

SCIENTIFIC OPINION

Scientific Opinion on monitoring for the emergence of possible new pandemic strains of influenza in animals¹

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ABSTRACT

Following the emergence in 2009 of the new pandemic H1N1 influenza virus, which contained gene segments from pig, bird and human influenza viruses, it was apparent that a better scientific understanding is required of influenza viruses to protect public and animal health. The latest scientific data on biological properties of the virus, transmissibility, host susceptibility and epidemiology has been evaluated in order to identify factors that could be monitored in animals and that would suggest a risk of emergence of a new pandemic influenza strains. Virological studies and animal models have highlighted the importance of individual virus proteins but virulence and transmissibility are polygenic effects and no single genetic marker can be reliably associated with increased pathogenicity or transmissibility. It was concluded that current monitoring of the influenza gene pool in humans has been able to provide an alert for the emergence of new human influenza strains of public health significance. In contrast, there is an incomplete view of the influenza virus strains circulating among pigs and birds at the global level. Interpretation of the origins and pandemic potential of influenza viruses do require knowledge of the influenza gene pools in both pigs and birds, as well as other animal species. It is recommended that there should be long term support for a passive monitoring network in pigs and birds in order to promote greater understanding of the evolution of influenza viruses at the global level. Maximum benefit can only be obtained by applying an integrated approach involving the medical and veterinary networks including development of harmonised tools and approaches, exchange of virus strains and sequence data and enhancing the coordination and dissemination of the findings from the human, swine and avian networks.

KEY WORDS

Influenza, pandemic (H1N1) 2009, emerging strains, animals, monitoring

¹ On request from the European Commission, Question No EFSA-Q-2009-00983, adopted on 24 February 2011.

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³ Acknowledgement: The Panel wishes to thank the members of the Working Group on monitoring for the emergence of possible new pandemic strains of influenza virus for the preparation of this opinion: Anette Bøtner, Ilaria Capua, Derek Gatherer, Jackie Katz, Philippe Lemey, Vicente Lopez, Isabella Monne, Elisabeth Mumford, Angus Nicoll, Mo Salman, Mike Sharp, Jan A. Stegeman and EFSA's staff members Per Have and Sandra Correia for the support provided to this EFSA scientific output.

SUMMARY

Following the emergence in 2009 of the new pandemic H1N1 influenza virus, which contained gene segments from pig, bird and human influenza viruses, it was apparent that a better scientific understanding is required of influenza viruses and, in particular, of the underlying factors that most strongly contribute to the emergence of influenza viruses with the final aim being to protect public and animal health.

Consequently, the European Commission requested EFSA's Scientific Panel for Animal Health and Welfare to deliver a Scientific Opinion on monitoring for the emergence in animals of possible new pandemic strains of influenza. Specifically, the Panel was asked i) to indicate the most important factors to be monitored in animals that would suggest a risk of emergence of a new pandemic influenza strain, ii) to assess possible options of monitoring for the presence of the most important factors that would suggest a risk of emergence of influenza viruses (potentially leading to a pandemic) in different animal populations, that could act as reservoirs, mixing vessels or otherwise contribute to the risk posed to humans and animals by influenza viruses and iii) to assess the possible predictability of the emergence of a new pandemic influenza strain by monitoring the molecular evolution and development of influenza viruses in different animal populations. Following discussion with the Commission, it was decided that this Scientific Opinion would focus on influenza in birds and pigs; it would not cover the potential role of influenza in other mammalian species (e.g. horse, dog, cat, marine mammals) that may contribute to the overall process of influenza strain evolution.

The Working Group assessed the latest scientific data and progress to present an overview of current understanding of molecular markers in influenza viruses that have been linked to biological properties of the virus, transmissibility of the virus, particularly between species, host susceptibility, epidemiology and monitoring in pigs, birds and humans.

On the basis of the Working Group's report, the Panel made several conclusions in relation to each of the terms of reference in the mandate. The principal conclusions relating to the most important factors to be monitored in animals that would suggest a risk of emergence of a new pandemic influenza strain were that only a subset of the 16 haemagglutinin and 9 neuraminidase subtypes of influenza A virus have been observed in humans, of which only H1, H2, H3, N1 and N2 have been shown to be involved in pandemic and seasonal influenza. Virological studies and pathogenesis studies in animal models have highlighted the importance of individual virus proteins, although these properties are inconsistent between strains. Further, virulence and transmissibility are not determined by the properties of a single gene or protein; they are polygenic and the specific gene constellation is also important. Consequently, no single genetic marker or genetic constellation can be reliably associated with increased pathogenicity or transmissibility of influenza virus strains in mammals and cannot therefore be used to monitor for the emergence of a new pandemic strain of influenza virus.

The Panel made several conclusions when considering options of monitoring for the presence of the most important factors that would suggest a risk of emergence of influenza viruses. It was concluded that current monitoring of the influenza gene pool in humans through the GISN has been able to provide an alert for the emergence of new human influenza strains of public health significance. In contrast, it is very likely that there is an incomplete view of the influenza virus strains circulating among pigs and birds at the global level. There is more information available for Europe and consistent information about influenza virus strains circulating in pigs in Europe has been obtained through the ESNIP projects, which therefore will extend the body of knowledge and information to promote a greater understanding of the epidemiology of SIVs and will establish a baseline to support alerts for the emergence of new reassortants. However, even in Europe, routine monitoring of poultry and wild birds for avian influenza virus subtypes is restricted to the H5 and H7 subtypes; monitoring for other subtypes is not mandatory and, consequently, knowledge on viruses that potentially could transmit to humans, such as H1, H2, and H3, is rather scarce. This gap limits understanding of their

epidemiology, molecular diversity and potential contribution to strains of public health concern. Consequently, even though it appears that there is insufficient coordination between medical and veterinary diagnostic systems in Europe to support the routine detection of swine or avian influenza viruses infecting humans, it was concluded that new strains of influenza virus of public health significance are more likely to be detected by monitoring human samples. However, interpretation of the origins and pandemic potential of such viruses would require knowledge of the influenza gene pools in both pigs and birds, as well as other animal species, when relevant.

The Panel concluded that, at present, it was not possible to predict the emergence of new pandemic influenza virus strains by monitoring the molecular evolution of the virus. The potential of phylogenetics has been demonstrated by revealing patterns of seasonality and tracking of the current 2009 H1N1 pandemic. However, further application of phylogenetics to monitor emergence and evolution of influenza viruses is constrained by the uneven representation in databases of influenza virus sequences from different species and geographical areas

The main recommendations were that there should be long term support for a passive monitoring network in pigs and birds based on current diagnostic practices in both pigs and birds, such as the ESNIP network, in order to promote greater understanding of the evolution of SIVs and AIVs at the global level and establish a baseline to support alerts for the emergence of new reassortants. Monitoring in birds should be extended to include AIVs that express the HA and NA subtypes that have been observed in man, particularly H1, H2 and H3. A risk-based approach focusing on domestic poultry, and animals in contact with them, should be used.

Maximum benefit would be obtained by applying an integrated approach involving the medical and veterinary networks. This should include the development and implementation of harmonised tools and approaches, exchange of virus strains and sequence data, and enhancing the coordination and dissemination of the findings from the human, swine and avian networks, as well as from other species, when relevant.

Monitoring should include typing of the H and N genes as a minimum, although full sequencing of the virus is the preferred option, as this will provide important additional information, including reassortant events. Depositing data in publicly accessible platforms for data analysis and interpretation in conjunction with epidemiological information would support and accelerate the general advancement of knowledge on the zoonotic and pandemic risk linked to influenza viruses.

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BACKGROUND AS PROVIDED BY THE COMMISSION⁴

The present influenza pandemic (H1N1) 2009 influenza virus is a new virus subtype of influenza A (H1N1) viruses that spreads from human to human and is causing a human influenza pandemic in accordance with the declaration made by the WHO on June 11 2009.

The pandemic virus contains gene segments from pig, bird and human influenza viruses in a combination that has never been observed before. Apart from humans, the virus has also infected pigs in Canada, Argentina, Australia, Singapore (pigs originating from Indonesia), Norway, UK (Northern Ireland), the Republic of Ireland and Iceland, and turkeys in Chile. The epidemiological situation appears to be in evolution.

In contrast, the classical swine influenza viruses circulate widely in many pig populations around the world, including the EU. In relation to these viruses, a reasonably comprehensive monitoring programme has been ongoing under EU research programmes in the context of research on influenza viruses. Valuable experience in this regard has been made available through networks of expertise such as OFFLU and research initiatives under a specific call for avian and human influenza: Framework 6 (FP6) projects, FP7 projects, and preparedness and capacity building for emerging epidemics, swine focused projects such as ESNIP 2 and influenza network enhancing projects such as FLU-LAB-NET. The EU has been particularly active in research on human influenza. Some examples are FLUPAN, which developed the first candidate H7N1 vaccine, and NOVAFLU, which developed the computer algorithm now incorporated by WHO in the vaccine strain selection process.

There is no evidence suggesting that the novel virus behaves in pigs in a different way from the other classical influenza viruses of pigs that only cause a mild respiratory disease.

As regards poultry, the pandemic influenza virus was identified in August 2009 in two turkey breeder holdings in Chile. The clinical symptoms had started in mid July with a sudden drop in egg laying and altered egg shells. No increased mortality was observed. Normal egg production was again reached after 20 days of the infection. The symptoms were very much like an infection with a low pathogenicity avian influenza (LPAI) virus. By the time of the virus detection in turkeys there had been extensive human to human transmission of the pandemic influenza virus in Chile, which makes occasional transmission from man to bird the most likely scenario. Some birds had been in contact with persons with respiratory disease.

Genetic sequencing of the haemagglutinin (HA) gene from the pandemic influenza virus isolated from the turkeys showed 99.5 % similarity to the Californian human strain and a 100 % match to the human strain circulating in Chile at that time. Mutations that might explain an increased capability of the virus to infect turkeys have not been detected, but work to further characterise the virus is needed. No turkey-to-human transmission has been reported so far.

However, the finding of the pandemic influenza virus in turkey holdings in Chile is unexpected as attempts to date in the USA and Europe to infect turkeys experimentally with the pandemic influenza virus have been unsuccessful. The significance of the pandemic (H1N1) 2009 influenza virus for different animal species remains unclear.

Surveillance for avian influenza (AI) is currently carried out in Member States (MS) in poultry and wild birds. The objectives for AI surveillance are currently laid down in the official guidelines adopted in 2007 by Commission Decision 2007/268/EC. Surveillance in poultry aims in particular at detecting sub-clinical infections with the LPAI of these subtypes, thereby complementing other early detection systems, in order to determine the genetic characteristics of influenza viruses and subsequently preventing possible mutation of these viruses to high pathogenicity avian influenza (HPAI). It should be noted that surveillance for the HPAI H5N1 subtype virus in wild bird populations, by testing live birds and those found dead, has become more important in order to protect domestic poultry from becoming infected.

⁴ As provided on November 13, 2009

In the longer term, there is a need for comprehensive monitoring of influenza virus genotypes in order to follow the current situation, and the emergence and evolution of possible virus reassortants (virus monitoring) in pigs and other animal species, with the final aim being to protect public and animal health.

In order to limit the emergence and spread of influenza viruses with pandemic potential in an effective and proportionate way, the risk manager will require a better scientific understanding of influenza viruses and, in particular, of the underlying factors that most strongly contribute to the emergence of influenza viruses. It is also necessary to develop better methods and criteria to assess the risk such viruses may pose to people and animals.

During recent years, it has emerged that cooperation between public health and animal health experts from different fields, such as virology and epidemiology, is necessary in order to address this complex issue. Furthermore, full and immediate sharing of research results and data between the scientific community and health authorities is essential in order to reap the full public health benefits of these research efforts. Scientific advice and risk assessment also support gathering and exchange of relevant information.

In general, the potential control measures to be taken in case of pandemic (H1N1) 2009 influenza outbreaks or infection(s) on farms should be proportionate to: i) the risk posed by animals, in particular pigs and different poultry species, in the transmission of the pandemic virus to humans, if any, compared to the role played by human-to-human transmission, and ii) the severity of disease in animals and humans.

From an animal disease control point of view it is considered that certain movement restrictions should be implemented for animals showing signs of influenza, such as clinical respiratory illness. The main measure should be movement controls of live animals to other farms. The farm movement controls (quarantine) should be in place until a certain number of days (i.e. seven) after the last clinical signs of disease have been observed in the epidemiological unit, and influenza is no longer considered a veterinary risk.

Pre-existing immunity induced due to a previous influenza infection or following conventional influenza vaccination may not protect animals, especially pigs, against infection with pandemic influenza virus, although it may provide partial protection. Partial protection has been observed in some experimental studies with piglets having maternal antibodies but this is not supported with sufficient challenge studies to provide confidence in these findings. Vaccines currently used in the EU or elsewhere to protect pigs against influenza may not be effective against the pandemic influenza virus. Therefore, it is unclear whether vaccination is an appropriate tool to control pandemic (H1N1) 2009 influenza virus in different animal species.

As regards food safety, the statements made by the OIE/WHO/FAO/WTO/ECDC/EFSA adequately address the issue of safety of meat such as pork and pork products for human consumption in relation to influenza.

However, the Commission is in need of further scientific advice and risk assessment, as regards the pandemic (H1N1) 2009 influenza virus in animals.

