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Interspecies and intraspecies transmission of influenza A viruses: viral, host and environmental factors

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Abstract

Influenza A viruses are enveloped viruses belonging to the family *Orthomyxoviridae* that encompasses four more genera: Influenza B, Influenza C, Isavirus and Thogotovirus. Type A viruses belong to the only genus that is highly infectious to a variety of mammalian and avian species. They are divided into subtypes based on two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). So far, 16 HA and 9 NA subtypes have been identified worldwide, making a possible combination of 144 subtypes between both proteins. Generally, individual viruses are host-specific, however, interspecies transmission of influenza A viruses is not uncommon. All of the HA and NA subtypes have been isolated from wild birds; however, infections in humans and other mammalian species are limited to a few subtypes. The replication of individual influenza A virus in a specific host is dependent on many factors including, viral proteins, host system and environmental conditions. In this review, the key findings that contribute to the transmission of influenza A viruses amongst different species are summarized.

Keywords: influenza, interspecies transmission, virus–host interaction

Introduction

Influenza A viruses are polymorphic with a diameter of approximately 100–120 nm. Like other *Orthomyxoviridae* viruses, their genome consists of eight negative sense, single stranded and segmented RNA strands. The eight RNA segments encode for at least 10 identified proteins recognized as: polymerase basic 1 (PB1 and PB1-F2 [expressed in some but not all influenza A viruses]), polymerase basic 2 (PB2), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M1 and M2) and non-structural (NS1/NEP and NS2) proteins. The two surface glycoproteins, HA and NA, play roles in virus entry and release from the host cells, respectively. The HA is a trimeric rod-shape molecule that binds to the host cell receptor and has the immunogenic epitope of the virus. It is inserted through its carboxy terminus to the viral membrane and holds the receptor binding domain (RBD) and the antigenic sites (AS) at the globular hydrophobic ends away from the viral surface (Palese and Shaw, 2007). For its full activity, the HA protein should be cleaved into two subunits recognized as HA1 and HA2 subunit molecules (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975).

The HA protein recognizes neuraminic acids (sialic acids (SA)) on the host cell surface and it is believed to be a host-specific recognition. Upon its binding to host cell receptor, the virus enters the host cell mainly through cathrin-mediated endocytosis (Matlin *et al.*, 1981; Skehel *et al.*, 1982; Bouvier and Palese, 2008). Viral RNA synthesis in the nucleus occurs through two events, replication and transcription. Both processes are catalyzed by the viral RNA polymerase complex made of three protein subunits: PB1, PB2 and PA (Neumann *et al.*,

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2004). The PB1 subunit plays a role in the sequential addition of nucleotides during the RNA chain elongation. It also possesses endonuclease activity which generates the capped primer required for mRNA synthesis (Hagen *et al.*, 1994; Cianci *et al.*, 1995; Neumann *et al.*, 2004). Capped primers originate from cellular pre-mRNA in an activity defined as 'cap-snatching' and involves PB2 cap-binding activity and PB1 endonuclease activity (Fechter *et al.*, 2003). No specific role has been assigned to the PA subunit; however, mutations in the PA gene impair replication and transcription (Fodor *et al.*, 2003; Huarte *et al.*, 2003). The two shortest mRNA encoded from segments 7 and 8 are further spliced in the nucleus. The unspliced form of mRNA from segment 7 encodes for M1 protein, while the spliced form encodes for M2 protein. Similarly, the unspliced mRNA of segment 8 produces NS1, while the spliced form produces NS2 protein (Palese and Shaw, 2007). The mRNAs are translated into proteins in the endoplasmic reticulum, folded and modified in the Golgi apparatus of the host cell cytoplasm. HA and NA proteins (envelope spike proteins) have apical sorting signal and become inserted in the lipid raft of the host cell. The rest of the proteins (non-envelope proteins) move to the nucleus for the formation of RNP complex with the vRNA (Basler, 2007; Bouvier and Palese, 2008). The export of RNPs from nucleus into the cytoplasm requires two viral proteins, M1 and NS2 (also known as nuclear export protein (NEP)).

Although the M1 protein is the key player in the budding of progeny viruses from infected cells, other proteins like HA and NA are also essential for this process (Gomez-Puertas *et al.*, 2000; Chen *et al.*, 2007). It is believed that both M1 and M2 proteins with the help of the cytoskeleton are needed to capture the RNPs at the site of assembly (Avalos *et al.*, 1997; McCown and Pekosz, 2005; Palese, 2007). The full component of eight RNA segments is required for correct assembly and packaging.

To prevent the aggregation of viruses at the release site, the enzymatic activity of the NA (tetramer) protein is needed to remove the SA linked to the glycoproteins at the cell membrane and the newly budded viral particles (Palese *et al.*, 1974). In addition to its role in cleaving SA, NA can sequester cellular plasminogen, which is then activated to plasmin, an enzyme that can cleave the HA protein without requiring multiple basic amino acids at the cleavage site (Goto *et al.*, 2001).

The adaptation of the influenza virus to replicate in a specific host is a multigenic trait. In this review, we summarize old and recent findings about the key determinants that control host range specificity of influenza A viruses.

Pathotypes and ecology of influenza A viruses

Type A influenza is the only genus of *Orthomyxoviridae* that has been shown to infect a vast variety of animals including humans, wild and domestic birds, swine, horses, seals, whales, canines, minks and others (Wright *et al.*, 2007). Infection with the influenza A virus results in a wide range of clinical outcomes, depending on virus strain, virus load, host species, host immunity and environmental factors.

Influenza in wild and domestic birds

Based on pathogenicity in chickens, influenza A viruses are classified into two main pathotypes: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (Alexander, 2007). Infection with LPAI (includes all subtypes) usually causes little or no disease, but it can sometimes result in high morbidity and low mortality. On the other hand, infection with HPAI viruses results in at least 75% mortality in the infected poultry and it is caused solely by some viruses of H5 and H7 subtypes (Swayne and Halverson, 2007). The HA protein of LPAI is characterized by a single arginine (basic amino acid) at the cleavage site and another basic amino acid at position 3 or 4 upstream from the cleavage site (depending on the virus subtype). Thus, the HA protein of LPAI viruses is limited to cleavage by extracellular proteases (trypsin-like) that are secreted by cells or bacteria at the site of infection (e.g. trachea and intestine). On the other hand, HPAI viruses possess multiple basic amino acids at the HA protein cleavage site, making them prone to cleavage by intracellular ubiquitous proteases, such as furin (Stieneke-Grober *et al.*, 1992; Vey *et al.*, 1992; Senne *et al.*, 1996).

Wild birds, especially *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls, terns and surfbirds), are the natural reservoirs for influenza A viruses. The most recent report showed that these viruses have been isolated from at least 105 wild bird species representing 26 families (Olsen *et al.*, 2006). In an older study performed by Stallknecht and Shane (1988), a virus isolation recovery rate of 10.9% (2317 of 21,318 sample) was observed in samples collected from wild birds. A 15.2% recovery rate was reported for *Anseriformes*, 2.9% for *Passeriformes* and 2.2% for *Charadriiformes*.

LPAI viruses of all subtypes have been isolated from wild birds in which they cause little or no disease. Few HPAI viruses have been isolated from wild birds, represented lately by HPAI H5N1 in Asia, Europe and Africa (Swayne and Halverson, 2007). Sharp *et al.* (1993) suggested that the waterfowls do not act as a reservoir for all avian influenza viruses and part of the influenza gene pool is maintained in shorebirds and gulls.

Infection of poultry with influenza A viruses has always been associated with the transmission of the viruses from wild birds (Alexander, 2007; Swayne and Halverson, 2007). Poultry infection with influenza A virus was first reported in Italy in 1878. The disease was first diagnosed as fowl cholera and the causative agent was not identified

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as influenza A virus until 1955 (Swayne and Halverson, 2007). Milder diseases (primarily respiratory disease) are caused by LPAI viruses and were first reported in the middle of the last century. The oldest existing LPAI virus (H10N7) was isolated in Germany from chickens in 1960 (Swayne and Halverson, 2007).

Chickens and turkeys are not considered natural reservoirs of influenza viruses; however, due to alterations in the natural ecosystems by humans (ways of raising and marketing poultry), new niches have been created for the occurrence and distribution of influenza infections amongst poultry (Swayne and Halverson, 2007).

