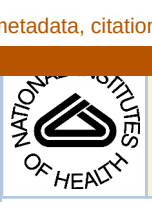

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Connecting the study of wild influenza with the potential for pandemic disease

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Abstract

Continuing outbreaks of pathogenic (H5N1) and pandemic (SOIVH1N1) influenza have underscored the need to understand the origin, characteristics, and evolution of novel influenza A virus (IAV) variants that pose a threat to human health. In the last 4–5 years, focus has been placed on the organization of large-scale surveillance programs to examine the phylogenetics of avian influenza virus (AIV) and host-virus relationships in domestic and wild animals. Here we review the current gaps in wild animal and environmental surveillance and the current understanding of genetic signatures in potentially pandemic strains.

1. Introduction

Nearly twenty years ago, in his landmark review of influenza, Rob Webster pointed out the probability that birds may serve as a source of all influenza A viruses (IAV) that become endemic in other species (1992). The emergence and maintenance of H5N1 lineages in wild and domestic birds and the 2009 novel pandemic strain of H1N1 virus with avian origins in humans have reinforced this view, yet shown the origin of epidemic virus to be complicated (Neumann et al., 2009; Shortridge et al., 1998). In many respects, recent influenza events emphasize the importance of understanding the ecology and evolution of IAV in wild animal vectors and viral reservoir species (Fouchier and Munster, 2009; Melville and Shortridge, 2006; Munster et al., 2007; Normile, 2006). Here, we review the recent literature in influenza with an emphasis on understanding i) how surveillance research in wild animals and the environment can benefit public health and ii) on how knowledge of the molecular determinants important in influenza evolution in wild species can inform pandemic preparedness.

Influenza viruses are normally classified by the antigenic properties of their highly variable major surface proteins, hemagglutinin (HA) and neuraminidase (NA). These two proteins are the primary targets of protective immunity in the host. Seventeen subtypes of hemagglutinin (HA: H1–H17) and 9 subtypes of neuramindase (NA: N1–N9) are described and all but one (H17 in bats (Tong et al., 2012)) and nearly all combinations have been isolated from wild birds (Olsen et al., 2006; Webster et al., 1992) although some more frequently than others. The influenza HA mediates viral binding to host cells and delivery of the viral genome into the cell cytoplasm while the NA assists in viral exit by cutting sialic acid ties to the host cell membrane. The viral genome of eight single-stranded negative sense RNA segments encodes 10+ proteins

depending on the strain. In addition to the HA and NA, three proteins form the polymerase complex (PB1, PB2, and PA) and bind the RNA segments with nucleoprotein (NP); matrix (M) and matrix 2 (M2) comprise the protein coat of the virus; and the non-structural (NS) and nuclear export protein (NEP) interact with cellular proteins and processes to assist viral replication and exit and avoid the host immune response. Several additional proteins have been identified in the PB1 and PA segments that are variably present through alternative transcriptional open reading frames, splicing, or secondary start codons. These include PB1-F2 and a suite of recently discovered PA forms (Jagger et al., 2012; Muramoto et al., 2012), all of which seem to impact virulence of infection and which demand further study.

Since the emergence of a highly pathogenic form of H5N1 avian influenza from a domestic goose in 1997, and its subsequent transmission to humans (de Jong et al., 1997), birds have received increased attention as the source of all natural IAV variants. On rare occasions, the highly pathogenic forms of IAV have been reported in wild birds -the first outbreak with mortality in wild birds being identified as an H5N3 influenza strain in common terns of South Africa in 1961 (Becker, 1966). However, retrospective analysis has identified avian origins for all segments of human pandemic viruses. This includes the “Spanish flu” of 1918, an H1N1 strain that was perhaps one of the greatest natural disasters in human history and is estimated to have contributed to the death of over 50 million people worldwide. Subsequent pandemic viruses though less severe have had enormous impact on human health and include an H2N2 virus in 1957, an H3N2 virus in 1968, and the pH1N1 virus, now endemic, in 2009. Each of these strains resulted from the reassortment of contemporary human strains with viruses derived from birds, but probably delivered through infection of an intermediate host such as the pig. Whether the 1918 virus moved into humans directly from an avian host is controversial. Regardless, the avian origin of all these viruses has spurred research into the avian host in hopes of understanding the characteristics and predictability of pandemic strains at their root.

Domestic and wild birds have been implicated as key agents for interspecies transmission to mammalian hosts of diverse taxa including whales, seals, pigs, horses, and also humans (Claas et al., 1998; Mandler et al., 1990; Reperant et al., 2009; Zhou et al., 2009). Phylogenetic analysis has even revealed that some gene segments belonging to previous human pandemic strains are still circulating in wild bird reservoirs. The NA genes of some H9N2 viruses isolated from migratory ducks in Hokkaido, Japan, clustered with those of H3N2 viruses responsible for causing the human pandemic of 1968 (Liu et al., 2003). Moreover, it has been speculated that the 3 parents of the triple reassortant virus that caused the 2009 H1N1 pandemic may have been assembled in one place by migratory birds (Gibbs et al., 2009). As such, increasing emphasis is now placed on understanding the evolution and molecular determinants of novel and pathogenic forms of influenza that originate from the IAV in birds.