TERMS OF REFERENCE⁵ AS PROVIDED BY THE COMMISSION

- To indicate the most important factors to be monitored in animals that would suggest a risk of emergence of a new pandemic influenza strain;

⁵ The terms of reference (ToRs) have been divided into two separate mandates by EFSA. Three ToRs are being addressed in this opinion, whereas the remaining 6 ToRs were included in the opinion on “current pandemic (H1N1) 2009 influenza and its potential implications for animal health” (EFSA-Q-2009-00935).

- To assess possible options of monitoring for the presence of the most important factors that would suggest a risk of emergence of influenza viruses (potentially leading to a pandemic) in different animal populations, that could act as reservoirs, mixing vessels or otherwise contribute to the risk posed to humans and animals by influenza viruses;
- To assess the possible predictability of the emergence of a new pandemic influenza strain by monitoring the molecular evolution and development of influenza viruses in different animal populations.

ASSESSMENT

1. Introduction

Influenza has been known for centuries to cause serious widespread disease in humans and historical evidence points to many cases of zoonotic outbreaks of influenza-like illness in horses, poultry or pigs with concomitant disease outbreaks in humans (Morens and Taubenberger, 2010). From the pandemic influenza of 1918 onwards, the etiological association of influenza viruses with disease in humans and animals has been convincingly established. However, the complex relationships of influenza virus ecology and evolution between its reservoirs in wild aquatic birds and a wide array of avian and mammalian host species remain a huge challenge in our efforts to prevent and mitigate influenza, and many gaps still persist in our understanding of the dynamic evolution of influenza viruses (Forrest and Webster, 2010).

Following discussion with the Commission, it was decided that this assessment will focus on influenza in birds and pigs but will not cover the potential role of influenza in other mammalian species (e.g. horse, dog, cat, marine mammals) that may contribute to the overall process of influenza strain evolution. Indeed, equine influenza viruses have been shown to cross the species barrier to dogs and perhaps pigs. Furthermore, in the context of this report, despite the successful experimental infection of human volunteers with equine influenza virus (EIV), there is currently little evidence that supports a zoonotic role for EIV (OFFLU Annual Report, 2010a).

2. Influenza A virus

Influenza viruses are segmented, negative, single-stranded RNA viruses that are placed in the family Orthomyxoviridae in three genera: Influenza virus A, B and C. Type A influenza viruses are further divided into subtypes based on the antigenic relationships in the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). A gene pool of influenza A viruses is maintained in aquatic birds, and various subtypes cause natural infections of birds, poultry and a large range of terrestrial and marine mammals, including humans. Influenza B is important in human seasonal influenza but it does not cause pandemics. Influenza C usually causes mild disease. However, influenza B and C viruses are normally not found in animals (Hay et al., 2001; Fouchier et al., 2003), therefore, these viruses will not be considered further in this Opinion.

On the basis of the antigenicity of their haemagglutinin and neuraminidase, 16 haemagglutinin subtypes (H1-H16) and nine neuraminidase subtypes (N1-N9) have so far been recognised. Each virus has one H and one N antigen subtype and almost every combination has been found (Webster et al., 1992). All 16 H subtypes are found in avian populations. However, only a subset has occurred in humans (H1, H2, H3, H5, H7, H9, H10), of which only the first three have transmitted easily in humans. Of the nine neuraminidase subtypes, again only a subset (N1, N2, N3, N7) has been found in humans, of which only the first two have been involved in pandemic and seasonal influenza.

The genome of influenza A virus is made up of eight segments coding for 10-11 proteins: PB2, PB1, PB1-F2, PA (viral RNA-dependent RNA polymerase complex), HA, NP (nucleoprotein), NA, M1 and M2 (matrix- and ion channel proteins), and NS1 and NS2 (non-structural proteins) (Das et al., 2010).

The haemagglutinin mediates binding to the host cell and subsequent fusion of viral and cellular membranes, followed by release of the viral ribonucleoprotein into the cytoplasm. The haemagglutinin together with neuraminidase are the major surface antigens and targets of host immunity. Antigenic drift of influenza viruses is derived from a high mutation rate and amino acid substitution in these surface antigens related to circumvention of the host immune pressure.

Influenza viruses are RNA viruses with their viral RNA polymerases lacking the proof-reading ability of DNA polymerases, so their mutation rate is high. This results in a proportion of each next generation of influenza viruses being mutants, with point mutations occurring in the H or N proteins

on the viral surface (Rambaut et al., 2008). Over time this may lead to “antigenic drift” from the ancestral makeup and the new variant can become dominant if it has an evolutionary advantage. This occurred for example when a new variant of the A(H3N2) virus (named A/Fujian(H3N2)) emerged in 2003-4 that was distinct from the preceding interpandemic strain (Russell et al., 2008).

Another characteristic of the influenza viruses is that the division of their RNA genome into eight segments allows mixing or reassortment of these segments when more than one type of influenza virus infects the same cell. When two influenza viruses, animal or human, infect the same animal or human at the same time (and potentially both enter one cell), these animals or humans can serve as a mixing vessel that may result in “antigenic shift”, with generations of novel influenza viruses having genes of both parent viruses and possibly new biological characteristics.

Hence, because of the deficiency in their RNA proof-reading enzymes and the reassortment mechanism, a seemingly endless variety of new viruses with potentially new properties are continually being engineered in nature, usually with gradual changes, but with an occasional abrupt change (drift and shift, respectively) (Morens et al., 2009). The larger, sudden changes may result in the emergence of a novel dominant human virus: a pandemic strain. An example of this is A(H2N2) replacing A(H1N1) (the 1957 pandemic), when the former overcame the acquired protective “historical” population immunity and out-competed the preceding influenza A viruses. Hence, these two mechanisms explain the perennial waves of epidemic influenza with varying pattern and severity from season to season and the ongoing risk of an occasional pandemic strain emerging (Taubenberger and Morens, 2006).

2.1. Transmissibility

A large array of viral, host and environmental factors influence intra- and inter-species transmissibility of influenza viruses. The HA receptor binding specificity, in conjunction with cellular receptor expression, influence host and tissue tropism, such as replication in the upper respiratory tract. However, other structural and non-structural proteins involved in replication and transcription of the viral genome also affect the host range, level of viral replication, temperature permissiveness and excreted titre (Neumann and Kawaoka, 2006; Yassine et al., 2010). The best example of the effect of a viral factor in biology involves the HA of avian influenza viruses and its influence on pathogenicity and transmission of these viruses.

Influenza A viruses infecting poultry can be divided into two distinct groups, low pathogenicity and high pathogenicity, on the basis of the severity of the disease they cause in 6-week-old chicks (OIE Manual, 2010). The low pathogenicity viruses (LPAIV) generally cause only mild disease in poultry. In contrast, the high pathogenicity viruses (HPAIV) cause a severe disease in which flock mortality in some susceptible species may be as high as 100 %. LPAIV can mutate to HPAIV by the acquisition of multiple basic amino acids (arginine and lysine) at their HA0 cleavage site. This well characterised modification extends the range of tissues that are permissive for the virus so that it replicates throughout the bird, and damages vital organs and tissues, which results in disease and death (Rott, 1992). Virus titres are also increased and enhance the transmission potential of infected birds.

Transmission and establishment of an infection in a population is also influenced by the prevailing specific immunity of the population. This effect can be assessed by estimates of the basic reproductive number (R_0), which has been used extensively to assess the transmission potential of influenza viruses in human populations. R_0 is defined as the average number of new infections caused by one infectious individual in a fully susceptible population. In order to establish itself in a population R_0 needs to exceed 1. For the 2009 pandemic H1N1 virus, estimates from the early observations of the pandemic were in the range of 1.2-2.0 (Fraser et al., 2009; WHO, 2009). In comparison, estimates of R_0 for the 1918 pandemic influenza virus varied from 1.3-3.8 (Chowell and Nishiura, 2008), whereas in poultry R_0 was estimated at 2.3-2.6 for H5N1 in Thailand (Tiensin et al., 2007). As soon as the number of immune individuals increases the effective reproductive number R_e in a population drops and an epidemic fades out as soon as R_e is below 1.

2.1.1. Cellular receptors

Influenza viruses bind to cellular receptors via specific binding between the viral haemagglutinin (HA) and N-acetylneuraminic acid. This receptor binding is also the underlying mechanism of *in vitro* haemagglutination assays seen with influenza viruses. Differential binding is observed depending on whether sialic acid (SA) is linked through the hydroxyl group of carbon-3 (α 2-3) or carbon-6 (α 2-6) of galactose. Avian viruses preferentially bind to receptors having the α 2-3 glycosidic linkage, whereas mammalian viruses preferentially bind to receptors with α 2-6 linkage. Mutations in the receptor-binding site of the virus may change the binding preference, such as the Asp222Gly (D222G) seen in the HA of pandemic H1N1 virus, which increases binding to α 2-3 receptors (Chutinimitkul et al., 2010; Liu et al., 2010). Whether the selection of the D222G mutation is a cause or a consequence of more severe lower respiratory tract infection is still unresolved (Kilander et al., 2010).

HA glycoproteins bind to certain SA isomers of host glycoproteins, supporting the role of HA-SA affinity in the host range. Avian viruses present conserved amino acid signatures in the receptor binding domain (RBD) and those avian HA proteins adapted to humans have mutations in several key residues of the RDB, namely 138, 190, 194, 225, 226 and 228 (H3 numbering) (Matrosovich et al., 2000), which increase binding from α 2-3 to α 2-6 SA. However, enhanced α 2-6 SA binding is not in itself sufficient for host switching and does not solely determine efficient human to human transmission (de Wit and Fouchier, 2008).

In birds, epithelial cells preferentially express α 2-3 linked receptors in both the intestinal and respiratory tract. However, some birds, such as Japanese quail (*Coturnix coturnix japonica*), also exhibit α 2-6 linked receptors (Wan and Perez, 2006).

In humans, receptors with α 2-6 are predominantly expressed in the upper respiratory tract but α 2-3 linked receptors are also expressed in the lower parts of ciliated bronchial epithelium and alveolar cells (Shinya et al., 2006). The relative distribution of α 2-6 and α 2-3 receptors in pigs is similar to that in man (Nelli et al., 2010; Van Poucke et al., 2010). These findings suggest that not only pigs but also humans may be co-infected by both avian and mammalian viruses, thus facilitating reassortment events between mammalian and avian influenza viruses considered to be an important step in the generation of viruses with pandemic potential.

Furthermore, the demonstration that α 2-3 and α 2-6 galactose linkages are also present in avian species, namely quails and chickens (Guo et al., 2007; Wan and Perez, 2006), suggests that certain gallinaceous species may also play a role in the adaptation or spread of the virus from its natural reservoir to either domestic poultry or mammalian species.

Receptor specificity is therefore an important characteristic of influenza virus strains (Nicholls et al., 2008). Furthermore, the ability to screen for a diverse set of sialic acid-containing glycans has shown that the mechanisms involved in receptor binding are complex, and several tools have been developed to screen for receptor specificity in relation to variations in glycan structure and composition (Stevens et al., 2006).

2.1.2. Replication

Following binding to the host cell sialic acid receptor, the virion is internalised in an endocytic vesicle where a low pH triggers an irreversible conformational change in the haemagglutinin molecule with dissociation of the HA1 and HA2 subunits. The N-terminal fusion peptide of HA2 induces fusion of viral and endosomal membranes and release of ribonucleoprotein (RNP) complex into the cytoplasm from where it is transported to the cell nucleus. Replication and transcription of the viral RNAs are carried out by the RNA polymerase complex in the nucleus of the cell and they are subsequently exported to the cell cytoplasm where viral mRNA is translated. Progeny virions assemble and bud at the plasma membrane (Neumann et al., 2009).

Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of the severity of the disease they cause. The very virulent viruses cause HPAI in which flock mortality in

some susceptible species may be as high as 100 %. These viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI.

The HA0 precursor proteins of LPAI viruses have a single arginine at the cleavage site and another at position -3 or -4. These viruses are limited to cleavage only by certain host proteases such as trypsin-like enzymes and are thus restricted to replication at sites in the host where such enzymes are found, such as the respiratory and intestinal tracts. The HA0 proteins of HPAI viruses possess multiple basic amino acids (arginine and lysine) at their HA0 cleavage sites, either as a result of apparent insertion or apparent substitution (Vey et al., 1992; Wood et al., 1993; Senne et al., 1996) and appear to be cleavable by a ubiquitous protease(s), probably one or more proprotein-processing subtilisin-related endoproteases of which furin is the leading candidate (Stieneke-Gröber et al., 1992). Thus, the HPAI viruses are able to replicate throughout the bird, damaging vital organs and tissues which results in disease and death (Rott, 1992).

2.1.3. Interspecies transmission

There are many dynamics that are involved in interspecies transmission of infectious agents, including transmission from animals to man, but in all situations this event requires the crossing of the host species barrier. The host species barrier is not a simple concept and can include not only biological factors but also behavioural factors. These processes have been modelled and reviewed recently (Lloyd-Smith et al., 2009, Appendix A).

Influenza A viruses infect a large variety of species, including humans, wild and domestic birds, pigs, horses, seals, whales, cats, ferrets, canines, minks and others. Generally, each strain shows a certain level of restriction to the host species in which it is circulating (Neumann and Kawaoka, 2006).

