005; Cheng *et al.* 2011; ProMED 2013, 2014), usually resulting in relatively mild disease. Their relatively low virulence in poultry provides an opportunity for undetected virus circulation. Interestingly, numerous reassortment events between H9N2 virus and other influenza virus subtypes have been reported (Li *et al.* 2003, 2005; Xu *et al.* 2007) and internal genes of many other zoonotic viruses, e.g. H7N9, H5N1 and H10N8, are derived from H9N2 viruses (Guan *et al.* 1999; Lam *et al.* 2013; Monne *et al.* 2013; Gu *et al.* 2014).

A major zoonotic outbreak caused by an avian H7N9 virus in several provinces of China has resulted in more than 400 laboratory-confirmed human infections and with more than 100 fatalities. Contrary to H5N1 viruses, the H7N9 viruses did not cause severe disease in poultry and therefore circulated undetected in poultry, making it more difficult to contain the outbreak and prevent further transmission to humans. Many recent studies have reported the occurrence of reassortment events between H7N9 viruses and H9N2 viruses circulating in domestic birds in China (Ke *et al.* 2014; Liu *et al.* 2014; Lu *et al.* 2014), highlighting the need for increased surveillance of poultry. Apart from H7N9 viruses, which stand out by the number of fatalities they have caused in humans, other H7 viruses have also sporadically caused zoonotic infections in the United Kingdom, United States, Canada, Mexico, the Netherlands and Italy (Campbell, Webster and Breese 1970; Taylor and Turner 1977; Fouchier *et al.* 2004; Tweed *et al.* 2004; Puzelli *et al.* 2005; Nguyen-Van-Tam *et al.* 2006; Editorial team 2007; Ostrowsky *et al.* 2012; Lopez-Martinez *et al.* 2013). In 1979, human infection was reported with an H7N7 virus of seals, which remains the only report of an influenza zoonotic infection coming from an animal host other than birds and pigs (Webster *et al.* 1981).

The severity of the disease caused by zoonotic influenza viruses is diverse. Symptoms range from conjunctivitis (often associated with the H7 subtype) to influenza-like symptoms, pneumonia, and acute respiratory distress syndrome to encephalitis. The distinction between HPAI and LPAI based on pathogenicity in poultry does not correlate with the severity of disease in humans. Whereas LPAI H7N9 virus can cause severe human illness, other LPAI or HPAI viruses of the H7 subtype have generally been associated with conjunctivitis or influenza-like illness.

GENERATION OF PANDEMIC INFLUENZA VIRUSES

Usually, zoonotic events are restricted to sporadic primary individual cases that are not transmitted between humans subsequently. However, on rare occasions, influenza viruses from the animal reservoir have acquired the ability to transmit between humans and as a consequence to start a pandemic. Thus, the critical distinction between zoonotic and pandemic influenza viruses lies in their transmission phenotypes. Four pandemics have been recorded in the last 100 years: the 1918 H1N1 Spanish pandemic, the 1957 H2N2 Asian pandemic, the 1968 H3N2 Hong Kong pandemic and the 2009 H1N1 (pH1N1) pandemic. These pandemic viruses were all highly transmissible between humans. Two main modes of transmission, which are not mutually exclusive, have been shown to support the spread of influenza viruses between humans: contact and airborne transmission. Contact transmission can either be direct, involving transfer of infectious particles by direct physical contact between an infected person and a naïve person, or indirect via contaminated surfaces or objects (fomites). On the other hand, airborne transmission occurs when infectious particles expelled by an infectious person travel via the air and directly settle on the respiratory tract mucosa of a susceptible person. Airborne transmission can occur via two modes. Respiratory droplets (also called droplet spray), of which the size exceed 5 μm, do not remain suspended in the air and are propelled at a distance of less than a meter but with sufficient momentum to directly hit the mucosa. On the other hand, small aerosols, which are defined a being smaller than 5 μm, have a slow settling velocity and can remain suspended in the air for minutes or hours. These infectious aerosols can be widely dispersed in air and be inhaled by susceptible persons who may be at some distance from the infectious source. Aerosols can also be the result of desiccation of respiratory droplets, and in such case are called droplet nuclei. There are conflicting opinions regarding the relative importance of contact and airborne transmission for the spread of influenza viruses among humans. However, the fact that all animal-origin influenza viruses that caused pandemics in the past were transmissible via the airborne route, point to this route being crucial for the pandemic potential of influenza viruses.

One fundamental question that needs to be addressed if we want to understand how influenza pandemics get started to ultimately prevent them or reduce their impact is how influenza viruses arising from the animal reservoir acquire the ability to become airborne transmissible. The fact that at least three of the four last pandemic influenza viruses (the ones of 1957, 1968 and 2009 as the origins of the 1918 H1N1 pandemic virus remain largely controversial; Gibbs, Armstrong and Gibbs 2001; Taubenberger *et al.* 2006; Smith *et al.* 2009; Worobey, Han and Rambaut 2014) occurred upon reassortment events between viruses of different origin established the paradigm according to which the principal mechanism of generation of pandemic viruses would be reassortment. Pigs were assumed to play an important role as intermediate hosts or 'mixing vessels' for human, avian and swine influenza viruses, because they can be infected readily by both avian and human influenza virus. However, recent research showed that the distribution of influenza virus receptors in the porcine respiratory tract is similar to that in humans, suggesting that humans are equally likely to constitute 'mixing vessels' (Nelli *et al.* 2010; Van Poucke *et al.* 2010). Although avian influenza virus receptors predominate in chickens, quail and other land-based birds, human influenza virus receptors are also present throughout the respiratory and enteric tracts of these birds (Guo *et al.* 2007), which could therefore serve as intermediate hosts for the reassortment or adaptation of influenza viruses. Moreover, the direct transmission of fully avian influenza viruses to humans (see above) established that avian viruses can infect humans without acquiring mammalian-origin influenza virus genes by reassortment in an intermediate host. Recent studies using animal models have also shown that fully avian influenza viruses have the ability to adapt to mammals to become airborne transmissible (Herfst *et al.* 2012; Sutton *et al.* 2014), suggesting that airborne transmissibility could also

be acquired without the contribution of mammalian-origin influenza virus genes. Therefore, many scenarios, which are not mutually exclusive, could support the transmission of influenza viruses from their original reservoir to humans and subsequent adaption to transmit via the airborne route (Fig. 2).