Surveillance research in wild birds holds the promise of informing public health preparedness for pandemic and seasonal influenza. Field surveillance studies to detect avian influenza viruses (AIV) in animal vectors was organized in the early 1970’s, culminating with detection of influenza virus from the cloacal swabs of wild ducks (Slemons et al., 1974). Into the 1990’s, research in the bird host centred on describing the viral natural history and its maintenance in waterfowl hosts. In response to the threat of Asian origin H5N1, sampling efforts have increased by an order of magnitude or more in the last 5 years, particularly in the U.S. and mainland China (Butler, 2012). These efforts have begun to tie the viral natural history and studies on viral evolution to the potential for generating novel pandemic viral strains. What is clear from past work is that the evolution and natural history of the virus is highly dependent on the epizootiology of infection in the avian host. It is hoped that understanding the virus in reservoir species such as gulls and ducks may help refine viral surveillance and identify unique virus for further study. However, large biases exist in the geographic distribution of sampling sites and most countries still have little or no organized surveillance. Countries where H5N1

is endemic, including Egypt, India, Bangladesh, Viet Nam and Indonesia often suffer from a lack of capacity to diagnose and characterize viruses in-country. These gaps in viral surveillance in the wild, the focus of the first half of this review, will need to be addressed to make the most of current surveillance research efforts.

Viruses that come out of surveillance work in wild animals is also enabling laboratory based studies to clarify the molecular determinants of interspecies transmission, virulence and pathogenicity, the focus of the second half of our review. Experimental work with potentially troublesome virus or viral segments before they become a problem, should enable the development of broadly or specific protective vaccines and therapeutics for intervention before a pandemic is started. Influenza is unique in some respects in that one or a combination of three mechanisms – point mutation, segment reassortment, or, less commonly, recombination may generate genomic diversity. While recombination events do not seem to occur frequently, (Boni et al., 2008; Hirst et al., 2004; Pasick et al., 2009, 2005) their impact could be large and deserves further study. High rates of mutation are produced by the viral RNA polymerase, which lacks proofreading ability during transcription of the genome. In essence, mutation renders the host infected with a population of similar viruses with varying levels of fitness in the given host. Selection of novel variants that possess enhanced fitness is responsible for drift in viral strains. Unfortunately, only consensus strain sequence is commonly reported so the importance and dynamics of variants in the host are poorly understood. This is one major gap that needs to be addressed. However, what seems to prove the biggest challenge for human health and a result of interspecies transmission is the ability for co-infecting viruses to swap segments (reassortment), producing novel strains that are antigenically distant from the original (i.e. – novel combinations of HA and NA as well as internal segments). This process allows the virus to ‘sidestep’ the immune system of the host and spread through populations (Webby and Webster, 2001; Webster et al., 1992). While some subtypes contain strains that are partially cross seroreactive, the HA sequence of influenza may differ by over 30% at the amino acid level and show limited cross reactivity in serological assays (Alexander, 2000; Dugan et al., 2008). As reassortment goes, viruses in pigs may be a major reservoir of human emergent strains because of the potential to mix with human subtypes (Hass et al., 2011; Shu et al., 1994). However, pigs may not be the only animals for which this mixing is likely to take place (see 2.2.2 “If pigs might swim” below). Studies in many other species indicate that interspecies transmission is relatively frequent (Capua and Alexander, 2002), but that epidemics are thought to almost always be self-limiting because viruses are not maintained or do not become endemic in alternative host species. The specific genetics governing host range are undoubtedly polygenic, but may depend on the co-evolution of viral gene products with host cellular machinery to produce a competitive virus capable of establishing infection through transmission. The steady frequency with which this occurs in humans (Morens et al., 2010) highlights that this is a difficult but achievable and possibly even a predictable event in nature. Understanding this dynamic in avian and other reservoir and spillover hosts holds promise to help define the criteria to look for in potentially pandemic virus. It is also possible that pandemics are the result of rare events that facilitate genesis of rare viruses that are challenging to predict. Several programs are underway to study whether IAV shows a ‘pandemic signature’, to test these competing theories, and to understand if study of wild IAV can inform public health risk for potential pandemic influenza. In the following sections, we explore the gaps needing work in wild animal surveillance and highlight advances in our molecular understanding that promises to improve public health preparedness for influenza.

2. What are we missing in influenza surveillance?

2.1 Gaps in wild bird surveillance

Field surveillance studies to detect IAV in animal vectors have been conducted for over forty years, beginning with detection in wild ducks (Slemons et al., 1974). In response to the threat

of Asian origin H5N1, sampling efforts increased by an order of magnitude since 2005 particularly in the U.S. and mainland China (Butler, 2012). In-depth wild bird surveillance has helped distil key concepts in IAV ecology including the role of i) aquatic wild birds as reservoirs, ii) migratory flyways as barriers to viral evolution, iii) young immuno-naïve birds as the hub of the wheel in IAV circulation, iv) fecal-oral transmission as the primary transmission route in ducks and v) warm temperatures and physico-chemical properties of aquatic habitat in limiting IAV infectivity. Now at the conclusion of continent-scale surveillance projects targeting wild birds (Deliberto et al., 2009; Ip et al., 2008), the questions arise: how has understanding of IAV advanced? And are there any critical gaps in understanding that remain?