Interspecies transmission occurs readily among avian species and, more seldom, from the avian to the mammalian host. However, the wide range of mammals that have been affected by H5N1 indicates that for certain viruses crossing the avian/mammalian species barrier may be easier than originally believed (Capua and Alexander, 2007). Other notable examples include transmission of swine influenza to turkeys (Pillai et al., 2009) and humans (Myers et al., 2007; Shinde et al., 2009), and avian H1N1 (Pensaert et al., 1981) and H9N2 (Cong et al., 2007) to pigs.

Adaptation of inter-species transmitted viruses to a new host species, for example, bird to mammal, usually requires a series of genetic adaptations to become successful, and these adaptations are the result of accumulating genetic changes that represent a complex polygenic viral trait (Forrest and Webster, 2010; Yassine et al., 2010), which may require several years to occur (Smith et al., 2009a).

2.2. Animal models for human disease

At present, determination of the relevance of the various molecular markers to pathogenesis and transmission in influenza virus can only be undertaken in animal models⁶. This approach has been illustrated best for influenza virus strains in avian species, where the intravenous pathogenicity index in 6-week-old chickens is used to discriminate low pathogenicity and high pathogenicity strains of influenza virus (OIE Manual, 2010).

Although a similar relationship has not been established for mammalian influenza viruses, various animals, such as cat, dog, ferret, guinea pig, hamster, mouse, pig, poultry and primates, have been used in studies to investigate aspects of the pathogenesis and transmission of influenza virus strains (Belser et al., 2009). However, as a model for influenza in man, the ferret is regarded as the most appropriate because: i) it is naturally susceptible to all strains of influenza virus without the need for

⁶ Animals that have been inoculated experimentally with strains of influenza virus are likely to develop clinical signs and may even die as a result of the infection. Animals should be killed, as well as those that are showing clinical signs indicating significant pain and distress, as soon as the scientific questions that have been posed have been answered. There is rarely a scientific need for animals to die.

prior adaptation, ii) clinical signs and illness parallel that which is produced in man with the same strains, and iii) the distribution of receptor types in the respiratory tract is similar to that in man (i.e. predominance of α 2-6 receptors in the upper tract and both α 2-3 and α 2-6 receptors in the lower tract). None of the other species fulfil all of these criteria.

i) Transmission

Studies in ferrets have demonstrated the different transmission properties of mammalian and avian influenza viruses. In particular, these studies indicate that although HA and NA play a critical role, other virus genes, including the PB2 gene, contribute to transmissibility in the ferret model (Belser et al., 2010). In this ferret model, mammalian viruses (e.g. H1N1, H3N2) can be transmitted by both direct contact and also indirectly via the airborne route (respiratory droplets), whereas avian viruses (e.g. H5N1, several H7 subtypes) generally are transmitted poorly, particularly by respiratory droplets (Katz et al., 2009). However, there are notable exceptions to this generality, such as an avian H7N2 influenza virus isolated from a human patient transmitted efficiently in ferrets that was shown to have acquired receptor-binding properties similar to human influenza viruses (Belser et al., 2008). Similarly, some avian H9N2 influenza viruses preferentially bind α 2-6 receptors, similar to human influenza viruses, although they appeared to transmit only by direct contact and not by an airborne route (Wan et al., 2008). This result reflects the reported observations of direct transmission to man of H9N2 viruses that recognise α 2-6 receptors (Butt et al., 2005; Lin et al., 2000).

A recent laboratory investigation demonstrated that reassortment between the 1997 HPAI H5N1 virus and human H3N2 virus generated reassortant combinations which can replicate in, but are not transmissible between, ferrets (Maines et al., 2006). This prompts questions about the potential of H5N1 viruses that are acquired from birds to adapt to, and be transmitted between, humans.

ii) Virulence/pathogenesis

Similarly, differences in virulence and pathogenesis of both mammalian and avian influenza viruses have been demonstrated in ferrets, which correlate with experience of the infection and disease in man (Belser et al., 2009). Again, there are notable exceptions to this generality. The HPAI H5N1 viruses usually cause severe disease in ferrets (Belser et al., 2008), but there are isolates, generally derived from avian sources, that result in asymptomatic or milder infections (Lu et al., 2003; Salomon et al., 2006). Interestingly, some isolates have caused lethal infections in ferrets either fed or intragastrically inoculated with virus-infected meat (Lipatov et al., 2009).

Studies on virulence have been extended in order to correlate different gene segments, constellations of genes and putative molecular markers with virulence and aspects of pathogenesis. These studies have reaffirmed and highlighted the importance of individual virus proteins, such as the HA, NA, polymerase and non-structural proteins, as well as their point mutations (see section 2.3 and 2.4). However, these studies have also emphasised that virulence and pathogenesis are not the properties of a single gene or protein. They are polygenic with the specific gene constellation being important (Katz et al., 2000), and the correlation between the molecular markers and the biological property is not absolute.

2.3. Molecular markers

A large number of mutations and molecular markers of the influenza virus genes has been linked to various properties of the virus, such as receptor binding, host and tissue tropism, virulence, modulation of the host immune response, and efficiency of replication and transmission (Arias et al., 2009; Belser et al., 2009). In most cases, however, the phenotypic manifestation of particular characteristics is polygenic and does not rely on single mutations (see section 2.2). The following examples of some of these reported factors illustrate the difficulties in identifying reliable markers for specific properties of influenza virus.

Changes in the RNP complex have long been implicated in the adaptation of avian influenza viruses to humans. The 1918, 1957, and 1968 pandemic viruses possessed a PB1 gene originating from an avian influenza virus (AIV). The repeated occurrence of this reassortment event suggests that an avian

PB1 segment can function in a mammalian adapted influenza virus, and avian PB1 may have a role in the emergence of a pandemic influenza virus (Chen et al., 2008).

The PB1 gene of most avian and human influenza A viruses encodes a second protein, PB1-F2, expressed by an alternative reading frame (Neumann et al., 2009). The protein is truncated in many classical swine influenza viruses, including the pandemic H1N1 2009 virus. PB1-F2 induces apoptosis, enhances pro-inflammatory responses and increases the frequency of secondary bacterial infections.

The role of PB2 in pathogenicity has also been linked to particular amino acid substitutions at positions 627 and 701 (E627K and D701N). The E627K mutation was reported to be a determinant of pathogenicity in human viruses and avian H5N1 viruses (Neumann et al., 2009). However, they were absent in early pandemic H1N1 viruses and introduction of these mutations by reverse genetics did not significantly alter replication or pathogenicity in mice and ferrets (Herfst et al., 2010).

The NS1 non-structural protein has the capacity to bind double-stranded RNA and thereby modulate the host interferon response to infection. Several mutations have been linked to the ability to bind ds-RNA and to modulate pathogenicity.

2.4. Reassortment

Since the genome of influenza A consists of eight separate RNA molecules (the genome segments), mixed infection by two viral strains within an individual can lead to reassortment. The rules that govern reassortment dynamics are largely unknown. For example, the currently circulating H5N1 viruses mainly reassort within the H5N1 subtype (different sublineages), while H9N2 viruses reassort with several other subtypes (Capua, 2010). The emergence and characteristics of progeny viruses are unpredictable and may therefore contain previously unknown genetic profiles.

The 2009 H1N1 pandemic provides an excellent case study in reassortment and its consequences. Of the eight segments in the pandemic H1N1 virus, six were derived from North American triple reassortant swine influenza viruses and the remaining two (M and N1) from Eurasian H1N1 swine influenza viruses. The 2009 H1N1 pandemic virus may therefore be seen as a result of a mixed infection event, most likely occurring in the latter part of the decade. Triple reassortant swine influenza viruses are themselves a product of earlier reassortment events, consisting of segments from “classic” swine influenza (NP, M, NS, H1, N1), human H3N2 seasonal influenza (PB1) and avian H1N1 (PB2, PA). The probable order of the reassortments resulting in triple reassortant viruses was: 1) a reassortment between human H3N2 and classic swine H1N1 to produce a swine H3N2; 2) a reassortment between this swine H3N2 and an avian H1N1 (delivering avian PA and PB2 segments); and 3) a reassortment between that product and classic swine H1N1. Since both human H3N2 and Eurasian H1N1 swine influenza had several segments of avian origin, it is possible to trace complex pathways for single gene segments through several reassortment events. For instance, the PB1 segment in the 2009 pandemic H1N1 has travelled from an avian source in 1968 into pandemic human H3N2, then onto porcine triple reassortant viruses via porcine H3N2 and finally back into humans as part of the pandemic H1N1 in 2009 (Smith et al., 2009b). Other reassortants between classic and Eurasian H1N1 swine influenzas, the “Thai 6+2” and “Thai 7+1” strain groups, have subsequently been discovered by computational mining of databases (Kingsford et al., 2009).

A “worst-case scenario” in pandemic influenza was often assumed to be a mixed infection of a human seasonal strain with a highly pathogenic avian subtype for which the human population has little pre-existing immunity, for example H5N1, producing reassortant progeny with the virulence of H5N1 and the ease of transmission of, for instance, H3N2. However, the 2009 pandemic H1N1 virus has demonstrated that new pandemics of influenza in man can come from other sources, in this case the reassortment of two porcine influenza strains. Thus, the most effective monitoring for new reassortments cannot be limited to humans at risk of mixed infection, such as poultry workers and people with backyard poultry, but should include other relevant susceptible species.

2.5. Phylogenetics

Molecular methods have become invaluable tools to trace the transmission patterns of infectious diseases. The historical information contained in viral gene sequences is contributing to a better insight into emergence and early transmission dynamics, even before systematic epidemiological surveillance has been initiated. In addition, molecular investigations can also reveal the genetic determinants of viral emergence, including adaptation to new hosts or increased transmissibility.

Thus, phylogenetics has an application at three levels of resolution: 1) assessment of the origins of current pandemic or seasonal viruses in humans, as has been recently performed for the 2009 H1N1 pandemic virus (Dawood et al., 2009; Fraser et al., 2009) and retrospectively for previous pandemics back to 1918 (Smith et al., 2009a); 2) assessment of the origins of influenza strains in intermediate hosts; and 3) deep phylogeny of influenza subtypes in their reservoir hosts (Gatherer, 2009b, 2010).

For many years, phylogenetic analysis has been the main tool in influenza molecular epidemiology. By reconstructing a phylogenetic tree, epidemiologists are able to study the evolutionary history of the viral strains and assess the epidemiological linkage between them. Recent developments in this field have aimed at incorporating additional information associated with the sampled sequences into phylogenetic models to infer, for example, temporal, demographic and geographic processes. Assuming that the rate of accumulation of substitutions has remained roughly constant throughout the evolutionary history, phylogenies can be rescaled from mutations into real time scales. The rate at which this molecular clock is ticking for rapidly evolving RNA viruses, such as influenza, can be calibrated by incorporating the time of sampling for the sequences (Drummond et al., 2003). Using coalescent-based methods, population genetic processes can subsequently be inferred from such time-scaled genealogies (Rodrigo and Felsenstein, 1999). Demographic inference, in particular, has become a popular tool in reconstructing the epidemic histories of viruses (Raghava et al., 2003).

These modern phylogenetic techniques allow for nuanced and flexible analysis of demographic and mutational patterns in the evolution of influenza. The power of fitting a statistical model of the evolutionary process to observed sequence data has clearly been demonstrated by a number of seminal studies, for example, by revealing patterns of influenza A H3N2 seasonality and characterizing the genomic dynamics of reassortment (Rambaut et al., 2008), or by providing genetic estimates for the pandemic potential of the 2009 H1N1 pandemic influenza virus (Fraser et al., 2009). The added value of time-measured phylogenies has also recently been demonstrated in order to disentangle the reassortment history of the H1N1 pandemic virus and for the elucidation of a relatively long unsampled history prior to its emergence (Smith et al., 2009b).

The most recent addition to the phylogenetic framework comes from advances in modelling the spatiotemporal diffusion dynamics. Novel models that consider geographic spread in discrete or continuous space can efficiently track an epidemic based on viral genetic data as it unfolds in time and space (Lemey et al., 2009a, 2010), such as the 2009 H1N1 pandemic (Lemey et al., 2009b). Such approaches may, for example, assist epidemiologists in assessing whether geographic range determines the likelihood for cross-species transmission.

Importantly, the model-based framework also opens up new opportunities for hypothesis testing by offering the possibility to incorporate more detailed geographic information, for example, air-traffic data, and to compare competing geographic models using model selection techniques. To fully appreciate the potential of these techniques, it is important to note that these probabilistic models are in essence general models of trait evolution. Host-species could, for example, be treated as a discrete state to estimate cross-species transmission throughout the evolutionary history and to test different potential predictors of such events. In addition, models for continuous traits may prove useful, for example, to investigate the relationship between genetic and antigenic evolution or to characterise the dynamics of properties that may be measured in animal models or specialised assays (e.g. glycan array technology). The integration of molecular data and such phenotypic data may, therefore, provide new opportunities for early warning of pandemic potential.

To harness the power of these state-of-the-art methods, in particular with respect to monitoring for emergence of new influenza strains, representative molecular data is required. Unfortunately, current databases are rather biased. For example, among influenza viruses circulating in aquatic wildfowl and shorebirds, H5 and H9 each have more than 1,000 non-identical sequences deposited in the NCBI Influenza Virus Resource database. At the other extreme, H8 and H12 to H16 inclusive have fewer than 100 each. Deep phylogenetic analysis of influenza A would therefore benefit from more extensive sampling and sequencing of under-represented subtypes.