Earlier and recent findings suggest that turkeys are more susceptible to influenza infections than chickens (Alexander, 2000; Pillai *et al.*, 2009). For example, the H7N2 LPAI virus isolated from Virginia in 2002 was more infectious in turkeys compared to chickens under laboratory conditions and was transmitted more easily amongst turkeys which also required lower doses to be infected (Swayne and Halverson, 2007). Influenza was reported in turkeys for the first time in 1963 in the USA (Alexander, 2000). Since then, various subtypes have been isolated from turkeys in many states within the USA. The isolation of these viruses was mostly reported from states that are heavily populated with turkeys and situated on migratory waterfowl flyways, such as California and Minnesota. In these states, outbreaks occurred every year since 1966, causing tremendous economic losses (Halverson *et al.*, 1998). In 1995, two major outbreaks were reported in turkeys in the USA. The first in Utah (H7N3) resulted in 40% mortality in young birds and was associated with co-infection with *Escherichia coli* or *Pasteurella multocida*. The other outbreak occurred in Minnesota (H9N2) causing economic losses estimated in millions of dollars in Minnesota alone (Halverson *et al.*, 1998). The elimination of outdoor raising of turkeys in the State of Minnesota in 1998 dramatically reduced the number of incidences of avian influenza.

Additionally, swine influenza viruses have been frequently isolated from turkeys in the USA since the early 1980s. The first report of swine influenza (H1N1) in turkeys was in 1981 (Mohan *et al.*, 1981). In 2003, a new form of swine influenza virus emerged in turkeys and was recognized as a triple reassortant (TR) H3N2 virus (Choi *et al.*, 2004). These viruses were first isolated from pigs in 1998 and then from turkeys in 2003. Infections with these viruses elicit no clinical signs, but are associated with a drastic drop or complete cessation in egg production in turkey breeder hens. In our *in vivo* experimental system, different H3N2 TR strains with high genetic similarity behaved differently in terms of transmission between both species (Yassine *et al.*, 2007). While some viruses were transmitted one or both ways between swine and turkeys, other viruses were not transmitted either way between the two species.

Despite their higher populations compared to domestic turkeys, primary influenza outbreak incidences in chickens are fewer compared to turkeys (Alexander, 2000). Major outbreaks in chickens in North America, like the Pennsylvania outbreak in 1983 and the Mexican outbreak in 1995, were due to HPAI viruses. Both outbreaks were very similar in their progress, where H5N2 was isolated as an LPAI virus prior to the outbreak, and mutated to a HPAI virus causing high mortality in the infected flocks (Alexander, 2000). On the other hand, the H7 subtype viruses were responsible for multiple outbreaks in poultry in the USA and other countries around the globe. From 1870 until the mid-20th century, fowl plague (HPAI caused by H7 subtype strains) occurred in many regions including several parts of Europe, North Africa, the Middle East, Asia, North and South America. Major recent outbreaks were recorded in Pakistan (H7N3; 1995–2003), the Netherlands (H7N7, 2003), Canada (H7N3, 2004) and North Korea (H7N7, 2005) (Alexander, 2000; CIDRAP, 2009a; Pasick *et al.*, 2009). Chickens were the primary host for the disease in all of these outbreaks (Swayne, 2008).

Between 1965 and 1966, chicken, turkeys and other avian species were diseased with clinical signs indistinguishable from those caused by fowl plague (H7 HPAI). Interestingly, viruses from these birds were not inhibited by antisera collected from fowl-plague recovered birds. The disease was first referred to as 'fowl plague-like' and was then identified as H5 HPAI (Swayne and Halverson, 2007; Swayne, 2008).

Since 1996, H5N1 has been the cause of the major ongoing pandemic of HPAI worldwide. This virus was first isolated from geese in 1996 in Guangdong Province, China (Swayne and Halverson, 2007). The virus then spread to infect various poultry species, and a broad range of non-poultry animals including humans (Sims and Brown, 2008). Between 2003 and 2004, outbreaks with H5N1 HPAI were reported almost concurrently in eight countries in South East Asia including China, Cambodia, Indonesia, Japan, Korea, Loa PDR, Thailand and Vietnam (Sims and Brown, 2008). The virus then spread over large portions of Asia, some countries in Europe and Africa. So far, the virus has evolved (genetic drift/genetic shift) to form at least 20 clades/subclades in the phylogenetic tree (Sims and Brown, 2008).

In addition to the above incidences of influenza in poultry, H9N2 LPAI has been a continuous problem in poultry since the mid-1990s worldwide. H9N2 viruses affected chickens, turkeys and domestic ducks in Germany, turkeys in the USA, ostriches in South Africa, pheasants in Ireland and chickens and quails in Italy, South East Asia and the Middle East (Alexander, 2007). Phylogenetic analysis of Asian-H9 subtype influenza isolated from poultry indicated wild bird-origin of the virus (Perez *et al.*, 2003). In Asia, H9N2 viruses have caused multiple outbreaks and established lineages in land-based poultry (Hossain *et al.*, 2008).

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Influenza in mammals

In contrast to birds and most mammalian species, humans are prone to infection with all three types of influenza viruses, A, B and C (95). Type C viruses are less common in human populations and cause milder disease compared to types A and B. Infections with type A and type B viruses result in mild to acute disease in human populations. Up to 142,000 hospitalizations are recorded in the USA alone every year as a result of influenza infection, with a mortality rate of ~1% (20,000–36,000) of all deaths in the USA (Thompson *et al.*, 2003, 2004; Subbaroa *et al.*, 2006).

Human influenza A viruses were first isolated in 1933. Historically, only viruses of H1, H2, H3, N1 and N2 subtypes have circulated widely in human populations (Subbaroa *et al.*, 2006). Three main pandemics occurred in the last century in humans, due to influenza A virus infections: Spanish influenza (1918; H1N1), Asian influenza (H2N2; 1957) and Hong Kong influenza (H3N2; 1968). The Spanish influenza was the most severe of all, resulting in deaths of 40–50 million people worldwide. Bacterial infections in the lungs were a prominent trait of fatal cases in the 1918 pandemic. Genetic analysis of H1N1 Spanish influenza viruses revealed avian-like genes with mutations for human adaptation (Taubenberger and Palese, 2006). Pandemics caused by H2N2 (1957) and H3N2 (1968) were less severe involving four million and one million deaths, respectively (Kilbourne, 2006). Genetic characterization revealed that both strains arose from reassortments between avian viruses and co-circulating human viruses. The H2N2 virus harbored HA, NA and PB1 of avian influenza and the rest of the genes were of circulating human H1N1 virus. On the other hand, the H3N2 virus harbored the HA and PB1 genes from avian influenza with the remaining genes from circulating H2N2 viruses (Subbaroa *et al.*, 2006).

For the last 15 years, the H5N1 HPAI virus represented one of the highest threats to human health worldwide. The first human disease caused by H5N1 was reported from Hong Kong in 1997, involving 18 cases, including 6 deaths (Peiris *et al.*, 2007). At the time of the preparation of this manuscript, at least 476 cases from 15 countries have been reported with H5N1 infection, resulting in the death of 283 people (WHO, 2009a). Seroepidemiological study performed after the 1997 outbreak in Hong Kong showed that some human infections with H5N1 could be asymptomatic or mildly symptomatic (Peiris *et al.*, 2007). Moreover, avian H7 and H9 subtype viruses have been transmitted to humans in Asia, Europe and North America. After the major outbreak of H7N7 in poultry in the Netherlands in 2003, 89 human cases were laboratory confirmed with the same subtype virus. Most of the infected humans developed conjunctivitis, few developed influenza-like illnesses and one veterinarian died from acute respiratory distress syndrome (ARDS) due to the infection (Koopmans *et al.*, 2004). Two people were also infected with H7N3 subtype virus after an outbreak in Canada in 2004, manifesting self-limited conjunctivitis and influenza illnesses (Tweed *et al.*, 2004; Belser *et al.*, 2009). Most of the above infections with the three subtypes (H5, H7 and H9) were due to direct contact of humans with infected poultry. Human to human transmission was suggested for three individuals in the Netherlands outbreak (Peiris *et al.*, 2007).