2.1.1 Hunting for a reservoir—Many of the dogmas of IAV continue to be the guiding principles that shape the way surveillance is conducted. Waterbirds belonging to the two orders Anseriformes (ducks, swans, geese) and Charadriiformes (shorebirds, gulls, auks) have long been recognized as the natural reservoirs of IAV (Webster et al., 1992) Within this ecologically diverse group the dabbling ducks (family: Anatidae), particularly mallards *(Anas platyrhynchos)* are believed to be a primary host. This notion stems from decades of field studies in North America (Alfonso et al., 1995; Hinshaw et al., 1980; Ip et al., 2008) and Europe (Munster et al., 2007; Wallensten et al., 2007) that report highest prevalence in mallards compared to other sympatric bird species. The role of mallards as a robust host for IAV is supported by experimental studies that demonstrate high titres of virus shed over extended periods by hatch year birds that remained asymptomatic (12 days: Jourdain et al., 2010; > 7 days: Keawcharoen et al., 2008). However, ecological context is important when considering findings from wildlife surveillance.

Mallards are the most ubiquitous waterbird species across their Holarctic distribution and are intensively managed in North America and Europe to ensure sizeable populations for hunting (Sedinger and Herzog, 2012). Large sample sizes are easy to obtain, especially from hunter-killed mallards, making the logistics of sampling this species easier than any other wild bird. Within the U.S., hunter-killed birds accounted for > 30% of all 78,300 Anatidae samples (positive and negative) deposited in the Influenza Research Database (IRD: accessed Sep 2012). Over 70% of hunter-harvested samples were collected from only 5 species of 51 Anatidae – mallard, green-winged teal (*Anas carolinensis*) northern shoveler (*Anas clypeata*), northern pintail (*Anas acuta*) and American wigeon (*Anas americana*), in descending order. This estimate of hunter-harvested Anatidae samples is conservative because it does not include the large-scale surveillance effort by the U.S. Department of Agriculture that sourced the majority of samples (68%) from hunter-shot birds (Deliberto et al., 2009), but highlights the dependency of surveillance on hunting as a source of inexpensive and readily-available samples, despite biases in species as well as seasonal timing, sex and age of hunted birds (Heitmeyer et al., 1993; Pace and Afton, 1999).

Species that are not viewed as ‘table birds’ and less sought after for consumption (i.e. northern shoveler, gadwall, *Anas strepera*) or non-game species (i.e. gulls, shorebirds, passerines) may present a challenge to obtain large sample sizes adequate for detection of IAV. Consideration of sample size is especially critical at wintering and stop-over sites where IAV prevalence is lower compared to breeding and fall staging grounds where prevalence reaches a peak in many aquatic birds (Guberti et al., 2007). Careful assessment of which species are high priority for surveillance demands a shift from opportunistic to sustained, long-term sampling plans that consider the diversity of wild bird species and spatio-temporal variation in infection patterns along the migratory flyway. Drawing on the expertise of ornithologists to capture non-game species may ensure sufficient sample sizes for species that may play an important yet undetermined role in hosting IAV strains with panzootic potential (Winker et al., 2008).

2.1.2 Charadriiformes: migrators and mixers—The current yardstick for measuring success of a surveillance study is detection of a large number of positive samples (Hoye et al., 2010b). This approach has perpetuated the focus on dabbling ducks, while the role of other water bird species in IAV ecology is overlooked. The product is a global bias towards sampling Anseriformes that account for the majority of wild bird samples collected in North America (78%), Europe (76%), Asia (35–58%) and Russia (47%) (Fig. 1). In contrast, sampling of Charadriiformes - another recognized reservoir for AIV, accounts for only 3–31 % of global surveillance efforts (Fig. 1). This sampling bias has led to over-representation of viral subtypes associated with ducks in surveillance and genomic data, including the ubiquitous H3N8 and H4N6. Virus pools of Anseriformes and Charadriiformes have long been recognised as distinct (Kawaoka et al., 1988; Sharp et al., 1993). Overlap occurs with promiscuous subtypes (H3, H6, H7), however the circulation of H9, H13 and H16 is generally limited to Charadriiformes (Krauss et al., 2004; Krauss and Webster, 2010). Uncovering the full viral diversity hosted by the wild bird reservoir, including Charadriiformes represents a more effective strategy for detecting influenza precursors with capacity to switch hosts and seed a human pandemic.