Archaeological or legacy samples are also of importance in addressing the issue of subtype extinction. Phylogenetic studies of avian influenza haemagglutinin and neuraminidase evolution (Gatherer, 2009a, 2010; Suzuki and Nei, 2002) produce trees which demonstrate the diversification of subtypes from their most recent common ancestor (MRCA). However, such tree building does not address the question of whether influenza was in fact less diverse in previous centuries, or whether there was always a proliferation of subtypes. In the first scenario, the date of the MRCA represents a genuine founder, in other words, a primordial AIV that either entered the avian population from a different host or which evolved from a different avian virus. In such a case, influenza A may be regarded as an emerging disease of the last millennium or so, which has relatively recently (i.e. in the past two centuries) also begun to spread to humans and other mammalian hosts.

In the second scenario, the MRCA would merely be one subtype among many in its time, and its apical position in the tree is simply an artefact of lineage extinction. The fact that the MRCAs of HA and NA are roughly equivalent in age (Gatherer, 2010) perhaps provides some weak support for the first option, but further sequencing would be needed to remove this question from the realms of speculation.

3. Epidemiology

It is intended in this section to outline some important epidemiological features that may influence overall monitoring and surveillance systems in man, pigs and birds. In particular, which data would be considered essential when combined with molecular/genetic information for phylogenetic analyses in order to improve our understanding of influenza virus biology and early alerts for the emergence of new strains.

3.1. Avian influenza viruses as precursors of pandemic strains

Wild birds, primarily wild ducks, gulls and shorebirds, are the natural hosts and reservoirs for all type A influenza viruses (Kawaoka et al., 1988; Stallknecht and Brown, 2007) and they provide a source of viral HA and NA subtypes antigenically novel to humans.

Direct transmission of avian viruses to humans is an infrequent event, and may result in a mild condition (viruses including H9N2, H7N7, H7N2, H7N3) or a severe condition (HPAI H5N1). All events described in recent times have resulted in only limited or no human to human transmission (Capua and Alexander, 2007).

Analysis of the historical and scientific data of previous pandemics may lead to a better understanding of the role of avian influenza viruses in the emergence of pandemic strains. Genetic analysis showed that the H2N2 1957 Asian pandemic virus acquired three gene segments from an avian virus (PB1, HA and NA) and kept five remaining gene segments from the H1N1 human strain circulating prior to 1957 (see Figure 1). Similarly, the 1968 H3N2 Hong Kong pandemic virus possessed two gene segments from an avian virus (PB1 and HA) and maintained six other genes from the H2N2 human strain that circulated in 1957-1968 (Webster et al., 1992). Molecular epidemiological studies also suggested that the avian derived genes of H2N2 and H3N2 pandemic viruses may have been generated through a series of multiple reassortment events and emerged over a period of years before the pandemic event (Smith et al., 2009a).

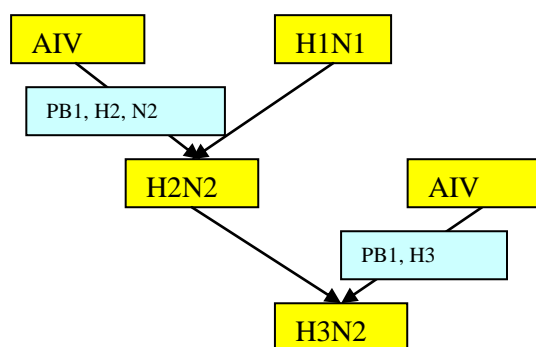


Figure 1. Reassortment events involved in generation of the pandemics of 1957 and 1968

Unlike the 1957 and 1968 pandemic viruses, genetic analysis of the 1918 pandemic virus suggests that all of its eight gene segments originated from the avian reservoir by a mechanism of adaptation of a pre-existing avian genome to humans (Taubenberger et al., 2005). However, it is not clear how long it took for an avian originated influenza virus to become adapted to mammals. Recent evolutionary studies have shown that the components of the 1918 pandemic strain were circulating in mammalian hosts at least 2 to 15 years before pandemic occurrence, thus suggesting a possible complex process of human adaptation and generation of precursor viruses preceding the actual pandemic (Smith et al., 2009a).

Although an antigenically novel HA subtype is a likely requirement for pandemic emergence, avian influenza viruses have usually not been transmitted efficiently from human to human. After at least a decade of continued circulation, HPAI H5N1 and LPAI H9N2 viruses continue to produce spill-over infections in humans but person to person transmission has been rare (Tarantola et al., 2010; Ungchusak et al., 2005; Yen et al., 2009).

Avian H1, H2 and H3 viruses have been of scarce interest to the veterinary community as they only cause mild disease and are not notifiable infections. The limited knowledge available on the epidemiology and molecular properties of these avian influenza subtypes, combined with their apparently scarce antigenic reactivity with human antibodies, increase the likelihood that these subtypes may find a way to donate the haemagglutinin gene again and contribute to the generation of the next pandemic virus. In addition to these considerations, the emergence of an H5N1 or H9N2 pandemic virus is a continuing concern. These two subtypes in poultry have spread across a large area of the world. LPAI H9N2 virus with dual or human-like receptor specificity has become endemic in poultry in many of the affected Asian countries and has resulted in sporadic human infections. H5N1 HPAI virus has now spread in poultry and wild birds throughout Asia and into Europe and Africa, thus posing an ongoing zoonotic threat.

Several reasons render the emergence of H5N1 a singular event in avian influenza history (Alexander and Brown, 2009) and the high pathogenicity to humans raises concerns about its pandemic potential. Both H5N1 and H9N2 are still evolving rapidly and their evolution into antigenically distinct clades, possibly driven in part by the use of poultry vaccines, makes it difficult to predict where H5N1 and H9N2 evolution is going and what to expect next.

Globalisation of trade, the increase in human consumption of poultry meat and the size of poultry farms may impact on the evolution of AI epidemiology. However, biosecurity within large scale commercial farms is generally adequate, at least in the EU, and AI outbreaks are infrequent. Nevertheless, the continued occurrence of spill-over events of avian influenza between wild birds and poultry indicate that gaps still exist (Kilpatrick et al., 2006; Tarantola et al., 2010; Terregino et al., 2007). A more important scenario involves live bird markets and rural farms, which have been demonstrated to play an integral role in the dynamics of influenza virus transmission and evolution.

They often bring together different species of animals, thereby facilitating reassortment of virus strains and interaction between susceptible hosts. Human infection with avian influenza viruses mainly results from intimate or prolonged contact with domestic birds (Vannier, 2007).

3.2. Swine

Influenza viruses of H1N1, H3N2 and H1N2 subtypes are endemic in pig populations worldwide, but there has been a clear genetic distinction between North American and Eurasian lineages of swine influenza viruses (SIVs) (Olsen et al., 2006). The predominant H1N1 viruses in Europe have an entirely avian genome and were introduced from wild ducks to pigs in 1979 (Pensaert et al., 1981). These “avian-like” H1N1 viruses have established a stable lineage and are currently co-circulating with H3N2 and H1N2 SIVs. The European swine H3N2 viruses contain haemagglutinin (HA) and neuraminidase (NA) genes similar to those of A/Hong Kong/68-like human influenza viruses, while the genes encoding internal proteins are of avian-like swine H1N1 origin. The dominant H1N2 viruses are considered triple reassortant viruses between a human H1N1 virus from the 1980s, from which they obtained the HA protein, the swine H3N2 virus, from which they obtained the NA, and the avian-like swine H1N1 virus, from which they inherited their internal genes (Brown et al., 1998). In North America, viruses of the classical swine H1N1 lineage were the dominant cause of influenza among pigs until the late 1990s. Beginning in 1998, H3N2 viruses with genes of classical swine, avian and/or human origin became established in pig populations. These viruses further reassorted with classical swine H1N1 viruses, leading to H1N2 and reassortant H1N1 viruses, and resulted in a very complex picture (Vincent et al., 2008). Thus, the SIVs in Europe differ significantly in their antigenic and genetic make-up from those circulating in North America, while still other variants are circulating in various Asian countries (Brockwell-Staats et al., 2009).

Upon experimental inoculation, all established subtypes (H1N1, H1N2, H3N2) and genotypes examined cause a typical respiratory infection with strong tropism for all respiratory tissues and no tropism shown for extra-pulmonary organs. Disease signs are mild and transitory. Several factors, even upon experimental inoculation, are known to influence the severity of disease, such as route and dose of inoculation, age and physiological status of the animals.

As mentioned in section 2.1.3, inter-species transmission of influenza viruses from other mammalian and avian species to pigs are regularly recorded. Accumulating evidence therefore suggests that pigs are indeed a mixing vessel with the potential for reassortments of influenza A viruses from many mammalian and avian species, and that the directional flow of virus goes both ways (i.e. to and from pigs; see Ma et al. (2009) for a review).

The mixing vessel hypothesis was first proposed 25 years ago by Scholtissek et al. (1985) based on observations that pigs were more readily infected by both avian and human viruses than seemed to be the case for direct transmission between birds and humans, thus creating a potential bridge for transfer of viruses between the latter, whilst also providing the basis for formation of reassortants with new properties and replicative potential. The hypothesis was further supported by the differential distribution of $\alpha 2-6$ and $\alpha 2-3$ cellular receptors in these species (see 2.1.1) and, although it has become clear that the receptor specificity alone does not determine host range, the underlying observations of pigs as a host for a wide range of influenza viruses still seem to be valid.

The most recent example of pigs contributing to the generation and emergence of new influenza viruses was the appearance of the pandemic H1N1 virus in 2009. Although the exact origin and pathways leading to the emergence of this pandemic virus in humans in the spring of 2009 remain enigmatic (Dawood et al., 2009; Kingsford et al., 2009; Smith et al., 2009b), it is beyond doubt that several swine influenza viruses circulating in various parts of the world are the nearest ancestors of this pandemic virus.

3.3. Humans

3.3.1. Seasonal influenza 1977 to 2008

Influenza virus types A and B cause acute respiratory illness. Although both types are able to cause epidemics, significant disease and deaths, type B infections are on the whole milder than A/H3N2 but not always milder than seasonal A/H1, and are thought to be confined to humans as hosts. They are more often detected in the context of localized outbreaks and are not associated with pandemics. In contrast, type A viruses, which on the whole cause more severe symptoms, are those responsible for the greater burden of human disease during seasonal epidemics and are responsible for the occasional worldwide pandemics. In Europe, influenza occurs in regular annual epidemics in the winter between week 40 in one year and week 20 in the next, and this is called the influenza season. However, in recent years, epidemics have rarely started in earnest in Europe until the Christmas to New Year holiday period. These then affect all European countries for one to two months and last in Europe for 3-4 months with a tendency to progress from West to East in most, but not all, seasons (Paget et al., 2007). Sporadic infections also occur outside of the influenza season, though the incidence is very low in the European summer months when infections may be the result of imported cases from equatorial areas (where transmission is more year round) and the southern hemisphere, where most infection takes place in the European “summer”. A global overview is always available from the WHO Global Influenza Programme [summaries](#). All age groups are affected but the incidence of infection is usually highest in children. The proportions of the affected groups seen in community surveillance vary from country to country (and to a lesser extent from year to year). However, the variation in the observed annual pattern is also influenced by care seeking behaviour and the availability of services, as well as the dominant viruses in the year and the level of population immunity. The pattern of disease is, however, different from that of infection as more severe disease is focused in certain risk groups, notably older people and persons with chronic illness. The European Centre for Disease Prevention and Control (ECDC) estimates that in each season, on average, there are about 38,500 deaths attributable to seasonal influenza in the EU/EEA countries (ECDC, 2010).

Following severe epidemics in the late 1990s, and a particularly severe millennium winter (1999-2000), the annual epidemics during the first years of the new century were milder. However, the extent of transmission and the severity of illness depends on the circulating strains, with some years being dominated by influenza A(H1N1) and other years by A(H3N2) viruses, which are more pathogenic among older people and account for higher numbers of deaths, as occurred in the 2008/9 season. Seasons dominated by influenza B viruses are uncommon but they are especially mild. In the 2007/8 winter, a new oseltamivir-resistant seasonal A(H1N1) virus, named A(H1N1)H274Y, seemingly emerged in Europe, and it came to predominate over non-resistant viruses (Meijer et al., 2009). The pattern of the current season and preceding years can be examined for the EU as a whole or by individual countries via the European Influenza Surveillance Network ([EISN](#)) website.

An important point that must be stated is that all the above data applies to the seasonal influenza mix from 1977 to 2008, the so-called old seasonal influenza (Nicoll and Sprenger, 2010). What is yet to be determined is how different the pattern of infection and disease will be in the “new” seasonal influenza, including the new pandemic A(H1N1). However, from the first view of ECDC’s Forward Look Risk Assessment it appears that the pattern will be more similar than different (ECDC, 2010).

3.3.2. Pandemic influenza

To date, only viruses of the H1, H2 and H3 subtypes are known to have caused pandemics and establish subsequent global circulation. Following each of the recorded pandemics, the new pandemic virus has tended to become the dominant virus in seasonal influenza and has even replaced the prevailing strain.

It is known that at least one past pandemic influenza subtype in humans has now disappeared: the H2N2 subtype, which gave rise to the 1957 pandemic and which circulated seasonally in humans for a decade afterwards. Additionally, H1N1 disappeared from human populations at the time of the 1957 pandemic and only re-emerged in 1977. Although seasonal subtypes may present considerable public health problems, the focus of interest for potential novel pandemic strains must be in the reservoir hosts (aquatic wildfowl and shorebirds) or intermediate hosts (principally pigs and domesticated poultry).