ANIMAL MODELS TO STUDY AIRBORNE TRANSMISSIBILITY OF INFLUENZA VIRUSES

Mapping the host, viral and environmental determinants of airborne transmission of influenza viruses has been on the research agenda already for many years. Ferrets are a well-established model for both transmission and pathogenesis of influenza viruses and have been used in influenza research since the early 1930s (Smith, Andrewes and Laidlaw 1933). There are two major advantages of the ferret model compared to other models, such as mice. First, ferrets are readily susceptible to infection with avian, swine and human influenza viruses without the need of prior adaptation. Moreover, depending on the influenza virus strain they are infected with, ferrets manifest symptoms of upper and lower respiratory tract (URT, LRT) disease and pathology similar to that observed in humans (Smith, Andrewes and Laidlaw 1933; Reuman, Keely and Schiff 1989). In the ferret transmission model, pandemic and seasonal influenza viruses isolated from humans are transmitted from an infected ferret to an uninfected ferret via the airborne route, whereas avian viruses are generally not (Linster *et al.* 2014). In addition to the ferret model, guinea pig transmission models were recently developed because they are more practical and less expensive (Lowen *et al.* 2006). Guinea pigs can also be readily infected with human and avian influenza viruses without adaptation but they display different symptoms upon influenza virus infection than ferrets and humans. Although influenza viruses can replicate to high titers in the respiratory tract of guinea pigs, they generally do not display severe disease upon infection with influenza viruses, even with strains that are pathogenic for humans and ferrets such as H5N1 viruses (Kwon, Lipatov and Swayne 2009). Despite these differences in symptoms and disease severity, similar general conclusions on the transmissibility of human and avian viruses can be reached using the ferret and guinea pig model (Bouvier and Lowen 2010). One of the great advantages of the guinea pig model is the possibility to cover areas of research difficult to study with the larger and more expensive ferret model. Guinea pigs can be kept in controlled climate chambers that are large enough to house multiple pairs of animals, to study the impact of environmental parameters such as temperature or humidity on influenza virus transmission (Lowen *et al.* 2007, 2008; Steel, Palese and Lowen 2011). The more economical and less demanding guinea pig model has also allowed increasing the number of replicates and therefore the statistical power of transmission experiments.

Although experimental setups may vary between different laboratories, they generally consist of placing inoculated donor animals in cages adjacent to cages housing naive recipient animals. To allow airflow from the donor to the recipient animal, and exclude transmission via direct contact or fomites, the two cages are separated by air-permeable barriers (Lowen *et al.* 2006; Maines *et al.* 2006, 2009; Munster *et al.* 2009). In general, the recipient animals are placed in the adjacent cage a day after inoculation of the donor animals to prevent exposure of the recipient animals to the inoculum. Donor animals are traditionally inoculated via the intranasal route, although aerosol inoculation has also been described for ferrets (Gustin *et al.* 2011) and guinea pigs (Mubareka *et al.* 2009) to better mimic natural infection. Nasal washes or swabs are collected frequently from the donor and recipient animals, and serology can be used to confirm infection of recipient animals.

One limitation of most experimental systems is that they do not allow discrimination between transmission via respiratory droplets and aerosols as both can occur over a short-range distances. Only a few studies have addressed the relative contribution of the different possible routes of transmission. An early study on transmission of influenza viruses using the ferret model made a strong case that transmission occurred primarily via aerosols (Andrewes and Glover 1941). It was demonstrated that virus transmission could occur when donor and recipient ferrets were up to 1,5 m apart in open wire cages, or even when the cages were separated by either straight or S- or U-shaped ducts. To rule out respiratory droplets transmission, recipient ferret cages were placed above the donor ferrets cages and transmission was still observed. Moreover, improvement of the ventilation of the room abolished transmission. More recently, Mubareka *et al.* (2009) used the guinea pig model to study the contribution of short- and long-range aerosol transmission. An H3N2 influenza virus was transmitted from inoculated to naïve guinea pigs over a distance of 80 or 107 cm and in an upward direction, suggesting an important role of aerosols in airborne virus transmission.

Another limitation of animal transmission models is the extrapolation to humans of the results of transmission experiments, which should be performed carefully. Indeed, mammalian species are diverse and some mammalian adaptation might be host specific. However, the detection in human strains of substitutions that have been selected upon adaptation to ferret (see below), as well as other arguments described above, pleads in favor of the appropriateness of the ferret model to study human influenza viruses.

ENVIRONMENTAL AND HOST FACTORS INFLUENCING AIRBORNE TRANSMISSIBILITY OF INFLUENZA VIRUSES

The environmental drivers of influenza virus airborne transmissibility are largely unknown. Several studies reported the impact of environmental factors on influenza virus survival, stability and transmissibility. Virus persistence in different environments is subject to variation in temperature and humidity. It is interesting to note that at constant humidity, avian influenza viruses remain viable in aerosols for longer time periods than human influenza viruses (Mitchell, Guerin and Robillard 1968). Moreover, biological decay of aerosolized influenza virus increased with relative humidity (Schaffer, Soergel and Straube 1976). This observation correlated with increased transmission efficiency at lower relative and absolute humidity (Lowen *et al.* 2007; Shaman, Goldstein and Lipsitch 2011). High temperatures have also been shown to be detrimental to airborne transmission efficacy (Lowen *et al.* 2008). The fact that cool and dry conditions enhance influenza survival and transmissibility might provide one explanation for the seasonality of influenza.

An important piece of missing information is about the relative contribution of specific host factors to the transmission efficiency of influenza viruses. The concept of superspreaders received considerable attention for other pathogens, such as measles virus and or SARS-CoV (Stein 2011), but little for influenza. Both respiratory droplets and aerosols can be expelled by coughing, sneezing, talking and during tidal breathing (Fabian

et al. 2008; Lindsley et al. 2010). Experimental evidence supports the fact that humans generate infectious particles in both respiratory droplets and aerosols (Lindsley et al. 2010) and that their generation is enhanced during influenza illness (Lindsley et al. 2012). Interestingly, Gustin et al. (2013) reported that ferrets infected with highly transmissible human influenza viruses exhaled and sneezed out more respiratory particles overall than those infected with poorly transmissible avian strains. Experimental data support the association between influenza virus airborne transmissibility between ferrets and the amount of vRNA-containing particles exhaled by the infected donor ferret (Lakdawala et al. 2011). However, Koster et al. showed that vRNA content in air samples is not an accurate surrogate marker for infectious airborne virus. Indeed, the longer the time between the inoculation of the donor and the exposure of the naïve ferret, the less efficient the transmission was, although vRNA levels remained relatively constant over 5 days post-inoculation (Koster et al. 2012). Capturing infectious virus-containing particles from exhaled breath, sneeze or cough remains technically problematic and prevents a more complete understanding of the aerobiology of influenza viruses (Milton et al. 2013).

Among healthy individuals, an important variation exists in the amount and size distribution of respiratory particles expelled while coughing or breathing (Edwards 2002; Lindsley et al. 2012). Moreover, concomitant infections, underlying respiratory diseases (e.g. asthma), age, overall immune competence, the level of host immune responses to controlling influenza virus infection, the nature of the natural resident flora and many other factors could influence the degree of virus shedding in aerosols and respiratory droplets and therefore the transmission efficiency. Children, who possess no or minimal immunity against influenza viruses, and immunocompromised individuals, who can shed virus for long periods of time at high titers, have already been pinpointed as good transmitters compared to healthy adults (Weinstock, Gubareva and Zuccotti 2003; Sato et al. 2005). However, it is important to conclude that considerable gaps remain in our knowledge of environmental and host factors important for influenza virus transmission.