In late April 2009, WHO announced the emergence of a novel influenza in humans (WHO, 2009b). Considering the highly contagious nature of H1N1 subtype influenza virus and the wide geographical prevalence, the first pandemic of the 21st Century was declared on 11 June 2009. As of January 2010, more than 208 countries/territories have been confirmed with pandemic H1N1 (pH1N1) influenza cases in humans (WHO, 2010a). In the USA alone, the Center for Disease Control and Prevention (CDC) estimated 39–80 million infected Americans along with 8–16 thousand deaths with pH1N1 virus, between April and December of 2009 (CNN, 2010). Interestingly, pH1N1 influenza accounted for more than 90% of the specimens tested positive for influenza A virus worldwide (WHO, 2010b). The new pH1N1 influenza virus contains a unique combination of gene segments not previously identified elsewhere. Although the origin of the new H1N1 virus is unclear, the new virus is genetically most closely related to TR swine influenza viruses currently circulating in the pig population. In particular, NA and M genes of the virus are closely related to those of Eurasian lineage of swine influenza viruses and the remaining six genes (HA, NP, NS, PA, PB1 and PB2) are similar to North American lineage viruses (Gibbs *et al.*, 2009).

While Spanish and other influenza pandemics caused a fatality rate of >2.5% and <0.1%, respectively, infection with the novel swine-origin virus was less severe with a fatality rate of <0.05% (Taubenberger and Morens, 2006; CIDRAP, 2009b; Donaldson *et al.*, 2009). Analyzing the household transmission of pH1N1 virus in the USA, it was shown that the transmissibility of the 2009 H1N1 influenza virus in households is lower than that seen in past pandemics. It was also shown that most transmissions occur soon before or after the onset of symptoms in a case patient (Cauchemez *et al.*, 2009).

pH1N1 virus has infected species other than humans including pigs, turkeys, cats and dogs (Swayne *et al.*, 2009; Ariel Pereda *et al.*, 2010; AVMA, 2010; Sponseller *et al.*, 2010). Experimentally, pH1N1 replicates similarly compared to other swine influenza viruses in pigs. On the contrary, turkeys were resistant to a natural route of infection, although increased susceptibility was demonstrated using the intrauterine inoculation route.

Pigs are susceptible to infection with both human and avian influenza viruses and have been hypothesized to serve as the intermediate host for interspecies transmission of these viruses. H1 and H3 subtypes are the most common influenza subtypes in pigs worldwide, but sporadic infections with other subtype viruses have been recorded (Ninomiya *et al.*, 2002; Ma *et al.*, 2007; Yu *et al.*,

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2008). Influenza was initially recognized in pigs in 1918, in a temporal and spatial coincidence with Spanish flu in humans (Subbaroa *et al*., 2006). The first isolation of the virus (H1N1) was in 1930 and it was later recognized as classical H1N1 lineage of swine viruses (cH1N1). Recent research has indicated that swine and human viruses of 1918 were closely related to each other, both genetically and antigenically (Reid *et al*., 2001; Kilbourne, 2006). cH1N1 swine viruses in the USA remained genetically and antigenically stable until the 1990s, when variants of cH1N1 viruses were isolated (Vincent *et al*., 2006; Wright *et al*., 2007). In Europe, classical H1N1 viruses disappeared in the late 1970s with the introduction of avian-like viruses. Avian-like viruses reassorted with human viruses in pigs resulting in new viruses harboring human-like HA and NA genes and avian-like internal genes (Campitelli *et al*., 1997; Dunham *et al*., 2009).

Reassortants between swine and human viruses have been reported in more than one incident (e.g. Japan (H1N2, 1978) and Europe (H1N2, 1987)) (Wright *et al*., 2007). H1N2 viruses are still widely circulating with H1N1 and H3N2 subtypes in pigs in Europe. In the USA, a new form of reassortant viruses (H3N2) emerged in swine populations in 1998. These viruses harbored genes from human (HA, NA and PB1), avian (PB2 and PA) and swine (NP, M and NS) lineage viruses (Webby *et al*., 2000). Interestingly, HA and PB1 genes share the same origin (human lineage), in a similar pattern to the 1957 and 1968 human pandemic viruses (Kawaoka *et al*., 1989). Human virus genes in the H3N2 viruses were similar to those contained in human vaccine strains used in the mid-1990s (Olsen, 2002). Further reassortments occurred between H3N2 TR and cH1N1 viruses resulting in the emergence of H1N2 viruses. Since then, double and/or triple reassortants of H1N1, H1N2, H3N1 and H3N2 have been isolated from the swine population in the USA (Lekcharoensuk *et al*., 2006; Wright *et al*., 2007; Yassine *et al*., 2007). Interestingly, more than one of these viruses has been isolated from species other than swine, including humans and turkeys. H1N1 TR viruses have been isolated from humans in several locations in the USA including Ohio, Wisconsin and Texas (Olsen, 2002; Olsen *et al*., 2002; Gray *et al*., 2007; Newman *et al*., 2008; Schnirring, 2008). An H3N2 TR virus was also isolated from a child in Canada in 2007 (Robinson *et al*., 2007).

Additionally, pigs have been infected with avian influenza viruses worldwide. Influenza viruses of H4 and H9 subtypes have been isolated from pigs in Canada and China, respectively (Karasin *et al*., 2000; Cong *et al*., 2007). Although H5N1 influenza viruses were isolated from pigs (Shi *et al*., 2008; Zhu *et al*., 2008), inoculation and feeding domestic pigs with H5N1 viruses from different origins (human, chicken, duck and whooper swan) or virus contaminated feed did not result in a severe disease. Virus replication was restricted to the respiratory tract and tonsils of pigs, which is in contrast to mouse and ferret where some of the viruses were highly pathogenic and replicated systemically. Additionally, the virus replication level was remarkably low compared to the replication of swine influenza viruses (Lipatov *et al*., 2008).

As with human and swine diseases, equine influenza has been a problem for many years. Two main subtypes have been recognized in horses, equine-1 (H7N7) and equine-2 (H3N8) (Timoney, 1996). The disease is usually more associated with H3N8 viruses, with symptoms similar to those observed in human and swine influenza infections, including fever, coughing, loss of appetite and muscular soreness (Wright *et al*., 2007). H7N7 was first isolated in 1956 and the last confirmed outbreak was reported in 1979. On the other hand, H3N8 was first isolated in 1963 and is still widespread in horse populations. It caused major outbreaks in the USA (1963), China (1989) and widespread epidemics in Europe and around the world (Timoney, 1996; Wright *et al*., 2007).

Dogs and other canine species can be infected with influenza A viruses. Equine H3N8 viruses were isolated from dogs (greyhounds) with respiratory disease in Florida, suggesting interspecies transmission of these viruses (Crawford *et al*., 2005). Serologic studies indicated that H3N8 virus has spread to the general dog population (Crawford *et al*., 2005). Avian H3N2 influenza viruses were also isolated from dogs with severe respiratory disease in Korea, in 2007 (Song *et al*., 2008).

Cats (domestic cats, tigers and Owston civets) were shown to be infected with influenza A virus. H5N1 virus was isolated from all three species and probable virus transmission was reported amongst tigers (Thanawongnuwech *et al*., 2005). Pandemic H1N1 viruses were also isolated from dogs and cats with influenza-like illness in the USA and around the globe (oregonvma, 2010). In the USA, death was associated with some cat infections with the pandemic H1N1 influenza A virus.

Minks are also prone to infection with influenza viruses, naturally and experimentally. Both human and avian viruses can be transmitted to and infect minks. In 1985, a mink died in Sweden due to an infection with the avian-like H10N4 subtype. In addition to avian viruses, minks were also experimentally infected with human, swine and equine viruses and the viruses were transmitted to cage mates (Wright *et al*., 2007).

Finally, influenza viruses were isolated from marine mammals including seals and whales and were in most cases avian-like viruses, indicating a possible transmission from sea-shore birds and water fowls (Hinshaw *et al*., 1986; Nielsen *et al*., 2001).

Interspecies and intraspecies transmission of influenza A viruses

The transmission of influenza viruses amongst and between species is influenced by many viral, host and

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environmental factors. Below is a summary of the most important factors affecting the transmission of influenza viruses.

Viral factors

The role of surface glycoproteins

Influenza host-range restriction is a multigenic trait, where most of the genes play a role in influenza virus adaptation to a specific host. The availability of a large number of virus sequences and the utilization of reverse genetics, site-directed mutagenesis (SDM) and other molecular techniques permitted the identification at the molecular level of the determinants of host-range specificity of influenza viruses.