Appreciation of the importance of pigs in the evolution of influenza pandemic strains has waxed and waned. Since the emergence of the 2009 pandemic A(H1N1), seemingly from pigs, the interest is currently high. The number and frequency of human infections with swine influenza in Europe is essentially unknown. Only a few infections have been confirmed in recent times (Adiego Sancho et al., 2009; Myers et al., 2007) and it seems likely there may be others that are undetected. It is also unclear whether there is any, or at least sufficient, communication between medical and veterinary diagnostic systems in Europe to support the detection of swine influenza viruses infecting humans, unless isolates are sent to National Influenza Centres or equivalent research laboratories.

4. Monitoring

The objectives of monitoring are to capture viral events (genetic, host range) that would signify an altered and expanded potential for replication in host populations considered relevant for acquiring pandemic potential. Like phylogenetics, monitoring can be seen as operating at three levels: 1) detection and assessment of the significance of molecular or antigenic changes in currently circulating strains that may greatly increase their pathogenicity; 2) assessment of similar changes in intermediate host strains, particularly in regard to reassortment events of the kind which resulted in the derivation of the 2009 H1N1 pandemic virus from triple reassortant swine influenza; and 3) assessment of the potential for appearance of novel subtypes in the reservoir host populations.

The third of these levels constitutes the most basic level of “early warning system”. The application of phylogenetics to data acquired through monitoring systems has allowed an assessment of past patterns of subtype diversification, and the historical distribution of diversification events in the avian haemagglutinin phylogenetic tree provides some circumstantial evidence for the suggestion that climate change may have been a factor (Gatherer, 2010). This is supported by some ecological studies on influenza in current avian populations (Gilbert et al., 2008). The mechanism by which this occurs may be related to disruption of avian migration patterns (Carey, 2009; Tingley et al., 2009) and consequent allopatric cladogenesis of the virus in isolated or marginal host populations, which is a well established phenomenon in population genetics (Mayr, 1942). This implies that the most appropriate aquatic wildfowl and wader populations for sampling would be those on the extremities of their species range, or those which are in recognised isolated habitats, especially if there is previous ecological evidence of niche disruption over the last century. Domestic poultry populations in proximity to these candidate wild avian populations could be sampled for evidence of transmission of any novel sequence variants. This can only be achieved by a multidisciplinary effort incorporating ecologists, veterinarians, virologists, epidemiologists and bioinformaticians. However, with careful choice of target populations by the ecologists, the expense need not be great.

If future pandemics arise as described above, this interval may provide the best opportunity for health authorities to intervene in order to mitigate the effects of a pandemic or even to abort its emergence (by early development of candidate vaccines). However, such an approach would require high-throughput characterisation of all eight gene segments of human virus isolates, even those that have unremarkable HA antigens, particularly from human viruses isolated in hotspots for zoonotic infections with avian influenza viruses. At present, global influenza surveillance in humans focuses attention primarily on haemagglutinin. Although this focus will continue to be required for strain selection for seasonal influenza vaccines, it is considered that this surveillance will not suffice for early warning of an incipient pandemic (Smith et al., 2009a).

4.1. Humans

Global influenza virological surveillance in humans is carried out by the Global Influenza Surveillance Network (GISN) coordinated by the WHO. The objectives of GISN are to monitor the circulation of influenza viruses and epidemics and to provide recommendations for influenza preparedness and response in certain areas, including vaccines, diagnosis, antivirals and continuing risk assessment. The Network also serves as a global alert mechanism for the emergence of influenza viruses with pandemic potential. Established in 1952, the GISN currently comprises 6 WHO Collaborating Centres (WHO CCs), 4 Essential Regulatory Laboratories (ERLs), 135 National Influenza Centres in 105 countries and 11 WHO H5 Reference Laboratories.

On a voluntary basis, the GISN has contributed greatly to the understanding of influenza viruses and their epidemiology, and has served as a scientific foundation for other control measures, including the twice annual update of the composition of influenza vaccines for the subsequent influenza season in the Northern and Southern Hemispheres. The value of the Network has been proved through the response to the 2009 influenza A(H1N1) pandemic, from the identification of the pandemic virus, to the global laboratory confirmation capacity, to the selection, development and making available of pandemic vaccine viruses for pandemic vaccine development and production.

Surveillance in the EU combines both primary care surveillance and virological surveillance but is relatively weak for severe cases, as seen in hospitals, and accounting for premature mortality. Virological surveillance contributes to the detection of new strains (such as the oseltamivir resistant viruses) and selection of vaccine candidate viruses matching the virus strains most likely to circulate in the coming season. The surveillance in national laboratories (called National Influenza Centres) in EU countries feeds into the GISN. This is comprised of the National Influenza Centres, including those that are part of the Community Network of Reference Laboratories for Human Influenza in Europe, which are managed and supported by the ECDC. These continuously report and share influenza viruses with the highly specialist WHO Collaborating Centres.

In Europe, a WHO Collaborating Centre is located in the UK (Mill Hill), where there is also the National Institute of Biological Standards and Controls (NIBSC), which further refines and prepares suitable viruses for passing onto industrial vaccine producers. Based on data arising from this surveillance, the WHO convenes specialist meetings each year at which it agrees on recommendations for the composition of the influenza vaccine for the next season. Separate meetings and recommendations are made for the Northern Hemisphere (which includes Europe) and the Southern Hemisphere. Current influenza vaccines are recommended to contain antigens protecting against two influenza A subtypes, H3N2 and H1N1, and one of the two lineages of the type B virus.

4.2. Pigs

Timely identification of new swine influenza virus (SIV) strains requires an adequate monitoring programme that presents a representative overview of the strains circulating among pigs.

At present, influenza is not a notifiable disease in pigs and the SIV strains that find their way into laboratories mostly originate from attempts to diagnose the cause of a clinical respiratory problem in pigs, which to some extent parallels the situation in the medical field. However, influenza virus isolation is attempted in only a very small proportion of pigs suffering from respiratory disease. Even so, serological surveys indicate that SIV is a common respiratory pathogen in pigs (Loeffen et al., 2003).

Research that involves monitoring influenza virus in pigs provides a second source of SIV strains. It also provides some indications, although fragmented, of the global distribution and spread of these strains. As a consequence, the current view of the strains circulating among pigs may be quite biased, and new SIV strains may be detected only after a considerable time has elapsed since their emergence, especially if they do not result in marked clinical problems in pigs. Late detection of new strains is also suggested by the work of Smith et al. (2009b) that indicates that the 2009 H1N1 pandemic virus may have circulated among pigs for a few years before the pandemic in humans started. However, this

work also suggests that monitoring systems have the potential to be refined to provide earlier detection of new strains.

It is also a challenge to gather SIV strains and deliver them into animal health laboratories in a timely manner and so detect the emergence of new strains or changes in geographical ranges. Ideally, this would require a global initiative to develop a flexible monitoring system adapted to the varying systems of pig husbandry across the world (as recommended in the OFFLU surveillance strategy; OFFLU, 2010b). Knowledge of the emergence of new strains in the past may help target future surveillance/monitoring programmes. Some steps towards this goal have been made in Europe by means of the consecutive research projects, ESNIP 1 and ESNIP 2 (ESNIP, 1999, 2006), which were developed to establish surveillance networks for swine influenza. The continuation of these projects through the current ESNIP 3 (ESNIP, 2010) will maintain and expand this network to include 21 participants from 11 EU MS with additional cooperation from partners in China and North America. Although this network is predominantly based on voluntary submission of diagnostic samples, and therefore mirrors the medical GISN on a smaller scale, it may provide a greater understanding of the epidemiology of SIVs at the global level and alert to the emergence of new reassortants. However, the project is based on research funding and therefore represents only a temporary solution to the long term threat to human and animal health.

4.3. Poultry/wild birds

As for pigs and man, timely identification of new influenza virus strains in birds requires an adequate monitoring programme that gives a representative overview of the strains circulating among birds. In contrast to pigs, some forms of AIV are notifiable and for many years there have been global statutory requirements that apply to high pathogenicity AIV subtypes H5 and H7. These requirements were extended within the EU in 2003 with the introduction of an annual serological survey for poultry to detect LPAIV of subtypes H5 and H7. Between 2006 and 2009, over 225,000 holdings were sampled. In the report for 2009 (SANCO, 2011), LPAIV was detected in only 90 holdings (0.26 %) with 52 holdings positive for the H5 subtype and 38 for the H7 subtype. The detection of H5 virus on 9.6 % and H7 virus on 42 % of the serologically positive holdings indicated ongoing infection.

Following the recognition of the global H5N1 crisis, the EU introduced further monitoring procedures to include compulsory surveillance for HPAIV in wild birds and the reporting of results. Between 2006 and 2009, more than 350,000 wild birds were sampled and tested for HPAIV in EU MS (SANCO, 2011). Generally, surveillance among wild birds in MS was carried out by actively sampling live birds (75 % of samples) and passively sampling sick or dead birds (25 % of samples). More than 1,000 (0.38 %) birds found dead or sick have tested positive for HPAIV of the H5N1 subtype, while only about five (0.006 %) healthy live birds tested positive during that 4-year period. This finding highlights that passive surveillance of sick and dead birds is more efficient at detecting H5N1 HPAI viruses. However, in 2009, LPAIV (H5 or H7 subtype) was detected mainly through active sampling of water fowl but it was found in only 162 birds (0.3 %).

Historical records and recent molecular analyses have highlighted the involvement of only H1, H2 and H3 subtypes in previous human pandemic events and the contribution of AIV genes to all of these pandemics (see Figure 1). The contribution of avian H1, H3 and N2 genes to these pandemic strains indicates that restricting the monitoring of AIV to the H5 and H7 subtypes will miss the emergence in birds of reassortants with potential public health importance. The extension of monitoring systems to embrace a wider range of subtypes (such as H1, H2, H3 and H9) will generate novel epidemiological, molecular and antigenic data which will improve our understanding of the dynamics of the influenza virus gene pool in wild and domestic birds and will be strategic for supporting both public and animal health.

Although wild birds play a key role in the perpetuation of AI viruses of all H subtypes, the huge seasonal variation in AI infection prevalence observed in wild birds, the high degree of adaptation of influenza viruses to their natural reservoirs and the need for huge economic efforts to implement an effective emerging virus surveillance makes it unlikely that wild birds will be a sustainable

surveillance target for capturing viral variants with pandemic potential. The higher contact rate existing between domestic poultry and mammals, including humans, and evidence that evolution occurs more rapidly in poultry species compared to wild aquatic birds (Widjaja et al., 2004), suggest that introduction of avian influenza viruses needs to be identified promptly in domestic animals.

Certainly, the emergence and global spread of the 2009 H1N1 pandemic virus suggests that it is imperative that we improve our surveillance, analytic and predictive capacities in order to optimise our public health response and manage the related animal health issues. The 2009 H1N1 pandemic virus emerged in an animal reservoir and contained a unique combination of genes from three species and two hemispheres. In a global environment, mapping gene movement across species and national borders and identifying mutations and gene constellations with pandemic potential or virulence determinants appears to be the first step required for examining the influenza gene pool as one evolving entity. This is in line with, and possibly the best example of, the “One Health” vision: a multidisciplinary collaborative approach to improve the health of humans, animals and the environment that is endorsed by the FAO, OIE and WHO (Capua and Alexander, 2010; Capua and Cattoli, 2010).

CONCLUSIONS AND RECOMMENDATIONS

TOR1: TO INDICATE THE MOST IMPORTANT FACTORS TO BE MONITORED IN ANIMALS THAT WOULD SUGGEST A RISK OF EMERGENCE OF A NEW PANDEMIC INFLUENZA STRAIN

CONCLUSIONS

- Only a subset of the 16 haemagglutinin (H1, H2, H3, H5, H7, H9, H10) and 9 neuraminidase (N1, N2, N3, N7) subtypes of influenza A virus have been observed in humans, of which only H1, H2, H3, N1 and N2 have been shown to be involved in pandemic and seasonal influenza.
- Receptor specificity is an important characteristic of influenza virus which influences host susceptibility.
- Mutations in the receptor-binding site of the virus may change binding preference.
- Recent findings show that not only pigs but also man and some gallinaceous avian species express α 2-3 and α 2-6 linked receptors. Therefore, each of these hosts may be co-infected by both avian and mammalian viruses, thus facilitating reassortment events between mammalian and avian influenza viruses and extending the number of species that need to be considered as “mixing vessels”.
- Most of the structural and non-structural influenza virus proteins involved in replication and transcription of the viral genome also affect the number of species that can be infected, level of viral replication, temperature permissiveness and excreted titre.
- New influenza strains emerge through natural reassortment and adaptation to their new host.
- Past experience has shown that reassortment events involving inter-species transmission are necessary steps in the evolution of new pandemic strains. However, it is not always clear in which species these events occur. Monitoring such events should therefore include important target species.
- Avian influenza viruses have played a role in the generation of the known human pandemic viruses, as in all instances at least one out of eight segments was donated by an avian virus.
- The rules that govern reassortment and the successful gene combination of a novel influenza strains are largely unknown.