VIRAL FACTORS DETERMINING AIRBORNE TRANSMISSIBILITY OF INFLUENZA VIRUSES

Learning from past pandemics

Retrospective analyses of how previous pandemic influenza viruses acquired the ability to infect humans and transmit between them have been very useful to understand the molecular basis of airborne transmissibility of influenza viruses. A common characteristic of human seasonal and pandemic viruses is that the receptor-binding site (RBS) of the hemagglutinin (HA) preferentially recognizes sialic acid receptors linked to galactose by α2,6-linkage (α2,6-SA) whereas avian influenza viruses preferentially bind α2,3-SA receptors (Stevens et al. 2006; van Riel et al. 2010; de Graaf and Fouchier 2014). Virus receptor specificity is thought to primarily determine host species restriction and cellular and tissue tropism as determined by differences in receptor distribution. In humans, α2,6-SA receptors are predominantly present on ciliated cells in the URT (oropharynx, nose, throat), while α2,3-SA receptors are mainly present on non-ciliated cells and type II pneumocytes of the LRT (bronchi, alveoli) (Shinya et al. 2006; van Riel et al. 2006). Studies on the three pandemic influenza viruses of the 20th century demonstrated that α 2,6-SA preference was a critical determinant not only of host adaptation but also of subsequent airborne transmission between mammals (Tumpey et al. 2007; Pappas et al. 2010; Roberts et al. 2011). Retrospective analyses of pandemic 1918 H1N1, H2N2 and H3N2 viruses showed that only one or two substitutions in the RBS of HA were required to confer α2,6-SA binding specificity (Matrosovich et al. 2000). Two substitutions, Q226L and G228S in the RBS of HA, were enough for avian H2 and H3 viruses to change from α2,3-SA to α2,6-SA specificity (Connor et al. 1994; Matrosovich et al. 2000). In contrast, the α2,6-SA preference of H1N1 was determined primarily by 190D and 225D (Matrosovich et al. 2000; Glaser et al. 2005). pH1N1 viruses also possessed 190D and 225D (Maines et al. 2009), although it has never been shown that these two amino acids were solely responsible for their α2,6-SA specificity. A D225G substitution, leading to dual receptor specificity for α2,3-SA and α2,6-SA, did not decrease the airborne transmissibility of the pH1N1 virus in ferrets and guinea pigs (Chutinimitkul et al. 2010). Attachment and subsequent viral replication in the URT of the donor, associated with α2,6-SA preference, is considered to be an important determinant for airborne transmissibility of influenza viruses. However, little is known whether the source of the exhaled virus from the donor is the epithelium in the URT or in the LRT or both, and whether shedding into respiratory droplets or aerosols depends on the location of viral replication. Although one could hypothesize that α2,6-SA preference would also facilitate infection in the recipient, it has been shown that respiratory droplets tend to remain trapped in the URT whereas aerosols can be inhaled into the LRT (Hinds 1982), suggesting α2,3-SA binders would also be able to penetrate directly in the LRT and start an infection.

The polymerase proteins that are part of the viral ribonucleoprotein complexes (vRNPs) are also known to carry host range restriction determinants (Naffakh et al. 2008; Mänz, Schwemmle and Brunotte 2013). For example, substitutions E627K and D701N in PB2 enhance polymerase activity and viral replication in mammalian cells at ~33°C, the temperature in the mammalian URT (Subbarao, London and Murphy 1993; Brown et al. 2001; Gabriel, Czudai-Matwich and Klenk 2013). These residues are commonly found in human influenza virus isolates and have been shown to support the transmission of human influenza viruses between mammals (Steel et al. 2009; Van Hoeven et al. 2009). However, these substitutions were not present in pH1N1 viruses and site-directed mutagenesis of the PB2 gene of pH1N1 virus did not affect airborne transmission (Herfst et al. 2010). It was shown subsequently that efficient replication of pH1N1 viruses in mammalian hosts was achieved by alternative substitution, T271A and the 590/591 SR polymorphism, in PB2 (Liu et al. 2012).

The matrix (M) gene segment was also found to be a critical factor for the transmission of pH1N1 in the guinea pig model (Chou et al. 2011). Other studies revealed a close interplay between the M and neuraminidase (NA) segments affecting virus morphology and transmissibility in mammalian hosts (Lakdawala et al. 2011; Ma et al. 2012; Campbell et al. 2014)

Most experiments performed on pandemic viruses have been loss of function (LOF) experiments. They were conducted using fully transmissible viruses and aimed to dissect what made them transmissible, by reverting mammalian adaptation substitutions and assessing their impact on transmission (Tumpey et al. 2007; Pappas et al. 2010; Roberts et al. 2011). These LOF experiments can give clues on determinants that are necessary for a given phenotype, but not on determinants that are strictly sufficient for this function. Although changes in receptor specificity and enhanced viral replication in mammalian hosts are prerequisites for cross-species transmission and host

adaptation, studies on airborne transmission of human and avian influenza viruses demonstrated that these traits were necessary but not sufficient to result in airborne transmission between mammals (Maines *et al.* 2006, 2011; Herfst *et al.* 2012; Imai *et al.* 2012), meaning that some pieces of the puzzle were still missing. Gain-of-function transmission (GOF-TXM) experiments are necessary when one aims to pinpoint the minimal molecular and phenotypic traits for a given phenotype, i.e. airborne transmission of influenza viruses. GOF-TXM experiments are also needed if we wish to investigate if viruses that have not yet acquired the potential of airborne transmission could acquire this trait in the future. Several recent GOF-TXM experiments conducted in different laboratories have improved our knowledge of the viral factors required for airborne transmission of influenza viruses. Moreover, these experiments showed that airborne transmission in mammals was not an exclusive property of the few virus subtypes that have caused human pandemics (H1, H2 and H3 influenza viruses). GOF-TXM experiments have indeed demonstrated that other virus subtypes, like H5N1, H9N2 and H7N1, can also overcome the natural barriers that prevent them from being airborne transmissible.

Airborne transmission of avian-origin H9 influenza viruses

An increasing number of contemporary avian H9N2 viruses possess α2,6-SAreceptor specificity as the result of a leucine at position 226 in the HA, supporting the ability to replicate efficiently in human respiratory epithelial cells and mammalian hosts (Matrosovich, Krauss and Webster 2001; Choi *et al.* 2004, 2; Wan and Perez 2007). Wan *et al.* studied eight avian H9N2 viruses isolated between 1997 and 2003, some of which possessed the Q226L substitution. Substitution Q226L tuned the specificity of H9N2 viruses toward α2,6-SA receptors but without eliminating binding to α2,3-SA receptors, and the Q226L substitution alone was not enough to support airborne transmission of these H9N2 viruses (Wan *et al.* 2008). Therefore, avian H9N2 viruses, including those that acquired α2–6 SA receptor specificity, still lack key features for efficient airborne transmission between mammals. Several studies aimed at dissecting the molecular constraints of airborne transmission of H9N2 viruses reported the acquisition of airborne transmissibility upon genetic reassortment with human influenza viruses, with or without the need of further adaptation (Sorrell *et al.* 2009; Kimble *et al.* 2011; Kitchen, Shackelton and Holmes 2014).