The HA protein is the major surface glycoprotein that mediates two important functions in virus replication: binding to the appropriate receptor on the host cell and fusion between viral and endosomal membranes. Four hundred to 500 trimeric HA spikes are found on each virus which enables simultaneous interaction of RBD with multiple copies of cell-surface receptors. The RBD that contains the SA binding residues is a shallow depression on the protein surface located on the membrane's distal end of the globular head (Matrosovich *et al.*, 2006b). Some residues at the RBD are rather conserved because of their essentiality in virus interaction with the receptor and others are variable within different subtypes and different lineage viruses. Residues 98, 131–134, 136–138, 153–155, 183–190, 194–195, 218–220, 224 and 226–228 have been postulated to be key amino acids that constitute or comprise the RBD. However, the exact location of the RBD depend on the individual HA subtype or specific strain. Amino acids at 134, 136 and 153 are preserved throughout the evolution of influenza viruses in different hosts. On the other hand, those at positions 190, 225, 226 and 228 are more variable and more prone to mutations. While human viruses usually have Asp190 (H1N1 and H3N2), Asp225 (H1N1), Leu/Ile/Val226 and Ser228 (H3N2 and H2N2), avian viruses harbor Glu190, Gly225, Gln226 and Gly228 at these positions (Matrosovich *et al.*, 2006b). In addition, avian viruses have conserved amino acids Ala138 and Leu194. Mutations at these positions could affect the binding of the avian viruses to their corresponding receptors. Interestingly, an H7N7 virus was isolated from domestic chickens, with a deletion in residues 221–228 of the HA molecule. Moreover, a laboratory generated mutant with deletion of residues 224–230 was still able to bind receptors and grow in embryonated chicken eggs (ECE) (Daniels *et al.*, 1987; Suarez *et al.*, 1999). This indicates a less critical role of residues at this site (220–230) for receptor binding, at least for some strains.

Generally, avian viruses are thought to preferentially bind to the *N*-acetylneuraminic acid α2,3-galactose (NeuAcα2,3Gal) form of SA receptors and human viruses preferentially bind to NeuAcα2,6Gal SA receptors. Hinshaw *et al.* (1983) were the first group to show the contribution of HA in determining host range specificity. They demonstrated that a reassortant between human (H3N2) and avian (H2N2) viruses, with HA being from human strain and the rest of the genes from the duck strain, failed to replicate in ducks. The introduction of double mutations (Leu226Gln and Ser228Gly) into the HA protein resulted in the replication of the above reassortant strain in duck intestine. A single Leu226Gln mutation was not sufficient to initiate replication in duck intestine. The Gln226 was found to be responsible for the binding to α2,3-linked SA and Gly228 enhanced the binding affinity (Naeve *et al.*, 1984; Vines *et al.*, 1998; Matrosovich *et al.*, 2000). Residues 228 and 229 were shown to be highly conserved in ducks and to a lesser extent residues 252 and 223 (Kawaoka *et al.*, 1988; Matrosovich *et al.*, 2006a).

Variations in receptor binding specificity were also observed within avian species themselves. Although gull viruses have higher binding affinity to α2,3-linked SA, few gull viruses were shown to infect and replicate in ducks. Interestingly, H13 subtype, which is the most prevalent subtype in gulls, has never been isolated from ducks (Sivanandan *et al.*, 1991; Laudert *et al.*, 1993).

Various subtypes isolated from chickens showed variability in receptor binding specificity. An H8N4 isolated from chicken in Ontario (1968) had duck-like receptor-binding specificity, while an H9N2 isolated from chicken in Wisconsin (1966) had human-like receptor specificity (Nobusawa *et al.*, 1991). The latter subtype (H9N2) has spread in many countries in Asia, Europe, Middle East and Africa since the early 1990s, affecting poultry production in all of these countries. Similar viruses were isolated from human and pigs in South East Asia, raising concerns about interspecies transmission of these viruses (Peiris *et al.*, 1999; Lu *et al.*, 2008; Yu *et al.*, 2008). Avian H9N2 viruses isolated from Hong Kong were shown to carry a human-like amino acid at residue 226 of the RBD (Leu instead of Gln) plus additional mutations at residues 183, 190 and 225 that are supposed to be conserved in avian viruses. These viruses showed an ability to bind human-like receptors (α2,6-linked SA) that also exist in chicken tracheal epithelial cells (Lin *et al.*, 2000; Matrosovich *et al.*, 2001; Ha *et al.*, 2002). Viruses isolated from pigs in China in 2006 also harbored Leu226, indicating a significant role played by this residue for the adaptation of avian H9N2 viruses in mammalian species (Guo *et al.*, 2004). In a recent study, using ferrets as a transmission model (Sorrell *et al.*, 2009), two mutations (at residues 226 and 198) occurred in the HA protein upon the adaptation of avian H9N2 in ferrets. Both mutations were shown to be required for transmission of the virus amongst ferrets. The same group (Wan and Perez, 2007) has previously shown that Leu226 on the RBD determines cell tropism and replication of H9N2 in human airway epithelial cells.

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The transmission of avian viruses to humans is not uncommon. Researchers speculate that an avian H1N1 virus with human adapted mutations was the cause of the Spanish influenza in 1918. Since 1996, the HPAI H5N1 virus has been isolated from humans in several countries over the globe. Few mutations at the RBD in both subtypes were shown to alter the receptor-binding specificity between α2,3 and α2,6-linked SA receptors. For the H1N1 (1918) viruses, Asp190 and Asp225 were important for binding of H1N1 (1918) virus to α2,6-linked SA receptors and also for the transmission of these viruses in the ferret model. A single mutation (Asp190Glu) decreased the ability of the virus to bind α2,6-linked SA receptors, while another single mutation (Asp225Gly) increased the ability of the virus to bind α2,3-linked SA receptors. Double mutations at the above two positions dramatically decreased the virus ability for binding human-like receptors, and affected transmission but not replication in the ferret model. Interestingly, swine H1N1 isolates from the 1930s expressed Asp190 at the RBD, emphasizing the role of Asp at this position for replication in mammals (Gamblin *et al.*, 2004).

Since 1996, researchers have been working to understand the mechanisms of the transmission of HPAI H5N1 from poultry to humans. Although the virus replicates efficiently in the infected humans, often leading to death, the virus has not yet adapted for human to human transmission. In 2003, H5N1 viruses were isolated from father and son in Hong Kong where the viruses had a replacement at residue 227 of the RBD. Such a mutation was shown in the same study to alter the receptor-binding specificity, where the human virus was able to bind both α2,3 and α2,6-liked SA receptors (Shinya *et al.*, 2005). A similar mutation (Ser227Asn) enabled an isolate from Vietnam-2004 to bind both α2,3 and α2,6-linked SA receptors (Stevens *et al.*, 2006).

In a study by Yamada *et al.* (2006), various avian and human isolates (H5N1) were analyzed for sequence similarity. Mutations were introduced based on the similarities/differences between isolates and receptor-binding specificity to either α2,3 and α2,6-liked SA receptors was evaluated. *In vitro* analysis revealed that when similar mutations were applied to viruses from different clades (I and II), variable results were obtained. Single mutations as Gln192Arg, Gly139Arg, Asn182Lys, or Asn193Lys, enabled a virus from clade I to switch binding specificity to α2,6-linked SA receptors. Double and triple mutants in residues 75, 123 and 167 (with or without Asn193Lys) were required to enhance binding to 2,6-linked SA receptors. On the other hand, only Gln192Arg and Asn193Lys mutations enabled a virus from clade II to support binding to α2,6-linked SA receptors. Other mutations, Gly139Arg and Asn182Lys, were not sufficient to enable binding to human receptors and abolished binding to α2,3-linked SA receptors. The specific structure/geometry of the HA molecule and the proximity/type of amino acid residues at the RBD, play a role in which mutations might be suitable for one strain, but not the other, in determining receptor-binding specificity.

In summary, different HA subtypes require various mutations at the RBD to switch binding specificity between α2,3 and α2,6-linked SA receptors. For H2 and H3, Gln226Leu and Gly228Ser are required to switch from avian- to human-binding specificity. In H1, Glu190Asp is critical for adaptation to human-like receptor binding. In H5, contradictory findings were recorded depending on the type of strain used in each study; however, mutations close to those in H3 might play a role in elevating the binding ability to α2,6-linked SA without abolishing the binding ability to avian-like receptors.

Receptor affinity can also be modulated by glycosylation and sialylation of the HA head domain. Human H1 viruses show hyperglycosylation compared to viruses from aquatic birds and pigs (Inkster *et al.*, 1993). Hyperglycosylation of the HA head was associated with increased virulence of H7 subtype viruses in chickens in Australia and Italy. It has been suggested that hyperglycosylation of HA, combined with compensating NA stalk deletion, modifies the progenitor aquatic bird virus prior to the development of virulence during virus evolution in chickens (Perdue *et al.*, 1995; Matrosovich *et al.*, 1999; Banks *et al.*, 2000, 2001; Baigent and McCauley, 2003).