Charadriiformes possess host traits that favour transmission, dispersal and hemispheric mixing of IAV suggesting they play a uniquely different role in the ecology of IAV compared to ducks (Gaidet et al., 2012). Shorebirds are highly gregarious along their migratory cycle and are true long-distance migrants connecting the northern and southern hemispheres (Gill Jr et al., 2009). This combination of host factors provides a mechanism for hemispheric reassortment of IAV and the movement of novel lineages that displace locally circulating strains. This is consistent with the higher frequency of hemispheric reassortment observed in IAV from shorebirds and gulls compared to other water bird hosts (Bahl et al., 2009; Dugan et al., 2008; Ramey et al., 2010; Widjaja et al., 2004). A growing number of studies have shown that the internal genes of virus isolated from Charadriformes in North America are of Eurasian origin, a pattern observed along the Pacific (Ramey et al., 2010; Wille et al., 2011a) and Atlantic coasts (Wille et al., 2011b) suggesting that wild birds belonging to this order are primary candidates for introduction of hemispheric reassortant virus. Enhanced surveillance of shorebirds and gulls may facilitate early detection of IAV strains imported from regions where highly pathogenic IAV is endemic or the incursion of novel segments into the endemic viral population.

A complete understanding of the global movements of IAV requires that Charadriiformes be incorporated into surveillance programs. Specifically, longer-term studies need to be established to compliment the site at Delaware Bay, U.S. that has yielded the bulk of virus samples from shorebirds, primarily from the narrow window of spring migration (Krauss et al., 2004; Krauss and Webster, 2010). Comprehensive sampling of Charadriiformes will necessitate greater international collaboration to target sites where migratory flyways overlap, allowing hemispheric reassortants to be more readily detected. Surveillance has thus far been North America- and Europe-centric (Butler, 2012) and rarely incorporates sampling sites in Eurasia despite the fact that breeding populations of migratory birds often span both hemispheres at northern latitudes (i.e. Beringian region, Arctic Russia). Long-standing political divides, the remoteness of sampling sites and lack of in-country diagnostic laboratories presents a challenge for conducting surveillance in Africa, the Middle East, Russia, South America and Asia, however researchers are increasingly making inroads (Fereidouni et al., 2010; Gaidet et al., 2012; Shestopalov et al., 2006). A commitment to capacity building and a mutual resolve to understand avian influenza dynamics across international boundaries may help to address this geographic bias.

2.1.3 Host ecology and migration—The influence of host ecology, behaviour and migration on transmission represents a large knowledge gap in our understanding of IAV in part because virology and ecology remain two disparate fields that rarely overlap during the

investigation of IAV in wild birds. Phylogeographic studies have distilled key concepts including the generalized pattern of gene flow from north to south along migratory flyways (Lam et al., 2012; Pearce et al., 2009) and relative separation of North American and Eurasian virus pools (Pearce et al., 2010; Ramey et al., 2011) despite evidence of migratory connectivity of wild birds between hemispheres (Flint et al., 2009; Winker and Gibson, 2010). Studies of virus evolution that rely on analysis of publically-available sequences can advance our broad understanding of IAV dynamics (Bahl et al., 2009; Lam et al., 2012) but mechanisms that drive virus gene flow in wild birds remain elusive without consideration of host ecological 'metadata'. However, tracking migratory animals can present a logistical and financial roadblock for studies that seek to investigate how host behaviour can promote or prevent transmission of pathogens (Altizer et al., 2011). Use of markers including ring-bands or more recent technology including satellite transmitters, geolocaters and stable isotope analysis of body tissues offers prospects for identifying the migratory behaviour and geographic origin of hosts if integrated with traditional surveillance.

Migration is common among wild birds from seasonal habitats; however there is variation in the propensity to migrate. Among the same species a continuum of migration strategies can exist with residency and long-distance migration on either ends of the spectrum (Alerstam et al., 2003). The effect of migration strategy on IAV dynamics has only recently been explored in detail, facilitated by the use of stable isotopes (Gunnarsson et al., 2012; Hill et al., 2012a) and trace element profiles (Fries et al., In press) in flight feathers. Using stable isotopes to identify the breeding origin of mallards, virus detected in resident mallards during winter in California became the predominant IAV circulating in locally-breeding mallards in summer, supporting the view that residents act as reservoirs (Hill et al., 2012a). In contrast, migrants introduced virus from northern breeding grounds including Alaska, but circulation of imported virus appeared to be limited. Virological studies have identified wild bird-mediated dispersal as the mechanism for the spread of Eurasian lineage HA subtypes along the Pacific Flyway resulting in an outbreak of H6N2 in poultry in California (Bahl et al., 2009; zu Dohna et al., 2009). A more nuanced understanding of the migration strategies of host species is key for predicting gene flow patterns and the introduction of Eurasian origin virus into agricultural regions that support farming of pigs or poultry.

The ability of migratory birds to spread IAV, particularly highly pathogenic subtypes, has been a divisive topic among researchers with evidence both for (Gaidet et al., 2008; Saad et al., 2007) and against (Gauthier-Clerc et al., 2007; van Gils et al., 2007). Central to this question is an understanding of how far birds can migrate before symptoms impact flight performance. Using satellite telemetry, the potential for 19 species of wild birds from Asia, Africa and Europe to spread HPAI was recently assessed by Gaidet et al (2010). Comparison of dispersal rates showed that the common teal (*Anas crecca*) had greatest potential to carry HPAI over 500km during the asymptomatic period of infection, yet the likelihood of this event was restricted to 5–15 days during spring or fall migration. Furthermore, co-mingling of satellite-tracked wild birds with domestic ducks - that can act as a reservoir for HPAI, days prior to migration was a predictor of wild bird outbreaks along the Central Asian Flyway between 2005–2010 (Newman et al., 2012). These studies highlight that dispersive potential is species-dependent, governed by flight performance, host pathobiology, virulence of IAV strains and spatio-temporal overlap with reservoir hosts. A shift to residency in some animal populations triggered by mild temperatures or dependency on agriculture or human resources may promote local circulation of more virulent strains (Altizer et al., 2011). The correlation between migration strategy of the host and virulence of transported pathogens is understudied in wild birds and warrants investigation in view of implications for IAV under climate change scenarios.