A reassortant virus containing the HA and NA of an H9N2 virus and the remaining genes of an H3N2 virus was initially not transmissible via the airborne route between ferrets, but acquired key mutations in the HA gene (Q226L, T189A and R192G) upon serial passaging in ferrets, rendering the virus airborne transmissible (Sorrell *et al.* 2009). This adaptation in ferrets also resulted in changes in the antigenic properties of the virus. These results suggested that avian H9N2 viruses require little adaptation in mammals following reassortment with a human influenza H3N2 virus to become airborne transmissible between ferrets. Similar reassortment experiments were performed with pH1N1 virus. In contrast to H3N2, a reassortant containing the wild-type avian H9N2 HA and NA with the internal genes of pH1N1 virus was readily transmissible without the need of further adaptation, pointing again toward the crucial role of internal genes in airborne transmission. However, when only the wild-type H9N2 HA was combined with the remaining genes of pH1N1 virus, airborne transmission was abolished, suggesting that an optimal balance between HA and NA activities was required for airborne transmission (Kimble *et al.* 2011). More recently, Kimble *et al.* (2014) showed that other reassortant viruses between pH1N1 and H9N2 were also airborne transmissible. Collectively, these results showed that avian H9N2 viruses and pH1N1 viruses, both of which have been isolated from pigs, are genetically compatible and can lead to the generation of novel reassortant viruses with airborne transmission potential in mammals with or without the need of further adaptation.

After the recent H7N9 outbreak, several studies have aimed to assess the potential risk of H9N2 virus to mammals, because they were the donors of the internal genes of many zoonotic influenza viruses. A total of 12 antigenically distinct H9N2 viruses, isolated from humans, birds or pigs in Asia between 1998 and 2009 were found to lack the ability of airborne transmission between ferrets (The SJCEIRS H9 Working Group 2013). However, Li *et al.* (2014) studied 35 H9N2 viruses isolated from poultry in 12 different regions of China between 2009 and 2013 and showed that all H9N2 viruses included in this study had a preference for α2,6-SA receptors. Moreover, they identified an I155T substitution in HA that played an important role in the binding of H9N2 viruses to α2,6-SA receptors. From a subset of nine H9N2 viruses belonging to different genotypes, six virus isolates were readily transmitted via the airborne route between ferrets. This is the first report of airborne transmission of avian H9N2 influenza viruses that are naturally circulating. Moreover, the rapid acquisition of additional mammalian adaptation substitutions, such as E627K and D701N in PB2, was detected upon infection of ferrets. These data indicate that recent H9N2 influenza viruses not only can acquire the ability to bind to α2,6-SAreceptors upon circulation in avian species, but also acquire the ability of airborne transmissibility between ferrets. This contrasts with the paradigm that mammalian intermediate hosts such as pigs are necessary to acquire such properties.

Collectively, the research on mammalian adaptation and airborne transmission of H9N2 viruses highlights the potential public health threat posed by these viruses. H9N2 viruses are evolving rapidly and acquire new phenotypic traits, that are passed on to other contemporary influenza virus subtypes via reassortment, and as such function as intermediates for these other virus subtypes to adapt for more efficient replication in and transmission between mammals.

Airborne transmission of avian-origin H5 influenza viruses

As HPAI H5N1 viruses remained a public health threat for over a decade, many studies have been performed in order to understand the molecular changes these viruses would require to become airborne transmissible between mammals. Although some mammalian adaptation markers were detected in human and avian HPAI H5N1 virus isolates (Watanabe *et al.* 2011), these changes did not result in sustained transmission between humans. Several early studies aiming to determine the requirements for airborne transmission of HPAI H5N1 virus failed to generate H5N1 viruses that were airborne transmissible. Some of these studies employed mammalian-adapted H5N1 viruses (Steel *et al.* 2009; Maines *et al.* 2011), or reassortants between H5N1 and human H3N2 and pH1N1 influenza viruses (Maines *et al.* 2006; Jackson *et al.* 2009; Schrauwen *et al.* 2013). However, more recent studies using H5N1 viruses and ferret and guinea pig transmission models brought us a step closer toward understanding influenza virus transmission via the airborne route (Chen *et al.* 2012; Herfst *et al.* 2012; Imai *et al.* 2012; Zhang *et al.* 2013; Linster *et al.* 2014).

Herfst *et al.* (2012) demonstrated that a fully avian HPAI H5N1 virus can become airborne transmissible between ferrets upon acquisition of only a handful of mutations. The introduction of amino acid substitutions Q226L and G228S in HA to switch the binding preference from α2,3-SAs to α2,6-SAs and E627K in PB2 of A/Indonesia/5/2005 (Indo05) to increase polymerase activity in mammalian cells was insufficient to confer airborne transmission of this virus between ferrets. However, airborne transmission of Indo05 was acquired upon adaptation to the URT by serial passaging in ferrets. Five substitutions were consistently found in all airborne-transmitted viruses: the three substitutions that were introduced initially, H110Y located at the trimer interface of HA and T160A that resulted in the loss of a potential N-linked glycosylation site at residues 158–160 of HA. Linster *et al.* (2014) subsequently showed that each one of the substitutions in the RBS (Q226L or G228S) alone was sufficient to support airborne transmission. The H110Y substitution in HA was shown to be important for transmission, and this substitution was associated with increased HA thermostability and a decreased pH threshold for HA-mediated membrane fusion (see below). Moreover, Linster *et al.* described a novel PB1 H99Y mutation, critical for airborne transmission of Indo05 H5N1 virus. The H99Y substitution in PB1, in concert with E627K in PB2, tuned the balance of transcription of different RNA species (vRNA, mRNA, cRNA) produced by the viral polymerase during infection of mammalian cells, resulting in increased virus replication.

Other recent studies demonstrated that H5N1 viruses can also acquire airborne transmissibility upon genetic reassortment with human influenza viruses, with or without the need of further adaptation (Chen *et al.* 2012; Imai *et al.* 2012; Zhang *et al.* 2013).

Imai *et al.* (2012) introduced random substitutions in the globular head of HA of A/Vietnam/1203/2004 (VN1203), and subsequently tested the viruses for α2,6-SA receptor-binding preference. Two substitutions in HA, Q226L and N224K, were selected to produce a reassortant virus carrying the mutated VN1203 HA and the remaining genes of pH1N1. Since this virus was not readily transmitted between ferrets, further adaptation was achieved by serial virus passaging in ferrets, to yield an airborne-transmissible virus. During adaptation to ferrets, two additional substitutions in HA were acquired: N160D and T318I. N156D resulted in the loss of the same putative N-linked glycosylation site that was lost through the T160A mutation as identified by Herfst *et al.* T318I is located close to the fusion peptide and was shown to have the same phenotype as H110Y identified by Herfst *et al.*

It was quite surprising that two independent studies, using different H5 HAs and different experimental procedures, came to similar conclusions on the critical substitutions in HA and the phenotypic traits leading to airborne transmission of H5N1 virus (Fig. 3). First, the affinity of both airborne Indo05 and VN1203 was tuned toward increased α2,6-SA receptor binding and decreased avian receptor binding, although 2,6-SA binding was relatively low compared to that of human H3 viruses. This shift was associated with differences in specificities for α2,6 sialosides (de Vries *et al.* 2014). The RBS of both airborne Indo05 and VN1203 was ~1 Å wider between the 130 and 220 loops compared to that of the wild-type RBS, similar to what has been observed with human influenza virus HAs (Lu *et al.* 2013; Xiong *et al.* 2013; Zhang *et al.* 2013).