The role played by the NA protein in determining host-range specificity is minor compared to that of the HA molecule. However, functional compatibility of receptor-binding properties with NA cleavage specificity, along with the stalk length of the NA protein, is critical. As is the case with the HA protein, the NA protein can specifically cleave SA in the form of α2,3 and/or α2,6 linkage. The mechanism of this specificity is not well characterized, but in one study it was shown that the specificity of the NA (N8) is associated with the amino acid at position 275, as well as glycosylation at residue 144 (Saito and Kawano, 1997). As another form of compatibility between HA and NA proteins, a reduction in NA specific activity is required in human viruses to maintain optimal balance with the HA protein that shows less affinity to its receptor compared to avian viruses (Baigent and McCauley, 2003).

Changes in the NA protein upon the transmission of influenza virus to a new host usually occurs to accommodate compatibility with the HA protein. For example, many of the NA stalk deletions have been recorded upon the transmission of wild bird viruses (especially duck) into chickens. This has been recorded for three major subtypes (H5, H7 and H9) that cause disease in chickens (Banks *et al.*, 2001; Sorrell and Perez, 2007; Steensels *et al.*, 2007; Hossain *et al.*, 2008). Types of gangliosides (with short and long sugar chains) that are distributed in the guts of chicken and ducks, and to which influenza viruses bind, may play a role in NA proteins acquiring stalk deletions when the virus is transmitted from ducks to chickens. NA stalk deletion was also observed in a swine H3N2 TR isolate from North Carolina in 2003 (Yassine *et al.*, 2008). A study by Luo *et al.* (1993) demonstrated

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that deletions up to 28 amino acids and insertions up to 41 amino acids of the NA stalk protein did not abolish the formation of progeny viruses *in vivo*. In a separate study (Mitnaul *et al.*, 2000), a human mutant influenza virus with a 24-amino acid NA stalk deletion, was shown to replicate like the wild-type strain in MDCK cells but not in ECE. Upon passaging several times in ECE, the virus acquired competence in two mechanisms: some viruses acquired insertions in the NA stalk from PB1, PB2, or NP protein, in a rare RNA/RNA recombination process, while others expressed mutations in the HA protein, affecting its binding to SA-linked receptors. Both mechanisms of adaptation require interaction between the NA and HA proteins, emphasizing balanced HA/NA activities for an efficient replication of influenza A viruses. On the other hand, the treatment of infected individuals with NA inhibitors has resulted in multiple mutations in the NA protein (e.g. Glu119V, Arg292Lys and His274Tyr) (Herlocher *et al.*, 2002; Ives *et al.*, 2002). Such mutations (especially Arg292Lys mutation) significantly compromised virus infectivity, pathogenicity and transmissibility (Herlocher *et al.*, 2002).

The role of ribonucleoproteins (RNP)

Contribution of RNPs in determining host-range specificity is maintained through three main mechanisms: (i) increasing genetic diversity from which variants with high adaptation and transmission potential are selected in the new host; (ii) proper interaction with host proteins, and thus generation of a suitable environment for efficient virus replication; and (iii) generation of escape mutants to avoid the innate immune system of the new host.

As is the case for the surface glycoproteins, adaptation through RNP occurs either directly in a new host, or in an intermediate host between the donor and recipient species (Naffakh *et al.*, 2008). Genetic analysis for the NP protein of the 1918 Spanish influenza revealed that its sequence is divergent from that of the wild bird viruses, indicating that the virus (H1N1) evolved for a long time in an intermediate host (distinct from wild birds) before emerging in the human population. In contrast, when a new PB1 gene was introduced (along with HA and NA genes) to the existing human virus genetic background by genetic reassortment in the 1957 (H2N2) and 1968 (H3N2) pandemics, its sequence was closer to that of avian viruses than the concurrent circulating human (H1N1) viruses (Naffakh *et al.*, 2008). Generally, the PB2, PA and NP proteins seem to share similar evolutionary pathways, suggesting co-evolution due to strong physical and functional interaction with each other (Naffakh *et al.*, 2008).

Out of 52 host-associated genetic signatures identified throughout the influenza viral genome (Chen *et al.*, 2006), 35 are linked with the RNP (2, 8, 10 and 15 for PB1, PB2, PA and NP, respectively). The contribution of RNPs in determining the host range of influenza virus was first shown in 1977 (Almond, 1977). Generating reassortant viruses using the traditional plaque assay method, it was revealed that the extended host range for one strain was conferred on the PB2 segment. Utilizing the same technique, reassortants were generated between human and avian viruses to identify genes and their amino acids' roles in replication of influenza viruses in different host systems. It was shown that the HA and NA genes alone (of human virus lineage) did not confer good replication of the virus in squirrel monkeys if the other six genes were of avian origin (Naffakh *et al.*, 2008). Later on, it was shown that the most significant genetic signature for host adaptation is located at residue 627 of PB2.

In human viruses, Lys is usually encoded at that residue, while most avian viruses have Glu at this position. Although the PB2 was of avian origin in the 1918 Spanish influenza virus, Lys was recognized at the PB2-627 residue (Taubenberger *et al.*, 2005). Such substitution was also observed in many of the human H5N1 isolates, where 23 out of 43 analyzed isolates had this substitution. Interestingly, Lys was also observed in H5N1 isolates from tigers in Thailand, but not in raccoon dog isolates from China which maintained avian-like Glu at this position (Maines *et al.*, 2005; Amonsin *et al.*, 2006; Naffakh *et al.*, 2008; Le *et al.*, 2009; Qi *et al.*, 2009). Moreover, during the H7N7 virus outbreak in the Netherlands in 2003, the same Glu627Lys substitution was found in the virus isolated from humans with fatal pneumonia, but not with cases of conjunctivitis in the same outbreak (Fouchier *et al.*, 2004). On the other hand, swine and equine isolates of avian-like lineages retained Glu at this position, suggesting less selective pressure for this residue in both species (Shinya *et al.*, 2007). The 2009 pandemic H1N1, which is thought to have originated from the swine influenza virus, contains avian-like residue at this position (Chen and Shih, 2009). In support of these findings, Glu627Lys substitution in PB2 gene of swine-origin H3N2 TR virus did not affect its replication in primary epithelial cells generated from pig and human tracheas (unpublished observations).

When PB2 from a duck strain (A/Mallard/78) was introduced to a human strain (A/Los Angeles/78), the reassortant had restricted growth in MDCK cells and squirrel monkeys. Revertants from the reassorted strain that were able to replicate in both systems showed a single substitution (Glu627Lys) that was responsible for the extended host range phenotype (Subbarao *et al.*, 1993). Using the ferret model to study influenza transmission, Lys at position 627 of PB2 protein was necessary and sufficient for respiratory droplet transmission, but not required for direct contact transmission, of 1918 H1N1 virus amongst ferrets (Van Hoeven *et al.*, 2009). Studying the pathogenesis of H5N1 (A/Vietnam/1203/04) and H7N7 (A/Netherlands/219/03) in a mouse model, it was shown that the PB2 gene alone, with or without other genes, contributed to virulence of the above two strains in mice. In both cases, substitution of Glu with that of Lys determined the outcome of infection in mice. However,

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the presence of either Glu or Lys at PB2-627 does not strictly correlate with the severity of human infection with H5N1 (Gao *et al.*, 1999).

In addition to its role in virulence and host range adaptation, PB2-627 plays a role in tissue tropism of the virus. Lys at position 627 of the PB2 protein enabled H5N1 virus to replicate in nasal turbinates and lungs of infected mice, whereas viruses with Glu at this position replicated poorly in nasal turbinates.

Human viruses with Lys at PB2-627 replicate well at 33°C, while avian viruses with the same substitution replicate poorly at the above temperature. Taking into account that avian viruses replicate in the intestinal tract of birds with a body temperature of 41°C, cold sensitivity of the polymerase proteins might limit the growth of avian viruses in the upper respiratory tract of humans and thus limit human-to-human transmission (Naffakh *et al.*, 2008).

Another remarkable residue to support the adaptation of avian influenza virus in humans is amino acid 375 of PB1. While Asn exists at this position in most avian species, most human viruses have Ser at this position. This substitution was the only conserved mutation observed in the PB1 gene (avian origin) in viruses from three pandemics in the last century (Kawaoka *et al.*, 1989; Taubenberger *et al.*, 2005). Additionally, a Gly375Ser modification was observed upon introduction of swine viruses to human (Kawaoka *et al.*, 1989). Nevertheless, PB1-375 cannot be classified as a genetic signature, where some human H3N2 viruses have Asn at this position, and Ser-375 is found in a considerable number of avian isolates (Taubenberger *et al.*, 2005).