- Most SIVs are reassortants of avian and mammalian viruses and have become stable lineages in pig populations. There are distinct variants in Europe, North America and Asia.
- Several molecular markers in influenza virus genes have been reported as being associated with certain biological properties, such as receptor binding, host and tissue tropism, virulence, and modulation of host immune response, as well as efficiency of replication and transmission. These associations have been inconsistent between strains.
- Studies in animal models have reaffirmed and highlighted the importance of individual virus proteins, such as the HA, NA, polymerase and non-structural proteins, and even point mutations within these molecules. They have also emphasised that the correlation between the molecular markers and biological properties is not absolute. Virulence and pathogenesis are not the properties of a single gene or protein. They are polygenic and the specific gene constellation is also important.
- Consequently, no single genetic marker or genetic constellation can be reliably associated with increased pathogenicity or transmissibility of influenza virus strains in mammals and cannot therefore be used to identify an emerging problem.

TOR2: TO ASSESS POSSIBLE OPTIONS OF MONITORING FOR THE PRESENCE OF THE MOST IMPORTANT FACTORS THAT WOULD SUGGEST A RISK OF EMERGENCE OF INFLUENZA VIRUSES (POTENTIALLY LEADING TO A PANDEMIC) IN DIFFERENT ANIMAL POPULATIONS, THAT COULD ACT AS RESERVOIRS, MIXING VESSELS OR OTHERWISE CONTRIBUTE TO THE RISK POSED TO HUMANS AND ANIMALS BY INFLUENZA VIRUSES

CONCLUSIONS

- Influenza A viruses are found in a variety of hosts, and are prone to interspecies transmission. These occur more readily among avian species than among mammalian species. Jumps of avian viruses to mammalian species and *vice versa* are detected relatively infrequently.
- Examples of incidental spill-over transmission from one mammalian species to another have been documented but becoming established and transmitting freely in that new species is considered a much rarer event.
- Transmission and adaptation to a new host population and subsequent establishment within that population may require changes in several genes over an extended period of time.
- Although several species have been used in studies to investigate pathogenesis and transmissibility of influenza virus in humans, the ferret is generally considered to be the model of choice as it most closely mimics the behaviour of influenza infection in man without the need for virus adaptation.
- This system, along with other laboratory-based investigations, therefore offers a potentially useful way to identify influenza A virus strains (including mutations) that might cause clinically important disease in man, although it should be noted that there can be important exceptions (e.g. not all HPAI H5N1 isolates are virulent in ferrets).
- Global influenza surveillance in humans focuses primarily on haemagglutinin, and this will continue to be required for strain selection for seasonal influenza vaccines. However, such surveillance will not suffice for early warning of an incipient pandemic.
- It appears that there is insufficient coordination between medical and veterinary diagnostic systems in Europe to support the routine detection of swine or avian influenza viruses infecting humans.
- Current monitoring through the GISN has been able to provide an alert for the emergence of new human influenza strains of public health significance.

- It is very likely that there is an incomplete view of the influenza virus strains circulating among pigs globally, although in Europe consistent information about circulating strains has been obtained through the ESNIP projects.
- The current ESNIP 3 network, based on passive surveillance, mirrors the medical GISN on a smaller scale. It will extend the body of knowledge and information to promote a greater understanding of the epidemiology of SIVs at the global level and will establish a baseline to support alerts for the emergence of new reassortants.
- Routine monitoring of poultry and wild birds for AIV subtypes, other than H5 and H7, is not mandatory and, consequently, knowledge on viruses such as H1, H2, and H3 is rather scarce. This gap limits understanding of their epidemiology, molecular diversity and potential contribution to strains of public health concern.
- The close and frequent contact between domestic poultry and mammals, including humans, and evidence that evolution occurs more rapidly in poultry species compared to wild aquatic birds, suggest that introduction of avian influenza viruses needs to be promptly identified in poultry.
- With the current state of knowledge, new reassortants of public health significance are more likely to be detected by monitoring human samples. Nevertheless, interpretation of the origins and pandemic potential would require knowledge of the influenza gene pools in both pigs and birds, as well as other animal species, when relevant.

TOR3: TO ASSESS THE POSSIBLE PREDICTABILITY OF THE EMERGENCE OF A NEW PANDEMIC INFLUENZA STRAIN BY MONITORING THE MOLECULAR EVOLUTION AND DEVELOPMENT OF INFLUENZA VIRUSES IN DIFFERENT ANIMAL POPULATIONS.

CONCLUSIONS

- The potential of applying phylogenetics to the influenza virus has been demonstrated by revealing patterns of seasonality and tracking of the current 2009 H1N1 pandemic.
- However, at present, further application for answering questions on emergence and evolution of influenza viruses is constrained by the uneven representation in databases of influenza virus sequences from different species and geographical areas.

RECOMMENDATIONS

- There should be long term support for a passive monitoring network based on current diagnostic practices, such as the ESNIP network, in order to promote greater understanding of the evolution of SIVs at the global level and establish a baseline to support alerts for the emergence of new reassortants.
- There should be long term support for an enhanced passive monitoring network, based on current diagnostic practices and regulatory requirements, in order to promote greater understanding of the evolution of AIVs at the global level and establish a baseline to support alerts for the emergence of new reassortants. Monitoring should be extended to include AIVs that express the HA and NA subtypes that have been observed in man, particularly H1, H2 and H3. A risk-based approach focusing on domestic poultry, and animals in contact with them, should be used.
- Maximum benefit from such networks should be obtained by applying an integrated approach. This should include the development and implementation of harmonised tools and approaches, exchange of virus strains and sequence data, and enhancing the coordination and

dissemination of the findings from the human, swine and avian networks, as well as from other species, when relevant.

- Monitoring should include typing of the H and N genes as a minimum, whereas strains that are not typed with currently used subtype reagents are of special concern and should be subjected to full genome sequencing.
- Full sequencing of the virus is the preferred option, as this will provide important additional information, including reassortant events. Depositing data in publicly accessible platforms for data analysis and interpretation in conjunction with epidemiological information would support and accelerate the general advancement of knowledge on the zoonotic and pandemic risk linked to influenza viruses.
- However, such an approach would require high-throughput characterisation of all eight gene segments of human virus isolates, even those that have unremarkable HA antigens, particularly for human viruses isolated from where zoonotic infections with avian influenza viruses are likely to occur.

REFERENCES

- Adiego Sancho B, Omenaca Teres M, Martinez Cuenca S, Rodrigo Val P, Sanchez Villanueva P, Casas I, Pozo F and Perez Brena P, 2009. Human case of swine influenza A (H1N1), Aragon, Spain, November 2008. *Eurosurveillance*, 14(7):pii=19120. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19120>
- Alexander DJ and Brown IH, 2009. History of highly pathogenic avian influenza. *Revue Scientifique et Technique del Office International des Epizooties*, 28, 19-38.
- Arias CF, Escalera-Zamudio M, Soto-Del Río MDLD, Cobián-Güemes AG, Isa P and López S, 2009. Molecular anatomy of 2009 influenza virus A (H1N1). *Archives of Medical Research*, 40, 643-654.
- Belser JA, Blixt O, Chen L, Pappas C, Maines TR, Van Hoeven N, Donis R, Busch J, McBride R, Paulson JC, Katz JM and Tumpey TM, 2008. Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 7558-7563.
- Belser JA, Szretter KJ, Katz JM and Tumpey TM, 2009. Use of animal models to understand the pandemic potential of highly pathogenic avian influenza viruses. In: *Advances in Virus Research*. Eds Maramorosch K, Shatkin AJ and Murphy FA. Academic Press, 55-97.
- Belser JA, Maines TR, Tumpey TM and Katz JM, 2010. Influenza A virus transmission: contributing factors and clinical implications. *Expert Reviews in Molecular Medicine*, 12, e39, doi:10.1017/S1462399410001705.
- Brockwell-Staats C, Webster RG and Webby RJ, 2009. Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). *Influenza and Other Respiratory Viruses*, 3, 207-213.
- Brown IH, Harris PA, McCauley JW and Alexander DJ, 1998. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. *Journal of General Virology*, 79, 2947-2955.
- Butt KM, Smith GJD, Chen H, Zhang LJ, Leung YHC, Xu KM, Lim W, Webster RG, Yuen KY, Peiris JSM and Guan Y, 2005. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *Journal of Clinical Microbiology*, 43, 5760-5767.
- Capua I, 2010. What have we learned from H5N1? *Influenza and Other Emerging Zoonotic Diseases at the Human-Animal Interface*, 2nd FAO-OIE-WHO Joint Scientific Consultation, Verona, Italy. Available from: <http://www.fao.org/docs/eims/upload//276301/ak745e00.pdf>
- Capua I and Alexander D, 2010. Perspectives on the global threat: the challenge of avian influenza viruses for the world's veterinary community. *Avian Diseases*, 54(1 Suppl.), 176-178.
- Capua I and Alexander DJ, 2007. Animal and human health implications of avian influenza infections. *Biosciences Reports*, 27, 359-372.
- Capua I and Cattoli G, 2010. One flu for One Health. *Emerging Infectious Diseases*, 16, 719.
- Carey C, 2009. The impacts of climate change on the annual cycles of birds. *Philosophical Transactions of the Royal Society of London B*, 364, 3321-3330.
- Chen L, Davis CT, Zhou H, Cox NJ, Donis RO, 2008. Genetic Compatibility and Virulence of Reassortants Derived from Contemporary Avian H5N1 and Human H3N2 Influenza A Viruses. *PLoS Pathog.* 4(5), e1000072.
- Chowell G and Nishiura H, 2008. Quantifying the transmission potential of pandemic influenza. *Physics of Life Reviews*, 5, 50-77.

- Chutinimitkul S, Herfst S, Steel J, Lowen AC, Ye J, van Riel D, Schrauwen EJA, Bestebroer TM, Koel B, Burke DF, Sutherland-Cash KH, Whittleston CS, Russell CA, Wales DJ, Smith DJ, Jonges M, Meijer A, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus ADME, Garcia-Sastre A, Perez DR and Fouchier RAM, 2010. Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. *Journal of Virology*, 84, 11802-11813.
- Cong YL, Pu J, Liu QF, Wang S, Zhang GZ, Zhang XL, Fan WX, Brown EG and Liu JH, 2007. Antigenic and genetic characterization of H9N2 swine influenza viruses in China. *Journal of General Virology*, 88, 2035-2041.
- Das K, Aramini JM, Ma L, Krug RM and Arnold E, 2010. Structures of influenza A proteins and insights into antiviral drug targets. *Nature Structure and Molecular Biology*, 17, 530-538.
- Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB and Uyeki TM, 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *New England Journal of Medicine*, 360, 2605-2615.
- De Wit E and Fouchier RA, 2008. Emerging influenza. *Journal of General Virology*, 41, 1-6.
- Drummond AJ, Pybus OG, Rambaut A, Forsberg R and Rodrigo AG, 2003. Measurably evolving populations. *Trends in Ecology and Evolution*, 18, 481-488.
- ECDC (European Centre for Disease Prevention and Control), 2010. ECDC forward look risk assessment - likely scenarios and uncertainties in the 2010/2011 influenza season in Europe and beyond. Available from:
http://ecdc.europa.eu/en/healthtopics/H1N1/Documents/1003_RA_forward_look_influenza.pdf
- ESNIP (European Surveillance Network for Influenza in Pigs), 1999. European Surveillance Network for Influenza in Pigs 1. Available from:
http://ec.europa.eu/research/health/infectious-diseases/emerging-epidemics/projects/108_en.html
- ESNIP (European Surveillance Network for Influenza in Pigs), 2006. European Surveillance Network for Influenza in Pigs 2. Available from:
http://ec.europa.eu/research/health/infectious-diseases/emerging-epidemics/projects/109_en.html
- ESNIP (European Surveillance Network for Influenza in Pigs), 2010. European Surveillance Network for Influenza in Pigs 3. Available from:
http://cordis.europa.eu/search/index.cfm?fuseaction=proj.document&PJ_LANG=EN&PJ_RCN=11742603&pid=0&q=9EAF4BAE8CCC9B9618C7F96527794465&type=sim
- Forrest HL and Webster RG, 2010. Perspectives on influenza evolution and the role of research. *Animal Health Research Reviews*, 11(Special Issue 01), 3-18.
- Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F, Ghani AC, Ferguson NM, Rambaut A, Pybus OG, Lopez-Gatell H, Alpuche-Aranda CM, Chapela IB, Zavala EP, Guevara DME, Checchi F, Garcia E, Hugonnet S and Roth C, 2009. The WHO Rapid Pandemic Assessment Collaboration, 2009. Pandemic potential of a strain of influenza A (H1N1): early findings. *Science*, 324, 1557-1561.
- Fouchier RAM, Osterhaus ADME, Brown IH, 2003. Animal influenza virus surveillance. *Vaccine*. 21(16), 1754-1757.
- Gatherer D, 2009a. On the origin of influenza A hemagglutinin. *Indian Journal of Microbiology*, 49, 352-357.
- Gatherer D, 2009b. The 2009 H1N1 influenza outbreak in its historical context. *Journal of Clinical Virology*. 45, 174-178.