Secondly, both airborne Indo05 and VN1203 possessed a putative lack of glycosylation site at position 158–160. It has been shown in previous studies that the removal of this specific glycosylation site increased the binding of H5N1 viruses to α2,6-SA and supported efficient replication of H5N1 viruses carrying the Q226L alone or in combination with G228S (Gao *et al.* 2009; Wang *et al.* 2010). It can be speculated this glycosylation site interferes sterically with binding to cellular receptors and that its removal tunes the receptor-binding properties and replication of viruses harboring the receptor-binding changes Q226/G228S.

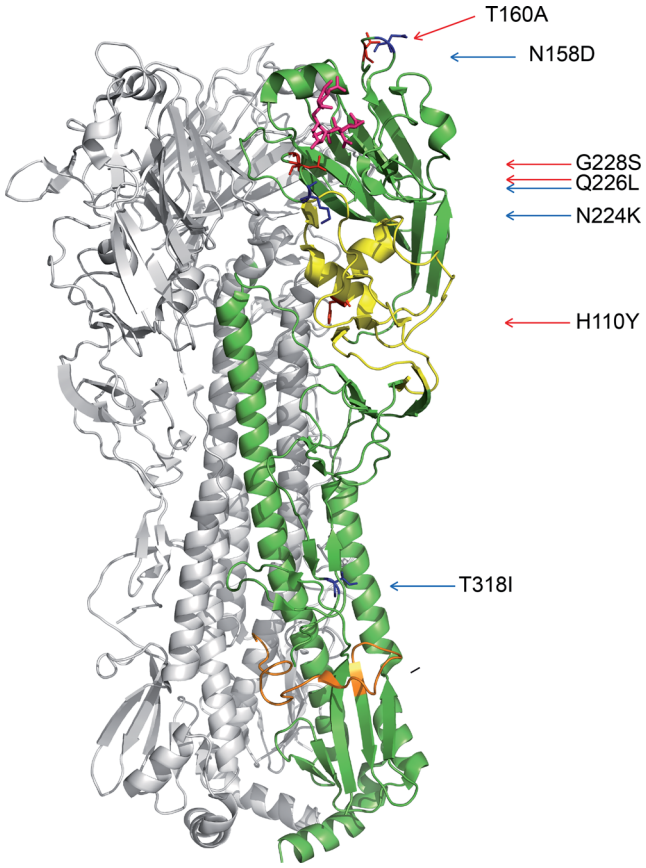

Figure 3. Substitutions associated with airborne transmission of H5N1 influenza virus between ferrets. Cartoon representation of the HA structure of A/Vietnam/1203/04 H5N1 influenza A virus (protein database code 4KDO). The ribbon structure of one HA monomer is colored in green, the fusion peptide and vestigial esterase subdomain are depicted in orange and yellow, respectively. The human receptor analog, LSTc, depicted in pink is docked into the RBS. The amino acid substitutions described by Herfst *et al.* are highlighted in red, and the mutations of Imai *et al.* in blue. Substitution Q226L was described by both groups. Substitutions T160A and N158D, H110Y and T318I, and Q226L/G228S and N224K/Q226L are thought to be functionally equivalent.

Moreover, both Linster *et al.* and Imai *et al.* highlighted a previously overlooked critical phenotypic trait for airborne transmission of H5 viruses: a decrease in the threshold pH at which membrane fusion is achieved by HA and that is correlated with an increased thermostability. After influenza viruses attachment to the cell surface by the binding of HA to SA, the virus particle is internalized by endocytosis. An irreversible conformational change of HA, which is triggered by a decrease of the pH in the endosome, mediates the fusion between the viral and the endosomal membranes, allowing the viral genetic material to be released in the cytoplasm. The pH at which the HA conformational change is triggered is specific to the HA and is important for virus infectivity. Viruses possessing an HA for which the conformational change is triggered at relatively high pH may be

more likely to lose infectivity upon exposure to low pH in the extracellular environment. Differences between the pH thresholds at which avian and human virus HAs induce membrane fusion have been suggested to point at this phenotype as a host range determinant (Galloway *et al.* 2013). The conformational change of HA from a non-fusogenic to a fusogenic state can also be triggered at neutral pH when the HA is exposed to increasing temperature (Carr, Chaudhry and Kim 1997). Both Linster *et al.* and Imai *et al.* showed that the introduction of substitutions leading to α2,6-SA preference increased the pH threshold for HA-mediated fusion and decreased the thermostability of HA. Amino acid substitutions H110Y and T318I in HA, found respectively by Linster *et al.* and Imai *et al.* upon adaptation to ferrets, both restored the pH threshold for fusion to the wild-type levels or even below. It still remains unknown whether the H110Y and T318I substitutions primarily compensate for the decreased pH stability caused by the substitutions responsible for α2,6-SA preference, or whether they reflect other stability phenotypes that are critical for airborne transmission of H5 influenza viruses, such as thermostability, stability in mucus, aerosols or respiratory droplets.

Chen *et al.* selected substitutions in HA of HPAI H5N1 virus using *in vitro* selection for binding to α2,6 sialosides. A Q196R substitution introduced in the HA of the clade 2.2 H5N1 virus A/egret/Egypt/1162/2006 (Egypt06) containing the Q226L and G228S substitutions, enabled contact transmission between ferrets. Of note, the HA of Egypt06 already lacks a glycosylation site around the RBS, affected by the N158D and T160A substitutions. Moreover, Chen *et al.* (2012) showed that an H5N1 reassortant virus containing HA with substitutions Q196R, Q226S and G228S, and the NA gene of a human H3N2 virus, was transmitted via the airborne route between ferrets. This data suggested that matched HA-NA functions may be important, in which a human virus NA may be required to accommodate for the α2,6-SA binding of HA.

In both the Imai *et al.* and Chen *et al.* studies, substitutions in HA associated with mammalian adaptation were necessary to achieve airborne transmission of H5N1 reassortants between ferrets. However, Zhang *et al.* (2013) showed that several H5N1 reassortants were transmitted via the airborne route between guinea pigs without the need for mammalian adaptation substitutions. Zhang *et al.* used H5 virus A/duck/Guangxi/35/2001 (Guangxi01), which already possessed dual receptor specificity for α2,3-SA and α2,6-SA, the T160A substitution that results in the loss of a glycosylation site in HA, and a D701N substitution in PB2 that increases virus replication in mammalian hosts. Here, it is noteworthy that all four H5N1 viruses that transmit in mammalian models lack the glycosylation site at position 158–160 (Chen *et al.* 2012; Herfst *et al.* 2012; Imai *et al.* 2012; Zhang *et al.* 2013). Zhang *et al.* showed that a reassortant virus containing the PA and NS genes of the pH1N1 virus and the remaining genes of Guangxi01 was transmissible via the airborne route between guinea pigs. The addition of the NP, M and NA genes from pH1N1 virus also enhanced the airborne transmissibility between guinea pigs. The results of Zhang *et al.* contrast with prior attempts to generate airborne-transmissible reassortant viruses between H5N1 and human viruses without introducing mammalian adaptation markers (Maines *et al.* 2006; Jackson *et al.* 2009; Imai *et al.* 2012; Schrauwen *et al.* 2013). This difference could be due, in part, to the use of different H5N1 viruses belonging to different lineages, viruses containing different mutations, and the use of different animal models. Moreover, it could also illustrate the need of a fine balance between the viral genes that could be specific to each virus.