2.1.5 Host immunity—Surveillance programs place an emphasis on collection of virus, while the host response to infection is often overlooked. Production of antibodies to limit and

overcome infection of IAV is central to the adaptive immune response in birds and should be incorporated into surveillance efforts to identify which species are involved in IAV circulation. Patterns of higher sero-prevalence relative to virus prevalence have been observed across many wild bird taxa, including geese (Hoye et al., 2010a), gulls (Toennessen et al., 2011) and ducks (De Marco et al., 2005, 2003). Investment in antibodies is a common strategy in the protection against IAV and partly explains the seasonal pattern of infection in waterbirds. Virus prevalence peaks after the breeding season in ducks, reflecting a build-up of young, immuno-naïve ducks in summer (Guberti et al., 2007; Hinshaw et al., 1985). Juvenile ducks are also the primary hosts at wintering sites (Ferro et al., 2010; Hill et al., 2012b) suggesting that exposure to novel strains occurs at both ends of the migratory flyway, a burden on the developing immune system of young birds. Despite collective evidence that immunity in juveniles drives the epidemic curve of IAV in nature, surveillance programs rarely place value on collection of paired swabs and serum from wild birds that may both inform and predict viral dynamics.

A neglected aspect that is likely to impact the scale and timing of infection among young birds on the breeding grounds is maternal antibody (MatAb) transfer. Ducklings or chicks may gain protection from influenza by MatAb, primarily class IgY, passed through the egg yolk (Liu and Higgins, 1990). Persistence of MatAb varies markedly from 3 to 40 days depending on life-history strategy and may directly correlate with length of incubation in birds (Lee et al., 2008; Tella et al., 2002). In common quail (*Coturnix coturnix*) MatAb wane at 15 days, however in the longer-lived Cory's shearwater (*Calonectris diomedea*) – a seabird that lays a single egg with a long incubation time, MatAb were still detectable in chicks at 30–40 days of age (Garnier et al., 2012). Under this hypothesis, long-lived birds such as seabirds are expected to produce longer lasting MatAb affording greater protection to juveniles. Maternal antibodies that provide immunity against IAV have been identified in gull eggs (Hammouda et al., 2011; Pearce-Duvet et al., 2009) but have not been investigated in other reservoir species. Waning immunity due to catabolism of MatAb may be responsible for the peak in IAV prevalence observed in ducklings weeks after they have left the nest and become flight capable. Further field and laboratory studies should be directed at understanding how characteristics of MatAb including temporal persistence and cross-protection may play a critical but unrecognized role in governing infection dynamics in juveniles and viral evolution across a range of reservoir species. Better understanding of MatAb dynamics may also help researchers to characterize the epidemic curve in wild birds and identify where and when to target surveillance efforts.

Antibody-mediated immunity is thought to drive antigenic evolution of avian influenza in humans (Ferguson et al., 2003) and wild birds (Dugan et al., 2008). However evidence for a causal relationship between antibody production and virus evolution in wild birds has never been conclusively drawn. The 26-year study by Krauss et al. (2004) has contributed towards understanding the temporal pattern of subtype turnover in wild birds. Yet our knowledge of virus evolution remains incomplete without understanding how the immune system acts as a selection pressure constantly modifying the virus pool in wild birds. MatAb confer protection against strains infecting the mother from prior seasons, while acquired immunity acts against currently circulating strains. No study has investigated the relationship between antibodies that develop in juveniles and the fate of targeted subtypes in subsequent seasons. Widely distributed subtypes in ducks; H3N8 and H4N6, predominate from year-to-year and are expected to evade the immune response of the host owing to antigenic drift, much like H1, H2 and H3 in human populations. Assessing rates of antigenic drift among H3N8 and H4N6 in wild birds may reveal the immunologic and genetic hallmarks of virus that have heightened fitness in the wild bird reservoir. Implications of evading host immunity include widespread and persistent circulation in wild birds and ultimately a heightened chance of spillover to non-reservoir species.

2.2 Gaps in wild mammal surveillance

2.2.1 Synanthropic mammals—Mammals closely associated with human settlement may represent a pathway for interspecies virus transmission and adaptation. More broadly, the increasing interface between animals and humans has led to emergence of infectious disease on a global scale (Daszak et al., 2000; Lebarbenchon et al., 2008). Emergence of highly pathogenic IAV is no exception, with the evolution of H5N1 facilitated by co-mingling of wild and domestic ducks on rice paddies (Gilbert et al., 2008; Hulse-Post et al., 2005) followed by spillover to humans involved in the market chain (Martin et al., 2011). Agricultural practices in Asia have been identified as critical to the spread of influenza; however other human activities that diminish barriers between host species are often overlooked by surveillance programs. Study of synanthropic wild mammals is a critical gap in our knowledge of how IAV evolves to exploit novel hosts brought into close contact with avian reservoir species. Isolation of a novel lineage of IAV from little yellow-shouldered bats (*Sturnira lilium*) resulting in designation of an H17 subtype highlights that wild mammals can act as a reservoir with the potential for spillover to sympatric domestic and agricultural animals (Tong et al., 2012). Investigation is needed to clarify which genetic markers and host combinations allow influenza to jump the species barrier, triggered by close contact between birds and mammals.