- Gatherer D, 2010. The Little Ice Age and the emergence of influenza A. *Medical Hypotheses*, 75, 359-362.
- Gilbert M, Slingenbergh J and Xiao X, 2008. Climate change and avian influenza. *Revue scientifique et technique (International Office of Epizootics)*, 27, 459-466.
- Guo C, Takahashi N, Yagi H, Kato K, Takahashi T, Yi S, Chen Y, Ito T, Otsuki K, Kida H, Kawaoka Y, Hidari KII, Miyamoto D, Suzuki T and Suzuki Y, 2007. The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors. *Glycobiology*, 17, 713-724.
- Hay AJ, Gregory V, Douglas AR, Lin YP, 2001. The evolution of human influenza viruses. *Philosophical Transactions of the Royal Society of London B*, 356(1416), 1861-1870.
- Herfst S, Chutinimitkul S, Ye J, de Wit E, Munster VJ, Schrauwen EJ, Bestebroer TM, Jonges M, Meijer A, Koopmans M, Rimmelzwaan GF, Osterhaus AD, Perez DR and Fouchier RA, 2010. Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission. *Journal of Virology*, 84, 3752-3758.
- Katz JM, Lu X, Tumpey TM, Smith CB, Shaw MW and Subbarao K, 2000. Molecular correlates of influenza A H5N1 virus pathogenesis in mice. *Journal of Virology*, 74, 10807-10810.
- Katz JM, Veguilla V, Belser JA, Maines TR, Van Hoeven N, Pappas C, Hancock K and Tumpey TM, 2009. The public health impact of avian influenza viruses. *Poultry Science*, 88, 872-879.
- Kawaoka Y, Chambers TM, Sladen WL, Webster RG, 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? *Virology*. 163(1), 247-250.
- Kilander A, Rykkvin R, Dudman SG and Hungnes O, 2010. Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010. *Eurosurveillance*, 15(9):pii=19498. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19498>.
- Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP and Daszak P, 2006. Predicting the global spread of H5N1 avian influenza. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 19368-19373.
- Kingsford C, Nagarajan N and Salzberg SL, 2009. 2009 Swine-origin influenza A (H1N1) resembles previous influenza isolates. *Public Library of Science One*, 4, e6402, doi:10.1371/journal.pone.0006402.
- Lemey P, Rambaut A, Drummond AJ and Suchard MA, 2009a. Bayesian phylogeography finds its roots. *Public Library of Science Computational Biology*, 5, e1000520, doi:10.1371/journal.pcbi.1000520.
- Lemey P, Suchard M and Rambaut A, 2009b. Reconstructing the initial global spread of a human influenza pandemic. *Public Library of Science Currents*, 1. Available from: <http://knol.google.com/k/reconstructing-the-initial-global-spread-of-a-human-influenza-pandemic#>
- Lemey P, Rambaut A, Welch JJ and Suchard MA, 2010. Phylogeography takes a relaxed random walk in continuous space and time. *Molecular Biology and Evolution*, 27, 1877-1885.
- Lin YP, Shaw M, Gregory V, Cameron K, Lim W, Klimov A, Subbarao K, Guan Y, Krauss S, Shortridge K, Webster R, Cox N and Hay A, 2000. Avian-to-human transmission of H9N2 subtype influenza A viruses: Relationship between H9N2 and H5N1 human isolates. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 9654-9658.
- Lipatov AS, Kwon YK, Pantin-Jackwood MJ and Swayne DE, 2009. Pathogenesis of H5N1 influenza virus infections in mice and ferret models differs according to respiratory tract or digestive system exposure. *Journal of Infectious Diseases*, 199, 717-725.

- Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W, Daniels R, Gregory V, Uhlenhorff J, Kiso M, Klenk H, Hay A, Feizi T and Matrosovich M, 2010. Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. *Journal of Virology*, 84, 12069-12074.
- Lloyd-Smith JO, George D, Pepin KM, Pitzer VE, Pulliam JRC, Dobson AP, Hudson PJ and Grenfell BT, 2009. Epidemic dynamics at the human-animal interface. *Science*, 326, 1362-1367.
- Loeffen WLA, Nodelijk G, Heinen PP, van Leengoed LAMG, Hunneman WA and Verheijden JHM, 2003. Estimating the incidence of influenza-virus infections in Dutch weaned piglets using blood samples from a cross-sectional study. *Veterinary Microbiology*, 91, 295-308.
- Lu XH, Cho D, Hall H, Rowe T, Mo IP, Sung HW, Kim WJ, Kang C, Cox N, Klimov A and Katz JM, 2003. Pathogenesis of and immunity to a new influenza A (H5N1) virus isolated from duck meat. *Avian Diseases*, 47(3 Suppl.), 1135-1140.
- Ma W, Lager KM, Vincent AL, Janke BH, Gramer MR and Richt JA, 2009. The role of swine in the generation of novel influenza viruses. *Zoonoses and Public Health*, 56, 326-337.
- Maines TR, Chen L, Matsuoka Y, Chen H, Rowe T, Ortin J, Falcón A, Hien NT, Mai LQ, Sedyaningsih ER, Harun S, Tumpey TM, Donis RO, Cox NJ, Subbarao K and Katz JM, 2006. Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 12121-12126.
- Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I and Kawaoka Y, 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *Journal of Virology*, 74, 8502-8512.
- Mayr E, 1942. *Systematics and the origin of species from the viewpoint of a zoologist*. Harvard University Press, New York, 334 pp.
- Meijer A, Lackenby A, Hungnes O, Lina B, van der Werf S, Schweiger B, Opp M, Paget J, van de Kasstele J, Hay A and Zambon M, 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season. *Emerging Infectious Diseases*, 15, 552-560.
- Morens DM and Taubenberger JK, 2010. Historical thoughts on influenza viral ecosystems, or behold a pale horse, dead dogs, failing fowl, and sick swine. *Influenza and Other Respiratory Viruses*, 4, 327-337.
- Morens DM, Taubenberger JK and Fauci AS, 2009. The persistent legacy of the 1918 influenza virus. *New England Journal of Medicine*, 361, 225-229.
- Myers KP, Olsen CW and Gray GC, 2007. Cases of swine influenza in humans: A review of the literature. *Clinical Infectious Diseases*, 44, 1084-1088.
- Nelli R, Kuchipudi S, White G, Perez B, Dunham S and Chang K, 2010. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. *BMC Veterinary Research*, 6, 4, doi:10.1186/1746-6148-6-4.
- Neumann G and Kawaoka Y, 2006. Host range and pathogenicity in the context of influenza pandemic. *Emerging Infectious Diseases*, 12, 881-886.
- Neumann G, Noda T, Kawaoka Y, 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature*. 459(7249), 931-939.
- Nicholls JM, Chan RW, Russell RJ, Air GM and Peiris JM, 2008. Evolving complexities of influenza virus and its receptors. *Trends in Microbiology*, 16, 149-157.

- Nicoll A and Sprenger M, 2010. The end of the pandemic - what will be the pattern of influenza in the 2010-11 European winter and beyond? *Eurosurveillance*, 15(32):pii=19637. Available from: <http://www.eurosurveillance.org/viewarticle.aspx?articleid=19637>
- OIE 2010. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Available from: <http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/>
- OFFLU (OIE/FAO Network of Expertise on Animal Influenzas), 2010a. OFFLU Annual Report 2010. Available from: http://www.offlu.net/OFFLU%20Site/OFFLU_Annual_Report_2010.pdf
- OFFLU (OIE/FAO Network of Expertise on Animal Influenzas), 2010b. OFFLU strategy document for surveillance and monitoring of influenzas in animals. Available from: http://www.offlu.net/OFFLU%20Site/OFFLUsurveillancePH1N1_180110.pdf
- Olsen C, Brown I, Easterday B and Van Reeth K, 2006. Swine influenza. In: *Diseases of swine*. Eds Straw BE, Zimmerman JJ, D'Allaire S and Taylor DJ. Iowa State University Press, Ames, IA, pp. 469-482.
- Paget J, Marquet R, Meijer A and van der Velden K, 2007. Influenza activity in Europe during eight seasons (1999-2007): an evaluation of the indicators used to measure activity and an assessment of the timing, length and course of peak activity (spread) across Europe. *BMC Infectious Diseases*, 7, 141, doi:10.1186/1471-2334-7-141.
- Pensaert M, Ottis K, Vandeputte J, Kaplan MM and Bachmann PA, 1981. Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man. *Bulletin of the World Health Organisation*, 59, 75-78.
- Pillai S, Pantin-Jackwood M, Jadhao S, Suarez D, Wang L, Yassine H, Saif Y and Lee C, 2009. Pathobiology of triple reassortant H3N2 influenza viruses in breeder turkeys and its potential implication for vaccine studies in turkeys. *Vaccine*, 27, 819-824.
- Raghava G, Searl S, Audley P, Barbe J and Barton G, 2003. OXBench: A benchmark for evaluation of protein multiple sequence alignment accuracy. *BMC Bioinformatics*, 4, 47, doi:10.1186/1471-2105-4-47.
- Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK and Holmes EC, 2008. The genomic and epidemiological dynamics of human influenza A virus. *Nature*, 453, 615-619.
- Rodrigo AG and Felsenstein J, 1999. Coalescent approaches to HIV population genetics. In: *The evolution of HIV*. Ed Crandall K. Johns Hopkins University Press, 223-271.
- Rott R, 1992. The pathogenic determinant of influenza virus. *Veterinary Microbiology*, 33(1-4), 303-310.
- Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, Gregory V, Gust ID, Hampson AW, Hay AJ, Hurt AC, de Jong JC, Kelso A, Klimov AI, Kageyama T, Komadina N, Lapedes AS, Lin YP, Mosterin A, Obuchi M, Odagiri T, Osterhaus ADME, Rimmelzwaan GF, Shaw MW, Skepner E, Stohr K, Tashiro M, Fouchier RAM and Smith DJ, 2008. The global circulation of seasonal influenza A (H3N2) viruses. *Science*, 320, 340-346.
- Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen H, Hulse-Post DJ, Humberd J, Trichet M, Rehg JE, Webby RJ, Webster RG and Hoffmann E, 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. *Journal of Experimental Medicine*, 203, 689-697.
- SANCO, 2011. Avian influenza surveillance. Available from: http://ec.europa.eu/food/animal/diseases/controlmeasures/avian/eu_resp_surveillance_en.htm
- Scholtissek C, Bürger H, Kistner O and Shortridge K, 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology*, 147, 287-294.

- Senne DA, Panigrahy B, Kawaoka Y, Pearson JE, Süß J, Lipkin M, Kida H and Webster RG, 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. *Avian Diseases*, 40, 425-437.
- Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, Lindstrom S, Gubareva LV, Deyde V, Garten RJ, Harris M, Gerber S, Vagasky S, Smith F, Pascoe N, Martin K, Dufficy D, Ritger K, Conover C, Quinlisk P, Klimov A, Bresee JS and Finelli L, 2009. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009. *New England Journal of Medicine*, 360, 2616-2625.
- Shinya K, Ebina M, Yamada S, Ono M, Kasai N and Kawaoka Y, 2006. Avian flu: Influenza virus receptors in the human airway. *Nature*, 440, 435-436.
- Smith G, Bahl J, Vijaykrishna D, Zhang J, Poon LLM, Chen H, Webster RG, Peiris JSM and Guan Y, 2009a. Dating the emergence of pandemic influenza viruses. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 11709-11712.
- Smith G, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghvani J, Bhatt S, Peiris JSM, Guan Y and Rambaut A, 2009b. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature*, 459, 1122-1125.
- Stallknecht DE, Brown JD, 2007. Wild birds and the epidemiology of avian influenza. *Journal of Wildlife Diseases*, 43(3_Supplement), S15-20.
- Stevens J, Blixt O, Paulson JC and Wilson IA, 2006. Glycan microarray technologies: tools to survey host specificity of influenza viruses. *Nature Reviews Microbiology*, 4, 857-864.
- Stieneke-Gröber A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W, 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO Journal*, 11(7), 2407-2414.
- Suzuki Y and Nei M, 2002. Origin and evolution of influenza virus hemagglutinin genes. *Molecular Biology and Evolution*, 19, 501-509.
- Tarantola A, Barboza P, Gauthier V, Ioos S, El Omeiri N and Gastellu-Etchegorry M, 2010. The influenza A(H5N1) epidemic at six and a half years: 500 notified human cases and more to come. *Eurosurveillance*, 15(29):pii=19619. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19619>
- Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG, 2005. Characterization of the 1918 influenza virus polymerase genes. *Nature*. 437(7060), 889-893.
- Taubenberger JK and Morens DM, 2006. 1918 Influenza: the mother of all pandemics. *Emerging Infectious Diseases*, 12, 15-22.
- Terregino C, De Nardi R, Guberti V, Scremin M, Raffini E, Martin AM, Cattoli G, Bonfanti L and Capua I, 2007. Active surveillance for avian influenza viruses in wild birds and backyard flocks in Northern Italy during 2004 to 2006. *Avian Pathology*, 36, 337-344.
- Tiensin T, Nielen M, Vernooij H, Songserm T, Kalpravidh W, Chotiprasatintara S, Chaisingh A, Wongkasemjit S, Chanachai K, Thanapongtham W, Srisuvan T and Stegeman A, 2007. Transmission of the highly pathogenic avian influenza virus H5N1 within flocks during the 2004 epidemic in Thailand. *Journal of Infectious Diseases*, 196, 1679-1684.
- Tingley MW, Monahan WB, Beissinger SR and Moritz C, 2009. Birds track their Grinnellian niche through a century of climate change. *Proceedings of the National Academy of Sciences of the United States of America*, 106 (Suppl. 2), 19637-19643.
- Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, Uiprasertkul M, Boonnak K, Pittayawonganon C, Cox NJ, Zaki SR, Thawatsupha P, Chittaganpitch M, Khontong