Airborne transmission of avian-origin H7 influenza viruses

Although some avian H7 influenza viruses can be efficiently transmitted by contact, avian H7 viruses are not readily airborne transmissible between mammals (Belser *et al.* 2008, 2013; Song *et al.* 2009; Sutton *et al.* 2014). Sutton *et al.* (2014) were the first to report the generation of an airborne-transmissible H7 influenza virus. They used the HPAI virus A/ostrich/Italy/2332/2000 (H7N1), for which there were no reported cases of human infection during the 1999–2000 H7N1 outbreak in Italy. This H7N1 virus, which possessed the PB2 E627K mutation, was initially not capable of airborne transmission but became transmissible between ferrets after serial passaging in the URT of ferrets. Transmission to naïve ferrets occurred relatively late after exposure, usually within 6–7 days. In contrast, the transmission kinetics of human seasonal or pandemic influenza virus is generally much faster, within 1–2 days after exposure. Similar to the study of Herfst *et al.* on H5N1 virus, no reassortment was required to generate the airborne-transmissible H7 virus. Studies comparing the airborne-transmissible H7N1 virus with the wild-type H7N1 revealed that adaptation of the H7N1 virus to ferrets did not substantially decrease the pathogenicity of the virus in this animal model. Consensus sequence analysis revealed a limited number of amino acid substitutions that were found consistently in the airborne exposed animals: PB2 T81I, HA K/R304R, NP V284M, and M1 R95K and R211Q.

Although the receptor specificity of the airborne-transmissible H7N1 virus was not investigated, the K/R304R substitution is located in the stalk of H7 HA, distally from the RBS, and is unlikely to alter receptor specificity. This contrasts with what has been found for H5N1 viruses (Maines *et al.* 2006; Herfst *et al.* 2012; Imai *et al.* 2012) and seasonal and pandemic viruses (Tumpey *et al.* 2007; Pappas *et al.* 2010; Roberts *et al.* 2011), for which a receptor specificity switch was necessary, although not sufficient, to confer airborne transmissibility. The relatively high propensity of H7 influenza viruses to cross the species barrier might explain why additional substitutions in the RBS were not required for this H7N1 virus to become airborne transmissible. Given its location, it is possible that the K/R304R substitution has an impact on the acid stability and thermostability of H7 HA, as observed for H5N1 viruses. However, it was previously shown that HA-stabilizing substitutions alone are not sufficient to yield airborne-transmissible H5N1 viruses (Shelton *et al.* 2013; Zaraket *et al.* 2013), and it would therefore be unlikely that the K/R304R substitution alone would be sufficient to result in airborne transmission of H7 virus.

Two substitutions in M1 were acquired during adaptation to ferrets. The role of the M gene segment in transmission has been shown previously for the pH1N1 virus (Chou *et al.* 2011; Ma *et al.* 2012; Campbell *et al.* 2014). In particular, a filamentous morphology due to a fine interplay between the M and NA gene has been found to be important for transmission of pH1N1 virus (Campbell *et al.* 2014). Interestingly, the R95K mutation, which was acquired by the H7N1 virus during adaptation to ferrets, may function the opposite way as it has been reported to change H3N2 and H1N1 virus morphology from filamentous to spherical (Bourmakina and García-Sastre 2003). However, further studies are required to verify a change in morphology upon acquisition of R95K in H7N1 virus and its effect on transmission. The other substitution acquired in M1 was R211Q, in the region responsible for binding to vRNPs. Two other substitutions in the vRNP-encoding genes PB2 T81I and NP V284M were observed in the airborne-transmissible H7N1 virus. Whereas polar amino acids

at position 81 are predominant in avian strains, non-polar amino acids at the same position are predominant in human strains (Miotto *et al.* 2010), suggesting that the T81I mutations in PB2 might reflect mammalian adaptation. The NP V294M substitution is in the C-terminal PB2 interaction domain and might be a compensatory mutation modulating NP-PB2 binding. Further studies are necessary to dissect the phenotypes associated with the H7N1 airborne-transmissible associated mutations.

The airborne transmissibility of early H7N9 strains has been determined rapidly after their emergence (Belser *et al.* 2013; Zhang *et al.* 2013; Richard *et al.* 2013; Watanabe *et al.* 2014; Xu *et al.* 2014). The different laboratories working on various H7N9 strains provided remarkably similar results: all human H7N9 viruses displayed limited ability of airborne transmission between ferrets. H7N9 virus transmission was more efficient than that of other avian influenza viruses, which are generally not transmitted via the airborne route. However, the number of naïve animals that became infected upon aerosol exposure was lower and the kinetics of transmission was slower than for human and pandemic influenza viruses (Itoh *et al.* 2009; Munster *et al.* 2009). Mammalian adaptation markers, known to tune the binding preference toward α2,6-SA receptor specificity (e.g. G186V and Q226L in HA) or increase virus replication in mammalian hosts at lower temperature (e.g. E627K and D701N in PB2) for other viruses, were detected in the vast majority of human H7N9 virus isolates (Wang *et al.* 2014). The role of PB2 E627K, as well as other functionally equivalent substitutions, in increasing virus replication of H7N9 viruses in mammalian hosts and cell lines has been confirmed (Zhang *et al.* 2014; Mok *et al.* 2014; Song *et al.* 2014).

As a result of changes in the RBS of HA, most H7N9 viruses were able to bind to α2,6-SAreceptors and had a higher affinity for α2,6-SA than other H7 influenza viruses while keeping a dual receptor specificity for both α2,3-SA and α2,6-SA (Dortmans *et al.* 2013; Shi *et al.* 2013; Xiong *et al.* 2013; Xu *et al.* 2013; Yang *et al.* 2013; Ramos *et al.* 2013). Shi *et al.* (2013) showed that the Q226L contributes to the binding of H7N9 viruses to α2,6-SA, but was not solely responsible for it, and that other substitutions in the RBS were also implicated in the α2,6-SA preference of H7N9 viruses. Using directly labeled viruses, it was shown that H7N9 virus attached to both the URT and the LRT tissues of humans and other mammals, to ciliated and non-ciliated cells, and to type-I and type-II pneumocytes (Siegers *et al.* 2014). The α2,6-SA receptor-binding pattern of human H7N9 viruses probably arises from the introduction of two bulky hydrophobic residues by the substitutions Q226L and G186V, which widens the separation between the 220-loop and the 130-loop by about 1 Å, as described above for human influenza viruses and airborne H5 (Xiong *et al.* 2013).

None of the H7N9 virus isolates described to date possess the G228S substitution, which has been found in combination with the Q226L substitution in pandemic and other airborne-transmissible viruses. The introduction of G228S in an H7N9 virus already containing Q226L resulted in an overall increase in binding to α2,3 and α2,6-SA, but did not switch the relative receptor specificity (Yang *et al.* 2013). Tharakaraman *et al.* (2013) showed that the G228S substitution resulted in increased binding of A/Anhui/1/13 to the apical surface of human trachea and alveolar epithelium. However, since other amino acid substitutions in HA, such as 190D and 225D (Matrosovich *et al.* 2000; Glaser *et al.* 2005) can also affect α2,6-SA binding preference, it is possible that alternative amino acid substitutions could improve the receptor-binding preference of the H7N9 viruses, thereby potentially increasing airborne transmissibility of H7N9 viruses.