Recent interest in free-ranging mammalian hosts has demonstrated that a larger than expected number of wild species are competent hosts for IAV. Wild house mice (*Mus musculus*) sampled after an outbreak of low pathogenic H5N8 at a game bird breeding facility tested sero-positive to IAV (Shriner et al., 2012). The possibility of spillover from migratory ducks sighted at the facility prompted the authors to experimentally infect house mice with mallard-origin IAV and demonstrate that replication occurs efficiently without adaptation of the virus to a mammalian host (Shriner et al., 2012). The ability of IAV to cross the species barrier and replicate in mammals without adaptation has also been demonstrated in ferrets experimentally infected with H1N9 and H6N1 (Driskell et al., 2012). Neither virus subtype showed an affinity for α-2,6 linked sialic acid (SA) receptors suggesting limited selection for mammalian adaptation in the laboratory setting. These cases highlight the relative ease with which IAV circulating in wild birds may spread among sympatric mammalian populations, however natural infections have rarely been documented. Pikas (*Ochotona curzoniae*) represent one of the few free-ranging mammals naturally-infected by IAV (highly pathogenic H5N1) circulating among migratory waterfowl at Qinghai Lake, China (Zhou et al., 2009). Lack of diseased or dead mammals at outbreak sites (pikas: Zhou *et al*., 2009) or limited clinical symptoms in experimentally infected mammals, depending on subtype (mice: Driskell et al., 2010; ferrets: Driskell et al., 2012; Hinshaw et al., 1981) may mask infection and contribute to a low rate of detection in wild mammals.

Co-ordinated sampling of wild birds and mammals may shed light on mechanisms that allow influenza to overcome the host barrier in nature, including which subtypes and host combinations are conducive to spillover. Species with abundant urban populations such as raccoons (*Procyon lotor*), European rabbits (*Oryctolagus cuniculus*) or bats that have the potential to interact with wild birds are a prime candidate. Raccoons sero-surveyed for IAV have shown exposure to subtypes commonly circulating in wild water birds (H1, H3, H4 and H10: Hall et al., 2008) and poultry (H5N1: Horimoto et al., 2011) providing evidence of interspecies transmission. Synanthropic rodents and bats are targets for surveillance by public health agencies because of the need to curb zoonotic transmission of mammal-borne pathogens, most notably hantavirus in rodents (Phan et al., 2011) and lyssaviruses in bats (Kuzmin et al., 2012). Trapping and abatement programs led by government agencies may be a source of a large number of samples from areas with concentrated human populations (i.e. urban and recreational parks). Expanding sampling of free-ranging mammals in conjunction with existing public health surveillance is imperative to monitor spread of AIV in view of how readily

interspecies transmission can occur when avian and mammalian populations overlap. Synanthropic mammals may be at highest risk of co-infection from avian and mammalian strains of influenza providing conditions suitable for reassortment in nature.

2.2.2 If pigs might swim: marine mammals as mixing vessels—Marine mammals are a particularly interesting and phylogenetically diverse group whose members comprise multiple lineages which underwent numerous independent re-invasions of the seas and rely on the marine environment for food. Marine mammals are globally distributed and found in nearly all coastal waterways and shorelines. The coastal environment provides an interface between marine and terrestrial habitats where avian reservoirs of influenza collide (sea ducks, gulls, and shorebirds) and overlap spatio-temporally with marine mammals, providing an opportunity for interspecies transmission of IAV. In general pinnipeds (seals, sea lions, and fur seals) are aggregate, seasonal breeders resulting in highly synchronized terrestrial parturition, which may lead to heightened interactions between birds, pinnipeds, domestic animals (dogs) and humans. Our understanding of IAV in marine mammals is predominantly from pinnipeds and has stemmed from sampling stranded animals that have washed ashore in populated areas, bio-monitoring of wild populations deemed to be of conservation concern, mortalities resulting from entanglement in fishing gear, and sampling associated with subsistance hunted animals. While previous reviews did not consider there to be strong evidence for a transmission pathway between marine mammals and humans (Alexander and Brown, 2000), there is increasing support of the transmission of zoonotics between marine mammals and humans (Hunt et al., 2008; Siembieda et al., 2008; Webster, 1981; Webster et al., 1981). Several IAV isolated from marine mammals have demonstrated a preference for infection and replication in mammalian hosts (Hinshaw et al., 1981; Lang et al., 1981; Webster et al., 1981) including documented infection in a technician (Webster, 1981). Further, the recently isolated H3N8 from harbor seals (*Phoca vitulina*) demonstrated for the first time naturally acquired mutations that indicate mammalian adaptations (Anthony et al., 2012). These findings highlight the importance of IAV surveillance in wild marine mammals in which evidence of frequent transmission is accumulating, but for which many gaps in understanding remain and may pose a public health risk.