In addition to those specific amino acid residues mentioned above, PB2-701, PB2-714, PA-97, PA-615, PA-624 and NP-319 have been associated with high replication and pathogenicity of a H7N7 (A/Seal/Massachussets/1/80) and many H5N1 isolates in mice and humans. The role played by these residues in host-range determinacy is more significant in combination with Glu627lys substitution (Naffakh *et al.*, 2008). In a recent study by Steel *et al.* (2009), it was shown that PB2-701Asn could compensate for the reduced transmission of two human viruses (H3N2 and H5N1) harboring avian-like PB2-627(Glu) in the guinea pig model. In support of this study, Gao *et al.* (2009) showed in a more recent publication that Asn at position 701 in the PB2 protein was a prerequisite but not sufficient for duck strain transmission in guinea pigs. Another mutation (Thr160Ala) in the HA molecule was also required to sustain the avian virus transmission in a guinea pig model. This mutation in the HA protein, which results in loss of glycosylation at 158–160 position, was responsible for HA binding to sialylated glycans and was critical for H5N1 virus transmission in guinea pigs (Gao *et al.*, 2009).

Although no specific role has been assigned to PA protein in the RNP complex, several studies have demonstrated its potential role in influenza virus adaptation to a new host. Serial passage of an LPAI H5N1 strain in mice resulted in non-conserved mutations in six viral genes. Of these, Thr to Iso substitution at position 97 of the PA protein played a key role in determining the enhanced virulence of the adapted strain in mice. Such mutation was also shown to enhance polymerase activity, and thus virus replication, in mammalian but not avian cells (Song *et al.*, 2009).

The molecular mechanisms involved in the above actions are still not fully understood; however, interactions amongst viral and host proteins play a critical role in viral adaptation and pathogenesis in the different host systems. For example, the crystal structure of the C-terminus domain of PB2 protein complexed with human importin α5 showed how the last 20 residues unfold to permit binding to the importin factor. The domain contains three surface residues (including Asp701) implicated in adaptation from avian to mammalian hosts (Tarendeau *et al.*, 2007). In a more recent study, adaptive mutations in PB2 (Asp701Asn) and in NP (Asn319Lys) enhanced the binding of these proteins to importin α1 in mammalian cells. This paralleled increased transport of PB2 and NP to the nucleus of the mammalian cells (Gabriel *et al.*, 2008). Tarendeau *et al.* (2008) demonstrated that Lys627Glu mutated domain did not show any structural difference, but a change in charge disrupted a striking basic patch on the domain. RNPs derived from avian viruses showed low levels of polymerase activity in human cells as compared to their counterparts of human lineage viruses. Such observations were associated with reduced binding of NP to PB1–PB2–PA complex and to the PB2 alone. Both deficiencies were restored upon introducing Lys to PB2-627 residue, suggesting a pivotal role of PB2 in molecular interactions of viral NP and other cellular proteins (Labadie *et al.*, 2007).

The roles of other internal proteins in host-range specificity are limited compared to those mentioned above. M protein of avian viruses was shown to restrict the replication of avian H2N2 and H3N8 in squirrel monkeys and pigs, respectively. Sequence analysis showed at least 10 sites with amino acids specific for avian and human influenza strains, but which of these contribute to host-range restriction is not known (Buckler-White *et al.*, 1986; Murphy *et al.*, 1989; Kida *et al.*, 1994). In a 2008 study at the CDC, it was shown that the replacement of M protein in an avian virus (H5N1) with that of a human virus (H3N2), not only diminished virus replication in mice, but also altered the tropism of the virus in the infected mice (Chen *et al.*, 2008). This might indicate a possible role of M protein in functional compatibility of surface glycoproteins (HA and NA) in influenza A viruses.

Determinants of pathogenicity have been identified for NS1 and PB1-F2 proteins, as an interferon antagonist and inducer of apoptosis, respectively. How both proteins contribute to host restriction is not fully understood (Neumann and Kawaoka, 2006; Conenello *et al.*, 2007). It is speculated that differences in NS sequences might

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influence their activities in different host systems. While most influenza viruses are sensitive to interferon (IFN) and tumour necrosis factor (TNF)-α, most HPAI H5N1 isolates from human and birds expressed resistance to both cytokines in porcine lung cells. This was associated with Glu at residue 92 of the NS1 protein (Seo *et al.*, 2002).

Host factors

Host receptors

Influenza replication in a specific host cell starts at the interaction of virus HA protein with the host cell receptors. Specific receptors for influenza viruses are still not well characterized; however, SA have been identified as receptor determinants for influenza viruses. SA are a family of nine-carbon acid sugars that occupy terminal positions on oligosaccharide chains of glycolipids and glycoproteins, forming exclusively α-glycosidic linkage (Matrosovich *et al.*, 2006b). More than 40 elements have been identified in this family which include two main species: *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). The Neu5Ac is the most common form and the biosynthetic precursor of other SA. Neu5Gc is found in all mammals except humans. Generally, SA are bound in the form of α2-3- or α2-6-linkage to the D-galactose (Gal), *N*-acetyl-D-galactosamine (GalNAc) and *N*-acetyl-D-glucosamine (GlcNAc) of the penultimate sugar (Matrosovich *et al.*, 2006b).

The distribution of these receptors varies between species and in the different organs of the same species. The use of linkage-specific lectins, like *Maackia amurensis* (MAA, specific for α2-3-linkage) and *Sambucus nigra* (SNA, specific for α2-6-linkage), has enabled the study of distribution of SA receptors in different species. Unfortunately, different studies have shown conflicting results due to the use of different lectins manufactured by different companies. Nevertheless, interesting findings have been reported in the last few years regarding the distribution of SA in different species. In a recent study (Kuchipudi *et al.*, 2009), Pekin ducks showed differential expression of receptors in different organs. α2-3-linked SA was exclusively expressed in the intestine, supporting earlier findings. Although tracheal epithelial cells were shown to express the α2-6-linkage of SA, their ratio was very low compared to that of α2-3-linkage (1 : 20). Interestingly, other organs (vascular endothelium and tubular cells in the kidney, endocardium and alveolar cells) were shown to express substantial amounts of both receptors. Like in ducks, chickens expressed mainly α2-3-linked SA on their intestinal wall; however, α2-6-linked SA were dominant in the tracheal epithelial cells in a ratio of 10 : 1 to the α2-3-linked form of SA. They also expressed both receptors in various amounts in different organs, with a dominance of α2-6-linkage in the kidney vascular epithelial cells. In turkeys, both types of receptors are expressed on tracheal cells in almost equal amounts, whereas the α2-3-linkage is dominant on turkey intestinal cells (Pillai *et al.*, 2009). Examining quail as an intermediate host for influenza zoonotic transmission, Wan and Perez (2006) reported the abundance of α2-6 as well as α2-3-linked SA receptors in both the trachea and intestine.

Humans, on the other hand, are known to express α2-6-linkages on tracheal epithelial cells. Recent findings (Nicholls *et al.*, 2007) have shown that α2-3-linked SA are also expressed on alveolar pneumocytes, which could explain the susceptibility of humans to H5N1 HPAI viruses, where such viruses replicated *ex vivo* in human lungs (Nelli *et al.*, 2010; Nicholls *et al.*, 2007; Chan *et al.*, 2009). Moreover, humans express α2-3-linked SA receptors on their ocular and lacrimal duct epithelial cells, which could explain the disease of conjunctivitis in many patients infected with avian influenza viruses, specifically H7 and H9 subtypes (Olofsson *et al.*, 2005).

Pigs express substantial amounts of both α2-3-Gal- and α2-6-Gal-linked SA receptors on their tracheal epithelial cells (Nelli *et al.*, 2010). In addition, pigs have been hypothesized to serve as a 'mixing vessel' where both avian and human viruses can co-infect epithelial cells, resulting in the generation of reassortant virus. Though pigs express both types of receptors, avian-like swine viruses showed a shift in receptor binding specificity over time. Until 1984, viruses isolated from pigs in Europe recognized both types of receptors, where those isolated after 1985 were strictly binding Neu5Ac(α2-6)Gal-linked receptors. Ser142Leu mutation was shown to contribute to the loss of binding to Neu5Ac(α2-3)Gal-linked receptors (Ito *et al.*, 1998). In a further study, Glu190Asp and Gly225Glu were shown to be likely possible for initial changes in receptor-binding specificity (Matrosovich *et al.*, 2000). Although still not proven experimentally, consistent results from two reports suggest that swine H1 and H3 viruses bind Neu5Gc not Neu5Ac receptor types (Matrosovich *et al.*, 2006b).