Studies on H5N1 virus have indicated that the glycosylation and stability patterns of HA are important for airborne transmissibility. Interestingly, H7N9 viruses already lack a glycosylation site at position 158–160, which may have contributed to their capacity to infect humans. Moreover, H7N9 viruses were shown to have a relatively unstable HA, for which the conformational change can be induced at relatively low temperatures and high pH as compared to human viruses or airborne H5N1 viruses (Richard *et al.* 2013). Identification of amino acid substitutions in the H7N9 HA that might increase the stability of HA and improve receptor binding to α2,6-SA receptors will be of importance. The fact that H7N9 viruses have been reassorting extensively with H9N2 viruses in China (Ke *et al.* 2014; Liu *et al.* 2014; Lu *et al.* 2014), and that some of these H9N2 viruses have been found to be readily transmissible between ferrets (see above), suggests that the evolution of H7N9 viruses should be monitored closely.

Airborne transmission of avian H1 influenza viruses

Recent studies have focused on assessing the risk posed by naturally circulating avian influenza viruses for potential zoonotic transmission to mammals. The H1N1 subtype is a logical candidate for such risk assessments, because this subtype has been responsible for the pandemics of 1918 and 2009 and has shown the ability to become endemic in humans and enzootic in pigs and poultry. In a recent study using 31 naturally circulating avian H1N1 isolates, one virus isolate (A/shorebird/DE/300/2009) was successfully transmitted via the airborne route between ferrets (Koçer *et al.* 2012). This is the first report of a wild-type avian H1N1 virus with the ability of airborne transmission between mammals, for which further analysis is needed to understand the molecular basis. Watanabe *et al.* (2014) recently conducted a study to assess the risk posed by naturally circulating avian H1 influenza viruses that contain genes that are closely related to the 1918 H1N1 pandemic virus. Phylogenetic analyses showed that avian influenza virus genes, encoding proteins closely related to those of the 1918 H1N1 pandemic virus, are present in the large influenza virus gene pool harbored by diverse avian hosts. Watanabe *et al.* generated a 1918-like virus that possessed gene segments with high homology to the 1918 H1N1 virus, but that originated from different avian viruses. Although the original 1918-like avian virus was not transmitted between ferrets via the airborne route, upon further introduction of substitutions E627K in PB2 and E190D and G225D in HA, the virus was recovered from one of the three ferrets exposed via the airborne route. Of note, the latter virus consisted of a mixed virus population possessing E or D at position 89 of HA (89ED) in addition to the HA E190D/G225D and PB2 E627K substitutions introduced to achieve increased virus attachment and virus replication in mammalian cells. Consensus sequence analysis revealed that additional substitutions in PB2 (A684D), PA (V253M) and HA (S113N) were acquired upon transmission. A subsequent transmission experiment was performed and the virus was transmitted via the airborne route to two out of three exposed ferrets. Virus recovered from the positive contact animals possessed additional mutations in HA (I187T) and in NP (T232I). Collectively, these results indicated that 10 substitutions in PB2, HA, PA and NP were associated with airborne transmission of 1918-like avian influenza viruses. Receptor-binding analysis confirmed that the E190D and G225D substitutions in HA were sufficient to switch the receptor specificity of the virus from α2,3-SA to α2,6-SA receptors. In agreement with findings on airborne transmission of H5N1 viruses, Watanabe *et al.* found that substitutions leading to α2–6 SA binding (E190D/G225D)

resulted in reduced HA thermostability, which was restored by additional HA substitutions acquired upon airborne transmission (E89D, E89D/S113N). Interestingly, the E89D and/or S113N mutations had no effect on the pH threshold of HA-mediated membrane fusion. This contrasts with previous reports on airborne-transmissible H5N1 viruses, for which increased acid stability correlated with increased thermostability. The enhanced polymerase activity of the airborne-transmissible 1918-like avian virus was only due to PB2 E627K mutation with no detectable contribution from the other substitutions in polymerase genes (PB2 A684D, PA V253M) acquired upon transmission. An analysis of avian influenza viruses from 1990 to 2011 showed that avian viruses possessing PB2, PB1, NP, M and NS proteins closely related to those of the 1918-like avian virus have predominantly circulated in North America and Europe.

Airborne transmission of mammalian H3 influenza viruses

Recent human cases of infection with swine-origin H3N2v virus in the United States have raised concern over the public health threat posed by these viruses. Since their emergence in pigs, triple-reassortant swine H3N2 viruses have been responsible for sporadic human cases (Shinde *et al.* 2009). Since 2011, however, the number of human cases has increased substantially (Freidl *et al.* 2014). H3N2v viruses possess five to six of the 'triple reassortant of internal genes' (TRIG) genes. The TRIG cassette refers to the following gene constellation: PB2 and PA of avian origin, PB1 of human origin and M, NP and NS of classical swine origin. The TRIG cassette has been first identified in swine influenza viruses in 1998 (Zhou *et al.* 1999) and hypothesized to have contributed to the maintenance of the H1 and H3 virus lineages in swine by conferring a competitive advantage over other swine influenza viruses gene constellations (Ma *et al.* 2010). The major difference between the 2011 H3N2v viruses and earlier H3N2v viruses isolated from human cases of infection is the M gene, which is derived from the pH1N1 virus (inherited from the eurasian classical swine lineage) in more recent strains (Centers for Disease Control and Prevention 2011). Pearce *et al.* (2012) analyzed the airborne transmission of four H3N2v influenza viruses isolated from humans in 2009, 2010 and 2011. Two H3N2v viruses that were isolated in 2010 and 2011 transmitted each to three out of three airborne exposed ferrets and another H3N2v virus from 2010 was transmitted to six out of six ferrets. The kinetics of virus transmission, detected as early as 1–3 days after exposure, was similar to that of human seasonal and pandemic viruses. In contrast, the H3N2v virus isolated in 2009 was transmitted to two of three exposed animals, but only after 5 or 7 days of exposure, suggesting that recent H3N2v viruses might have increased airborne transmission potential between ferrets as compared to older ones. The H3N2v viruses possessed characteristic residues found in human-adapted seasonal H3N2 viruses, such as 190D, 226V/I/L and 228S in HA and as a consequence displayed binding properties similar to those of human-adapted seasonal influenza viruses. The binding preference of H3N2v influenza viruses for α2,6-SA receptors is likely to be one of the determinants of airborne transmission. However, the identification of potential additional determinants in other viral genes is necessary to fully understand the relatively efficient transmission of H3N2v viruses between ferrets. Common to all classical swine viruses, the H3N2v do not possess the polymerase activity enhancing E627K and D701N substitutions (Steel *et al.* 2009), but rather the SR polymorphism at positions 590/591 (Liu *et al.* 2012), similar to the PB2 gene of pH1N1 viruses (Garten *et al.* 2009). The 2011 H3N2v virus possessed the M segment of the pH1N1 virus. Although it has been shown that the M segment of the pH1N1 virus was important for airborne transmission between guinea pigs (Chou *et al.* 2011), the transmission of the 2011 H3N2v virus was similar to the transmission of 2010 H3N2v viruses. Thus, it remains unknown whether the acquisition of the M gene from pH1N1 virus enhanced the airborne transmissibility of H3N2v viruses and therefore their ability to spread and infect humans.