- R, Simmerman JM and Chunsutthiwat S, 2005. Probable person-to-person transmission of avian influenza A (H5N1). *New England Journal of Medicine*, 352, 333-340.
- Van Poucke SG, Nicholls JM, Nauwynck HJ and Van Reeth K, 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. *Journal of Virology*, 7, 38, doi:10.1186/1743-422X-7-38.
- Vannier P, 2007. Avian influenza; routes of transmission: lessons and thoughts drawn out of the past and present situation in the world and in the European Union. *Pathologie Biologie*, 55, 273-276.
- Vey M, Orlich M, Adler S, Klenk HD, Rott R and Garten W, 1992. Hemagglutinin activation of pathogenic avian influenza viruses of serotype H7 requires the protease recognition motif R-X-K/R-R. *Virology*, 188, 408-413.
- Vincent AL, Ma W, Lager KM, Janke BH and Richt JA, 2008. Chapter 3 - Swine Influenza Viruses: A North American Perspective. Academic Press, pp. 127-154.
- Wan H and Perez DR, 2006. Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. *Virology*, 346, 278-286.
- Wan H, Sorrell EM, Song H, Hossain MJ, Ramirez-Nieto G, Monne I, Stevens J, Cattoli G, Capua I, Chen L, Donis RO, Busch J, Paulson JC, Brockwell C, Webby R, Blanco J, Al-Natour MQ and Perez DR, 2008. Replication and transmission of H9N2 influenza viruses in ferrets: Evaluation of pandemic potential. *Public Library of Science One*, 3, e2923, doi:10.1371/journal.pone.0002923.
- Webster RG, Bean WJ, Gorman OT, Chambers TM and Kawaoka Y, 1992. Evolution and ecology of influenza A viruses. *Microbiology and Molecular Biology Reviews*, 56, 152-179.
- WHO (World Health Organization), 2009. Weekly epidemiological record, 13 November 2009, Transmission dynamics and impact of pandemic influenza A (H1N1) 2009 virus. 84, 477-484. Available from: <http://www.who.int/wer/2009/wer8446.pdf>
- Widjaja L, Krauss SL, Webby RJ, Xie T and Webster RG, 2004. Matrix gene of influenza A viruses isolated from wild aquatic birds: ecology and emergence of influenza A viruses. *Journal of Virology*, 78, 8771-8779.
- Wood GW, McCauley JW, Bashiruddin JB and Alexander DJ, 1993. Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. *Archives of Virology*, 130, 209-217.
- Yassine HM, Lee C, Gourapura R and Saif YM, 2010. Interspecies and intraspecies transmission of influenza A viruses: viral, host and environmental factors. *Animal Health Research Reviews*, 11(Special Issue 01), 53-72.
- Yen H, Aldridge JR, Boon ACM, Ilyushina NA, Salomon R, Hulse-Post DJ, Marjuki H, Franks J, Boltz DA, Bush D, Lipatov AS, Webby RJ, Rehg JE and Webster RG, 2009. Changes in H5N1 influenza virus hemagglutinin receptor binding domain affect systemic spread. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 286-291.

APPENDIX A. UNDERSTANDING INFLUENZA A AND THE HOST SPECIES BARRIER

1. INTRODUCTION

With the ever increasing connectedness of distant human populations and the omnipresent threat of animal diseases spreading to humans, interest is growing for determining the threat that is posed due to our associations with animals and on how we can minimise the resulting risks.

There are many dynamics that can be responsible for animal disease being transmitted to humans but in all situations, in order for disease to transmit from animals to humans, the crossing of the host species barrier is required.

The host species barrier is not a simple concept and can include not only biological factors but also behavioural factors. It is known, for example, that in some cases the primary barrier impeding disease transmission between species is lack of adequate contact. The host barrier can take many forms and the form this barrier takes is critical for understanding how disease transmits between species. Therefore, this section will describe, in terms of the host barrier, when linking models is beneficial to form control strategies and what types of models should be linked in order to model multi-species dynamics, as well as how these models should be linked and the possible implications for modelling multi-species dynamics. Lloyd-Smith [15] recently reviewed all models used to study zoonotic disease dynamics and provided a good, comprehensive review of what has been accomplished.

Obligate zoonoses are organisms that never achieve an R_0 in humans of > 1 . Thus, new cases are dependent on spill-over from the animal reservoir. Some zoonotic diseases can be classified as obligate zoonoses, and common examples include avian influenza (H5N1) and brucellosis. When considering obligate zoonoses, the dynamics within humans do not need to be explicitly modelled. Depending on the questions of interest, researchers have considered the force of infection, which is directly proportional to the number of cases in the animal reservoir, in order to determine human impact [12]. Alternatively, population density can be used to determine spatial infection patterns that result from various spatial distributions of organisms or migration patterns of the host species [19]. Models of spill-over have also been used to study food-borne and vector-borne pathogens [15]. These systems can reasonably be represented using mathematical models as evolution is not a key feature and the disease spread within the human host is generally highly dependent on the dynamics within the animal population at all stages.

2. STAGGERING CHAINS WITH EVOLUTION

When a pathogen initially infects a new host species, it is often ill adapted to that organism in terms of its ability to survive in the new host and transmit to other members of the recipient species [16]. It is thought that through multiple introductions, the pathogen will have increased opportunities and thus higher probability of adaptation to the recipient species [1]. As a virus adapts to a new host through staggering chains of transmission, its fitness in the donor host is usually reduced [16]. Since adaptations to a recipient host are likely to be deleterious to the donor host, such mutations are likely to be driven either towards extinction or adaptation. Critically, for modelling emergence, this leads to a situation in which competent disease is not passed through a semi-permeable membrane but rather only one, or possibly a few, successful introductions are expected.

In the case of swine and human influenza, the barrier has traditionally been thought to be strong, so that many mutations are required for establishment of a swine influenza lineage in humans and it is extremely unlikely that all of these will be acquired in one recombination event [10, 14, 23, 27]. As evidence that swine influenza viruses do not easily establish lineages in the human host, we see frequent cases of individuals with occupational exposure to swine contracting swine influenza and occasionally passing it on through very close contact, usually with a spouse, although, despite these isolated and frequent events, pandemic influenza has only emerged 10 to 20 times in the past 250 years.

Models of staggering chains with evolution in a stochastic spatial model have probably not been found in the literature for a number of reasons. Firstly, virus evolution is poorly understood and modelling of evolution is in its infancy. This is compounded by the fact that there is generally no data to inform parameters associated with mutated viruses, or how R_0 will change in each species through the evolution process. Secondly, the evolutionary events that lead to emergence are thought to be extremely rare, especially when multiple mutations are required [10, 14, 23, 27] that cause computational problems for time consuming simulation models. So, when evolution cannot be separated from the emergence process, only a few simple deterministic models have been used to study steady states and bifurcation points (for example, see Coburn et al. [5] and Iwami et al. [11]). Thirdly, dynamics in which a competent virus is seeded once or only a few times, lead to nearly independent epidemics in the host and recipient species so that only the initial probability that the epidemic is sparked is highly dependent on the donor species dynamics [7]. Since the seeding of one or a few viruses with $R_0 > 1$ leads to between-host dynamics that are almost independent and because of the difficulties associated with attempting to predict the probability of a virus with $R_0 > 1$ infecting a human, most modellers choose to initiate their model with one infected human and ignore both animal dynamics as well as host barrier dynamics.

Almost all influenza models found in the literature were developed under the assumption that between-host dynamics were not contributing significantly to new cases. Some of these models focused specifically on early containment of an influenza outbreak and these were generally directed from other models in the literature, since they considered a relatively small spatial scale (for example, see Ferguson et al. [8]). It has largely been accepted that containment must occur at a local scale, since once a virus has spread in a major city, control measures can only delay spread for short periods of time and containment is not realistic [6].

REFERENCES

- [1] Anita R, Regoes R, Koella J and Bergstrom C, 2003. The role of evolution in the emergence of infectious diseases. *Nature*, 426, 658-661.
- [2] Brookes S, Irvine R, Nunez A, Clifford D, Essen S, Brown I, Van Reeth K, Kuntz-Simon G, Loeffen W, Foni E, Larsen L, Matrosovich M, Bublot M, Maldonado J, Beer M and Cattoli G, 2009. Influenza A (H1N1) infection in pigs. *Veterinary Record*, 164, 760-761.
- [3] Burroughs T, Knobler S and Lederberg J, 2002. The emergence of zoonotic diseases: Understanding the impact on animal and human health. The National Academies Press, Washington DC, 176 pp.
- [4] Cleaveland S, Laurenson M and Taylor L, 2001. Diseases of humans and their domestic mammals: Pathogen characteristics, host range and the risk of emergence. *Philosophical Transactions: Biological Sciences London B*, 356, 991-999.
- [5] Coburn B, Wagner B and Blower S, 2009. Modeling influenza epidemics and pandemics: Insights into the future of swine flu (H1N1). *BMC Medicine*, 7, 30, doi:10.1186/1741-7015-7-30.
- [6] Cooper B, Pitman R, Edmunds W and Gay N, 2006. The international spread of pandemic influenza. *Public Library of Science, Medicine*, 3, 845-855.
- [7] Dobson A, 2004. Population dynamics of pathogens with multiple host species. *The American Naturalist*, 164, S64-S78.
- [8] Ferguson N, Cummings D, Cauchemez S, Fraser C, Riley S, Meeyai A, Iamsirithaworn S and Burke D, 2005. Strategies for containing an emerging influenza pandemic in southeast Asia. *Nature*, 437, 209-214.
- [9] Gray G and Baker W, 2007. The importance of including swine and poultry workers in influenza vaccination programs. *Clinical Pharmacology and Therapeutics*, 82, 638-641.

- [10] Gray G and Kayali G, 2008. Facing pandemic influenza threats: the importance of including poultry and swine workers in preparedness plans. *Poultry Science*, 88, 880-884.
- [11] Iwami S, Takeuchi Y and Liu X, 2007. Avian-human influenza epidemic model. *Mathematical Biosciences*, 207, 1-25.
- [12] Keeling M and Gilligan C, 2000. Bubonic plague: a metapopulation model of a zoonosis. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, 267, 2219-2230.
- [13] Kuiken T, Holmes E, McCauley J, Rimmelzwaan G, Williams C and Grenfell B, 2006. Host species barriers to influenza virus infections. *Science*, 312, 394-397.
- [14] Lipatov A, Govorkova E, Webby R, Ozaki H, Peiris M, Guan Y, Poon L and Webster R, 2004. Influenza: emergence and control. *Journal of Virology*, 78, 8951-8959.
- [15] Lloyd-Smith J, George D, Pepin K, Pitzer V, Pulliam J, Dobson A, Hudson P and Grenfell B, 2009. Epidemic dynamics at the human-animal interface. *Science*, 326, 1362-1367.
- [16] Parrish C, Holmes E, Morens D, Park E, Burke D, Calisher C, Saif L and Daszak P, 2008. Cross-species virus transmission and the emergence of new epidemic diseases. *Microbiology and Molecular Biology Reviews*, 72, 457-470.
- [17] Rambaut A and Holmes E, 2009. The early molecular epidemiology of the swine-origin A/H1N1 human influenza pandemic. *Public Library of Science, Currents: Influenza*, doi:10.1371/currents.RRN1003.
- [18] Ramirez A, Capuano A, Wellman D, Leshner K, Setterquist S and Gray G, 2006. Preventing zoonotic influenza virus infection. *Emerging Infectious Disease*, 12, 996-1000.
- [19] Rao D, Chernyakhovsky A and Rao V, 2009. Modeling and analysis of global epidemiology of avian influenza. *Environmental Modelling and Software*, 24, 124-134.
- [20] Shinya K, Ebina M, Yamada S, Ono M, Kasai N and Kawaoka Y, 2006. Avian flu: influenza virus receptors in the human airway. *Nature*, 440, 435-436.
- [21] Smith G, Vijaykrishna D, Bahl J, Lycett S, Worobey M, Pybus O, Ma S, Cheung C, Raghwani J, Bhatt S, Peiris J, Guan Y and Rambaut A, 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature*, 459, 1122-1126.
- [22] Van Poucke S, Nicholls J, Nauwynck H and Van Reeth K, 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and associations with sialic acid distribution. *Virology Journal*, 7, 38, doi:10.1186/1743-422X-7-38.
- [23] Van Reeth K, 2007. Avian and swine influenza viruses: Our current understanding of the zoonotic risk. *Veterinary Research*, 38, 243-260.
- [24] Van Reeth K, 2009. Swine influenza - recent developments. Available from: <http://www.swinecast.com/dr-kristien-van-reeth-swine-influenza-recent-developments>
- [25] Van-Tam J and Sellwood C, 2010. Introduction to pandemic influenza. Cambridge University Press, Cambridge, UK, 217 pp.
- [26] Webster RG, 2002. Evolution and ecology of influenza A viruses. *Vaccine*, 20, S16-S20.
- [27] Zimmer S and Burke D, 2009. Historical perspective - emergence of influenza A (H1N1) viruses. *The New England Journal of Medicine*, 361, 279-285.

GLOSSARY

Allopatric cladogenesis: speciation that occurs when one species is separated into two groups by some physical barrier, resulting from, for example, climate change, a geological event, or a human-induced change in the environment. Once separated, each group may accumulate genetic changes that eventually may lead to new species being established.

Pandemic: An epidemic that becomes very widespread and affects a whole region, a continent, or the world. An epidemic is a sudden outbreak that affects more than the expected number of cases of disease occurring in a community or region during a given period of time.