Since the first isolation of IAV in swine (H1N1) (Shope, 1931), it has become evident that multiple subtypes of IAV of either avian or human descent can infect pigs (Guan et al., 1996; Karasin et al., 2000; Peiris et al., 2001). Similar to pigs, multiple IAV have been isolated from marine mammals (Greig, 2011; Hinshaw et al., 1986). Additionally, avian-like (H3N8, H3N3, H4N5, H4N6, H7N7) and human influenza A (H3N2) and B viruses have been isolated from several species of marine mammals found within the coastal environment (Anthony et al., 2012; Blanc et al., 2009; Mandler et al., 1990; Ohishi et al., 2004, 2006, 2002; Osterhaus et al., 2000). The susceptibility of marine mammals to infection of both avian and human influenza viruses may, in part, be due to the type and distribution of SA receptors in tissues including the respiratory tract. Anthony et al. (2012) reported the presence of α-2,6 linked SA and to a lesser degree α-2,3 linked SA receptors within the respiratory track of harbor seals. These findings are in contrast to an earlier study that found only α-2,3 linked SA receptors present in respiratory tracks of “seals and whales” (Ito et al. 1999). Attachment of avianorigin H7N7 was predominately found in the upper respiratory tracts of harbor and grey (*Halichoerus grypus*) seals (Ramis et al., 2012) which corresponds to the distribution of α-2,3 linked SA receptors in seals (Anthony et al., 2012; Ito et al., 1999) whereas attachment of human H3N2 influenza was observed in the bronchiolar and alveolar epithelium of harbor porpoise and to a lesser degree in harbor seals (Ramis et al., 2012). These findings suggest that the location of infection within the respiratory track may differ for human and avian influenza viruses in marine mammals. Further, the observed anatomical differences between marine mammal groups (cetacean, pinnipeds) may lead to differences in susceptibility to IAV in these mammals.

In addition to genetic reassortment between avian and human influenza viruses, adaptation of an AIV leading to efficient infection in a mammalian host may also lead to influenza pandemics (Webster et al., 1992). There is evidence of such adaptation in the IAV isolated from marine mammals. In September 2011 an unusually high number of seals were observed stranded along the coast of New England. The mortalities were associated with an infection of avian-origin H3N8 possessing recent mutations suggesting adaptations to the mammalian host (D701N in PB2 and binding to α-2,6 linked SA receptors). A total of 37 amino acid substitutions distinguished this seal H3N8 virus from other avian H3N8 viruses (Anthony et al., 2012); the exact role of the rest of the mutations in adaptation is not yet understood. However, this was not the first occurrence of an H3N8 virus in marine mammals. An H3N8 subtype was isolated from one harp seal caught in fishing gear off Cape Cod between December 2005 and August 2007, with no significant pathology reported (Bogomolni et al., 2008). The H3N8 has drawn attention to the role of marine mammals in the ecology and evolution of IAV; however, the H3N8 virus was not the first case of IAV isolated from a marine mammal.

2.2.2.1 The long history of IA V in marine mammals: The first IAV (H1N3) isolated from a marine mammal was from a baleen whale (family Balaenopteridae) (Lvov et al., 1978). Shortly thereafter, a mass die off of harbor seals, impacting an estimated 20% of the population near Cape Cod was attributed to severe pneumonia and H7N7 infection (Webster et al., 1981). Most seals affected by the die off were young of the year, suggesting that the state or maturity of the immune system plays a role in susceptibility to IAV. The seal H7N7 was antigenically and genetically similar to avian H7N7 but showed greater ability to infect, replicate and produce pneumonia in a broad range of experimentally infected mammals compared to domestic birds (Kida et al., 1982; Lang et al., 1981; Murphy et al., 1983; Webster et al., 1981). Infection and disease observed in seals following experimental exposure to H7N7 differed between species with harbor seals exhibiting disease similar to naturally infected seals (Webster et al., 1981). Grey seals had no indication of infection; whereas harp seals showed no clinical signs of disease but had pathological changes and virus recovered from some seals with surviving seals becoming sero-positive (Geraci et al., 1984). Following the H7N7 mass mortality, harbor, grey, hooded (*Cystophora cristata*) and harp (*Pagophilus groenlandicus*) seals and pups were culled from eastern Canadian waters to assess the presence of IAV and antibodies (Geraci et al., 1984). While no viruses were isolated from any seals, antibodies to H7N7 were found in 3 adult grey seals, while the other species were sero-negative (Geraci et al., 1984). These findings raise a number of questions about the susceptibility to IAV infection between marine mammal species and age groups. From a public health perspective, the observation of conjunctivitis in a person working with the experimentally infected seals may be the most profound (Webster, 1981). Following the experimentally infected seal sneezing on a technician, seal H7N7 was recovered from the conjunctiva, suggesting that H7N7 may be transmitted between marine mammals and humans (Webster, 1981; Webster et al., 1981).