Equine (H3 and H7 subtype) and seal (H7 subtype) influenza viruses are more like avian viruses than mammalian viruses. H3 equine viruses have stricter receptor specificity compared to their counterparts from duck origin. They only agglutinate erythrocytes with Neu5Ac(α2-3)Gal-terminated receptors, whereas some duck viruses can agglutinate Neu5Ac(α2-6)Gal-containing erythrocytes. This was supported by the finding that horses express solely α2-3-linked SA receptors on their tracheal wall (Suzuki *et al.*, 2000). Influenza viruses isolated from seals are mostly of avian origin, but some of these display a lower binding affinity to Neu5Ac(α2-3)Gal-containing receptors without increase in binding affinity towards Neu5Ac(α2-6)Gal (Matrosovich *et al.*, 2006b). Dogs are also susceptible to influenza infection of avian (H5) and equine (H3) origin. α2-3-linked SA were shown to be present in dogs'

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trachea (Maas *et al*., 2007), which supports the susceptibility of dogs to both avian and equine influenza viruses.

The actual virus binding to its receptor is greatly affected by many factors that control the chemistry of binding between molecules. Human viruses bind to Neu5Ac(α2-6)Gal(β1-4)GlcNAc (6′SLN) more strongly than to Neu5Ac(α2-6)Gal(β1-4)Glc (6′SL). In contrast, avian viruses bind to both forms, 3′SL and 3′SLN (Mochalova *et al*., 2003). Avian viruses bind strongly to Neu5Ac rather than the Neu5Gc (Ito *et al*., 2000). Moreover, duck and shore bird viruses bind with greater affinity to Neu5Ac(α2-3)Gal(β1-3)GlcNAc containing receptors than Neu5Ac(α2-3)Gal(β1-4)GlcNAc. Chicken viruses bind more strongly to gangliosides with longer sugar chains compared to duck viruses which bind strongly to those with short sugar chains.

In summary, influenza virus binding to its receptor(s) on the host cell is a complex interaction including several factors on both sides of the reaction. There are currently more questions than answers to explain the specificity of binding of influenza viruses to host cell receptors. Further investigations is needed on the biological, chemical and physical aspects of the virus binding, to understand this process better.

Host immune system

Immunity against influenza virus in a specific host is determined by many factors including the presence of natural barriers that prevent virus replication, vaccination or pre-infection of the host with homologous and/or heterologous virus and genetic makeup of the host.

The respiratory tract secretions in Chimpanzees contain mucins that can bind specifically to influenza viruses and prevent the virus entry to the airway epithelial cells. This could explain the reasons for relative resistance of Chimpanzees to experimental respiratory exposure to human influenza viruses (Kuiken *et al*., 2006).

In birds, influenza virus infection can directly affect the immune response, but the insights about the roles of the specific innate and adaptive immune response mediators, such as the Mx protein, interferons and cytokines remain limited (Suarez and Schultz-Cherry, 2000). Wild ducks are the principle reservoir of influenza A viruses that can be transmitted to domestic poultry and mammals including humans (Kim *et al*., 2009). Further evidence revealed that the intestinal tract of ducks (crypts of Lieberkuhn in the large intestine) is the major replication site of influenza viruses (Kida *et al*., 1980). The species diversity of ducks may also play an important role in the pathogenicity of influenza viruses (Kim *et al*., 2009). It is noteworthy that a lack of adequate specific secondary immune responses in ducks following re-infection, may be responsible for ducks being susceptible to all influenza subtypes, and thus serve as a potential reservoir (Kida *et al*., 1980). In one exhaustive study on influenza viral pathogenesis and immune responses in 2-week-old chickens, turkeys, quails and ducks infected with eight influenza viruses of H5 subtype (by intramuscular, intranasal and contact routes), six of the viruses were highly pathogenic for chickens and turkeys, but only two viruses produced consistent serological responses in ducks (Alexander *et al*., 1986). This study confirmed the contribution of host factors within and between bird species in pathogenicity as well as transmission of influenza viruses.

Because influenza is an acute infection, cell-mediated immune responses through anti-influenza specific cytotoxic lymphocytes often fail to provide adequate protection, suggesting important roles for the innate and humoral immune responses in effective viral clearance. Most of the available information regarding immune responses to influenza viruses has been based on experiments in inbred mice, which many times are not relevant in other species.

In a recent study, infection of several laboratory inbred mice with H1N1 (PR8) and H7N7 (SC35M) viruses resulted in different weight loss kinetics and survival rate after infection (Srivastava *et al*., 2009). Additionally, the inbred mouse strains (C57BL/6J, DBA/2J, FVB/NJ, CBA/J, BALB/cByJ, (C57BL/6J×DBA/2J) F1, DBA/2NHsd and A/JOlaHsD (A/J)) showed distinct susceptibility (LD_{50}) to infection and expressed differential virus replication titers and cytokine production upon infection. The mechanisms for such variations have not been identified and similar studies should be done in other species. It is worth mentioning that two of the mice strains were deficient for *Mx1*, a major effector of interferon response to inhibit virus replication, and the other strains carried mutated *Mx1*. Whether these variations alone or together with other elements explain the above findings requires further investigation (Srivastava *et al*., 2009).

Mx proteins are GTPases that are expressed in the nucleus and cytoplasm of rodent cells, but mostly in the cytoplasm of other vertebrate species. The expression of Mx protein in mice was associated with a high degree of resistance to influenza infections, *in vivo* and *in vitro* (Haller *et al*., 2007). The antiviral activity of Mx proteins is reported in many species, including mammals, birds and fishes. They are polymorphic in most species, and thus, they have different modes of action in different species. Their mechanism of action is not fully understood; however, it is suggested that Mx proteins bind to essential viral components (mostly NP) during virus replication and block their function (Haller *et al*., 2007). In an earlier study, the expression of chicken and duck *Mx* genes in avian and mammalian cell lines (including mouse 3T3 cells) demonstrated no antiviral activity for several viruses including influenza. The *Mx* of chicken in that study was cloned from the White Leghorn breed in Germany (Bernasconi *et al*., 1995; Ko *et al*., 2002). In a later study, Ko *et al*. (2002) demonstrated the existence of a highly polymorphic *Mx* gene in different chicken breeds. Some Mx proteins expressed resistance to influenza and other

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viruses, and such activity was associated with residue 631 of the Mx protein. On the other hand, based on the above findings, duck Mx protein lacks antiviral activity. A comparison study like the one performed in chickens should be done on ducks before drawing any conclusions.

The duck immune system is quite different from that of chickens and other poultry species. Ducks have been reported to produce poor antibody responses and to express low HI antibody activity to natural and experimental infection with avian influenza viruses. The inability of ducks to produce the hemagglutinating antibody is also associated with other deficiencies in duck antibodies, including precipitation, complement activation and opsonization. The presence of only two constant regions in the heavy chain of the smaller IgY form in ducks, probably eliminates the functions associated with Fc region of the antibody molecule, including hemagglutination (Suarez and Schultz-Cherry, 2000). In one recent study, duck and chicken peripheral blood mononuclear cells expressed distinct levels of cytokines upon infection with H11N9 LPAI (Adams *et al.*, 2009). How such a difference in host response between the two species is related to the subclinical and persistent infections observed in ducks is something to be further investigated.

Influenza viruses from wild birds (including ducks) show a lower rate of evolution compared to those from poultry and mammals (Suarez, 2000). This might be explained by the difference in immune pressure (lesser in ducks compared to chickens and humans) exerted on the replicating viruses in different hosts. When the virus jumps from one species to another (example from ducks to chickens) it becomes more prone to mutations, and thus, antigenic drift might result in more transmissible and pathogenic viruses. On the other hand, the pre-existing immunity in the host due to prior immunization or natural infection might protect the host against clinical signs from LPAI or HPAI virus infection, but not necessarily against viral shedding. In such situations, there is a probability of generation of many mutants in the shed material (quasispecies), with potentially different behavioral characteristics than the parental strain.