From September to December 2011, an outbreak of H3N8 influenza viruses caused the death of over 160 harbor seals on the cost of New England, United States. The eight gene segments of the H3N8 virus responsible for the seal outbreak were all closely related to avian H3N8 influenza viruses isolated from waterfowl, suggesting a direct transmission from wild birds to seals. However, differences between the most closely related avian H3N8 virus and seal H3N8 viruses included known mammalian adaptation mutations (PB2 D701N) and mutations shared with human, canine and equine H3N8 viruses, indicative of adaptation to mammalian hosts (Anthony *et al.* 2012). An analysis comparing the biological properties of one seal H3N8 virus isolate and avian H3N8 isolates revealed that the seal isolate had increased binding affinity for α2,6-SA as compared to the avian isolates (Karlsson *et al.* 2014). However, the seal virus did not possess classical receptor switching substitutions, such as Q222L or G228S. It still remains unclear whether substitutions in the head region of HA, which differentiate the seal virus from avian viruses (I58M, S141N, V159L and V238I), might be responsible for the increased α2,6-SA receptor-binding preference of seal H3N8 viruses. Interestingly, the seal H3N8 virus isolate was transmitted via respiratory droplets or aerosols to two out of three exposed ferrets, 5–7 days after exposure. Consensus sequence analysis revealed the presence of an additional substitution in HA, A134T, in viruses recovered from a positive airborne contact ferret. This A134T substitution was previously shown to alter the preference of H5N1 viruses from α2,3-SA to α2,6-SA receptors (Imai *et al.* 2010). Further studies are necessary to determine the molecular basis of the airborne transmission of the seal H3N8 viruses, and to understand whether knowledge accumulated on the transmission of avian influenza viruses between mammals can be extrapolated to influenza viruses arising from marine mammals.

GAIN OF FUNCTION EXPERIMENTS CONTRIBUTING TO UNDERSTANDING OF INFLUENZA VIRUS TRANSMISSION

In the five years since the first publication on so-called gain-of-function studies on transmission of avian influenza viruses between mammals (Sorrell *et al.* 2009), the influenza research community has generated a wealth of knowledge on influenza virus transmission, as summarized above. This knowledge has accumulated as the result of many multidisciplinary investigations, including virus surveillance studies in humans and animals, observational studies, bioinformatics, phylogeny, modeling, virus characterization *in vitro* using isolated virus genes or attenuated and wild-type and engineered viruses, and animal experiments with natural virus isolates and viruses modified using reverse genetics. Over the last three years, there has been a fierce debate about the use of GOF-TXM experiments on influenza virus transmission, in which viruses are modified using reverse genetics or by repeated passage *in vitro* and in animals, to increase our basic understanding of the airborne transmission of (animal) influenza viruses. Although some have contested the value

of such GOF-TXM studies in the light of potential risks (Wain-Hobson 2013; Lipsitch and Galvani 2014), the summary of data above makes clear that some of the most convincing evidence to identify determinants of airborne transmission of influenza viruses has come from GOF-TXM studies, and additional public health benefits of such work for surveillance, vaccine strain selection and pandemic preparedness have been discussed recently (Davis *et al.* 2014; Schultz-Cherry *et al.* 2014). In particular for the viruses that have not (yet) acquired the ability of airborne transmission between mammals in nature (e.g. H9N2, H5N1, H7N1), GOF-TXM studies are the only tool to assess whether particular mutations or changes in biological traits could increase their transmissibility in the future. In addition to the already remarkable short-term gains in knowledge and public health benefits, there may also be a longer term impact of the research that is unanticipated and may accrue over time. As signatories of Scientists for Science (http://www.scientistsforscience.org/, date last accessed 11 January 2015), we are confident that this biomedical research on potentially dangerous pathogens can be performed safely and is essential for a comprehensive understanding of influenza virus pathogenesis, transmission, prevention and treatment. We propose that it is sensible and acceptable to continue this important research program, provided that any laboratory participating in this type of research adopts comparable biosafety and biosecurity conditions to those that are currently in place (Imai *et al.* 2012; Zhang *et al.* 2013; Linster *et al.* 2014; Sutton *et al.* 2014), as under such conditions the research can be performed with negligible risks to humans and the environment (Fouchier 2015).

CONCLUDING REMARKS

It is without any doubt that influenza viruses of subtypes H1N1, H2N2 and H3N2 can be transmitted via respiratory droplets or aerosols between humans and other mammals, as it has been shown during previous pandemics and outbreaks in animals, and in the laboratory using animal models. Changes in receptor specificity as determined by HA (and perhaps NA) and polymerase activity have been shown to be crucial for the airborne transmission of viruses of these subtypes. GOF-TXM studies using avian viruses of subtypes H1, H5, H7 and H9 have shown that similar changes in phenotype, caused by the same or functionally equivalent mutations, can result in the acquisition of airborne transmissibility in animal model systems. More importantly, these studies have shown that previously unrecognized or unappreciated traits, such as HA stability, HA/NA balance and previously unidentified substitutions in polymerase, can contribute to airborne transmission. These studies have also shown that the genetic compatibility between some of these virus subtypes and mammal-adapted viruses of subtypes H1N1 and H3N2 allows some animal viruses to acquire airborne transmissibility upon reassortment. Collectively, these studies helped to pinpoint critical determinants of airborne transmission of avian viruses, that can aid in molecular surveillance studies, to aid influenza surveillance and preparedness (Centers for Disease Control and Prevention 2015; Davis *et al.* 2014; Schultz-Cherry *et al.* 2014). These studies have also helped to identify further research needs to strengthen surveillance and risk assessment (Russell *et al.* 2012).

It is important to note that influenza virus evolution is still a rather unpredictable process, complicated e.g. by the wide variety of influenza virus hosts, the genetic heterogeneity of virus strains that are circulating and potential epistatic interactions (Wain-Hobson 2013). However, rather than resting with the thought that virus evolution is unpredictable, we should build an even stronger virus research agenda. Addressing what the impact is of epistatic interactions will be crucial to improve predictability of influenza virus transmission. Further investigation of virus subtypes beyond H1-H3, H5-H7 and H9 would be needed to this end, as well as taking a closer look at the apparently unique properties of H9N2 virus genes to contribute to reassortment, zoonoses and virus transmission. Given the threat posed by H7N9 viruses, GOF-TXM research to investigate the effect of amino acid substitutions that affect receptor specificity and stability of HA should be conducted. Given the problem of statistical power for some virus transmission studies, animal models should be improved or alternative (e.g. *in vitro* or mechanical) models should be developed to study virus transmission more quantitatively. In addition to these—relatively short-term—goals to better characterize viral factors, more attention should be paid to host and environmental factors, preferably linking laboratory investigations with analytical and observational studies on humans that are infected with various influenza viruses. More information about (airborne) transmission of other human respiratory viruses would also be welcomed, because—ultimately—we would like to increase the predictability of which influenza viruses, coronaviruses and paramyxoviruses, out of the hundreds that are circulating in animal reservoirs are most likely to cause the next pandemic(s), to prevent such pandemics from happening altogether, or to mitigate their impact if they cannot be prevented.

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