Environmental factors

In addition to host- and virus-related factors, the transmission of influenza viruses depends strongly on environmental conditions. These include virus load (in air and on fomites), mode of transmission, stability of the virus in nature, host behavior, social organizations and weather conditions. Three modes of transmission have been investigated: droplet, airborne and contact transmission. The first requires direct spray of large droplets via sneezing or coughing onto mucosal surfaces of a susceptible host. Airborne transmission occurs through droplet nuclei (<10 μm), where such small droplets can remain airborne for several minutes and can reach the alveolar region of the host. Contact transmission, on the other hand, occurs indirectly through secretions/fecal droplets on fomites, or directly through physical contact between the donor and the recipient host. Which of the above modes is responsible for most influenza virus transmission remains highly controversial. However, the virus inactivation rate in the three modes, which depends on the surrounding environmental conditions, is the key player for virus transmission (Weber and Stilianakis, 2008b).

Ducks, geese and shorebirds are the natural habitat of the virus, and they shed large amounts of virus in their feces. Several studies have evaluated virus survival rates in water (in laboratory and nature) of different salinity, pH and temperature. Expectedly, the virus infectivity rate was inversely proportional to salinity and pH in water. In one study, the virus was isolated after 100 days of inoculation (10^6 $TCID_{50}$) into water of 17°C, 0 ppt and pH 8.2 (Stallknecht *et al.*, 1990). Zhang *et al.* (2006) isolated influenza virus genes from Siberian lake ice, and it was assumed that the virus can be preserved in the ice in between the migration seasons. Interestingly, LPAI viruses showed higher survival rates than HPAI viruses in water, contradicting the claim that high virulence should be positively correlated with durability outside the host (Walther and Ewald, 2004; Weber and Stilianakis, 2008a). Lakes, ponds and pond sediments could act as a reservoir of influenza viruses, where the virus can survive for several days in such environments. This would enable the transmission of influenza viruses amongst wild birds in migration seasons and to commercial poultry in proximity to such water resources. In 2007, an outbreak occurred on a multiple age broiler breeder operation in Saskatchewan, Canada. The use of water from a nearby lake where wild aquatic birds have been observed was linked to the emergence of the H7N3 outbreak (Berhane *et al.*, 2009).

Sneezing and coughing produce droplet particles up to 2000 μm in size. For the most part, expelled droplets are too large (>200 μm) and settle down quickly (<60 cm), and thus, not available for inhalation. In general, the settling time of spherical particles of unit density is: 10 s for 100 μm (diameter), 4 min for 20 μm, 17 min for 10 μm, 62 min for 5 μm and those less than 3 μm essentially do not settle (Tellier, 2006). Hence, particles less than 10 μm can be transmitted by aerosol and are easily respired and can enter the alveolar region of the host.

Infectivity through droplet (large droplets and droplet nuclei) transmission depends on the region of deposition and the minimum number of viruses required to initiate an infection. In humans, the nasal infectious dose (ID_{50}) is estimated at 100–1000 $TCID_{50}$ whereas airborne ID_{50} is in the range of 0.6–3 $TCID_{50}$. One $TCID_{50}$ corresponds to 100–650 virion particles, and droplets between 1 and 10 μm can hold between 10^3 and 10^7 virions. This indicates that a dose of $\sim$0.6 $TCID_{50}$ can fit into one

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droplet that is able to infect the alveolar region of the host (Weber and Stilianakis, 2008a). To an extent, this could explain the ability of HPAI H5N1 influenza viruses to infect humans, where avian-like receptors (α2,3-linked SA) are expressed in the human lungs. This is taking into consideration that most of the human infections were of those having close contact with infected poultry, and the virus is usually shed in high titers from nasal secretions, fecal droplets and feather follicles (Mumford *et al*., 2007). Aerosol transmission of HPAI H5N1 was also demonstrated in the laboratory between geese and quails, but not between geese and chickens (Webster *et al*., 2002).

Airborne and droplet transmissions, however, are highly affected by temperature, relative humidity (RH), sunlight UV radiation and open air factors (OAF). Most studies indicate a higher survival rate of influenza virus at lower RH. In an early study, the maximum survival time varied between 1 h at 80% RH to 24 h at 20% RH (Loosli *et al*., 1943). Although these findings were supported by recent research (Lowen *et al*., 2007), it contradicts observations from more than one area in the globe. In some areas in India, Senegal and Brazil, peak influenza activity coincides with periods of high rain and humidity (Weber and Stilianakis, 2008b). This implies that outbreaks or seasonal influenza are not exclusively controlled by RH, but by many other factors as indicated above. Using the guinea pig as a host model, it was shown that cold (5°C) and dry (20–35% RH) conditions favored the transmission of influenza virus in an indoor setting. Additionally, infected guinea pigs at 4°C shed virus for 40 more hours at 5°C compared to 20°C. The last observation was not linked to impairment of the immune system between groups, but was related to the slow speed of mucociliary (physical barrier) clearance of the virus at 4°C and thus its spread in the respiratory tract (Lowen *et al*., 2007). In another study by the same group, a high temperature of 30°C was shown to inhibit aerosol transmission at different RH (20–80%); however, contact transmission was equally efficient at 20°C and 30°C (Lowen *et al*., 2008). In another recent study, it was shown that absolute humidity (AH) rather than RH modulates influenza survival, transmission and seasonality. However, the report was based on the analysis of previously published observations and needs validation through laboratory and epidemiological studies (Shaman and Kohn, 2009). The sun's UV radiation and OAF are other contributors to virus inactivation in aerosol state. In areas of higher latitudes, winter UV-radiation rates were shown to be low and allowed aerosolized virions to survive for days (Sagripanti and Lytle, 2007). As for OAF, outside air has been proven to inactivate microorganisms faster than inside air under the same temperature and RH conditions (Druett and May, 1968).

Contact transmission depends on the load and survival of the pathogen on inanimate products. Porosity is the major factor influencing the survival rate of the virus, where an increase of porosity enhances virus inactivation on hard objects. The influenza virus can survive more than 48 h on stainless steel and plastic objects, but cannot be detected after 12 h on papers and cloth tissues at 35–40% humidity (Bean *et al*., 1982). Interestingly, human influenza viruses showed a low inactivation rate on banknotes; where an H3N2 virus (A/Moscow/10/99) showed no considerable inactivation after 10 days. Nasal mucus is thought to enhance virus survival in this case (Thomas *et al*., 2008). In case of humans, the highest inactivation rate was observed on hands, where it appears to be the bottleneck for contact transmission via fomites (Weber and Stilianakis, 2008c).

In summary, the influenza survival rate varies between different modes of transmission. Aerosolized influenza viruses have a half life of several hours in low RH and low to moderate temperatures. The virus can survive for several days on fomites, depending on their porosity and the surrounding environmental conditions. Persistence could be very high in water, especially in cold areas/seasons.

Summary

Ecosystems and infrastructures of human life have been dramatically changed in the last two centuries. Man can travel around the world and transfer pathogens between the East and West in a matter of hours. The analysis of influenza and pneumonia mortality data between 1996 and 2005 at the CDC revealed that a combination between international and national air travel influences the time of influenza introduction in November into the USA (Brownstein *et al*., 2006). In another study on 13,000 H3N2 influenza isolates during 2002–2007, it was shown that most of the new strains originated in East and Southeast Asia (Russell *et al*., 2008).

Influenza viruses continue to evolve and emerge in new hosts, causing disease in dozens of animal species. Expression of influenza receptors is not unique to every host, but rather a mixture that enables many animals to act as mixing vessels including humans.

Countering any new influenza pandemic requires multiple efforts in more than one aspect. It will be a matter of a few days before any newly emerged influenza virus can spread over the globe. Applying strict security measures is the key point in diminishing the spread of the virus. Any breach in the above aspect can lead to a disaster for human and animal populations. Some pandemic influenza plans recommend the use of surgical but not the N95 masks as a part of personal protective equipment for routine patient care. Considering the ability of influenza viruses for aerosol transmission, the above measures are not safe in pandemics. Additionally, influenza was shown to cause conjunctivitis in more than one epidemic. Human ocular and lacrimal epithelial cells express avian-like receptors, and neglecting eye protection would enable avian viruses to replicate and evolve

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in human populations causing serious problems. Few mutations in the HA gene could occur very quickly and new antigenic variant strains can be generated due to antigenic drift/shift mechanisms. The availability of systems and the power to develop new vaccines in large amounts and distribute them rapidly is a crucial aspect in fighting new influenza pandemics. Continuing the surveillance for new influenza variants in different animal species and understanding their mechanism of evolution and ability to cross species barriers are important in establishing better plans to fight the influenza virus.

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