A second epizootic of seal pneumonia occurred from 1982 to 1983 in New England and was associated with an H4N5 influenza virus, the first time this virus had been isolated outside of birds (Hinshaw et al., 1984). Other subtypes including H4N6 and H3N3 have also been isolated from seals that died of pneumonia along the Cape Cod peninsula in 1991 and 1992 (Callan et al., 1995). Antigenic and genetic analyses showed that all genes were of avian origin (Callan et al., 1995; Hinshaw et al., 1984; Webster et al., 1981). The repeated outbreak of pneumonia associated with IAV of avian origin suggests transmission between avian host and seals, highlighting the importance of surveillance studies in these populations to gain a better understanding of interspecies transmission of IAV.

It is not surprising that much of what is known about IAV in marine mammals is based on samples from amphibious pinnipeds. However, influenza has been isolated from stranded cetaceans (dolphins and whales). Two influenza viruses (H13N2 and H13N9), demonstrating

dual infection, were isolated from an obviously emaciated and ill pilot whale (*Globicephala melaena*) stranded along the New England Coast. Phylogenetic analyses suggest the viruses originated from gulls (Hinshaw et al., 1986) and similar to other IAV isolated from seals (Lang et al., 1981; Webster et al., 1981), viral replication was observed in ferrets following intranasal inoculation (Hinshaw et al., 1986). The repeated infections in marine mammals during the last 30–40 years in New England and the associated sampling and laboratory studies have been the foundation of our understanding of IAV in marine mammals. However, the role of marine mammals in the ecology and evolution of influenza, outside of these mass mortalities and in other parts of the world remain limited.

2.2.2.2 Looking back to understand the present: These outbreaks in marine mammals and their implications for public health have prompted several retrospective studies assessing the seroprevalance of IAV antibodies. Serological evidence of exposure to avian- and human-origin IAV have been reported in several species of marine mammals from across the globe (Blanc et al., 2009; Ohishi et al., 2004, 2006, 2002); however, sampling effort has varied greatly. When subtype has been determined, H3 has been most frequently reported (Table 1). Serological evidence of exposure to IAV has also been reported in cetaceans (Dall's porpoise, *Phocoenoides dalli* and Minke whale) hunted in the Western Pacific Ocean (Ohishi et al., 2006). The presence of antibodies in the Minke whale may be of importance as this species performs large annual migrations (Kasamatu et al., 1995) that may provide a mechanism for the introduction of IAV to new regions, species or individuals (Altizer et al., 2011).

Based on serological evidence, Caspian (*Pusa caspica*), ringed and Baikal (*Pusa sibirica*) seals were exposed to human-origin H3N2 (Ohishi et al., 2004, 2002) further supporting a possible transmission route between marine mammals and humans. Seals from Hokkaido, Japan were sero-positive for H3 and H6 subtypes (neuraminidase was not determined) between 1998 and 2005. In all years, sero-positive seals included juveniles which the authors suggest is evidence of sporadic infections in this population (Fujii et al., 2007). Based on this evidence, the authors propose that seals may be a reservoir for IAV of human origin with implications for public health.

The seroprevalence of IAV in marine mammals of the arctic has been of particular interest, in part because this region is sensitive to climate change and also due to the reliance of subsistence hunters on these populations. Interspecies transmission of IAV has been documented through routes of ingestion and inhalation of aerosolized viruses in other species. Therefore, the presence of IAV in marine mammals hunted for human consumption has a direct implication for public health. Antibodies to influenza A have been found in many arctic species of marine mammals including beluga (*Delphinapterus leucas*), ringed seal, harp seals, hooded seals, and walrus (*Odobenus rosmarus*) (Calle et al., 2002; Danner and McGregor, 1998; Nielsen et al., 2001; Stuen et al., 1994). While other species including narwhals (*Monodon monoceros*), bowhead whale (*Balaena mysticetus*), bearded seals (*Erignathus barbatus*) and one population of walruses (Calle et al., 2002, 2008; Nielsen et al., 2001) have not shown antibodies. These studies were based on retrospective serological samples and viral isolates were not collected. Therefore, genetic analysis could not be performed, leaving open the question of the role of IAV in the arctic environment and its potential impact on public health.

Surprisingly antibodies to influenza B, normally a human only virus and the isolation of influenza B virus in harbor seals has been reported (Blanc et al., 2009; Osterhaus et al., 2000). These observations further highlight the need for systematic and prospective surveillance of influenza in wild marine mammals. The current knowledge of influenza virus in marine mammals has been built upon opportunistic and relatively low sampling, in part due to logistical and permit constrains of handling and sampling marine mammals required under the U.S. Marine Mammal Protection Act (MMPA, 1972). Therefore, prospective surveillance

of influenza in marine mammals will require the collaboration with government agencies and non-profits to facilitate adequate sampling in order to better understand the influence of these mammals on the ecology and evolution of influenza viruses and the potential impacts on public health.

2.3 The gaps in environmental surveillance

2.3.1 Environment as an intermediate "host"—The role of environmental persistence of virus in the overall ecology of influenza is undoubtedly a critical one and likely plays a significant role in species specificity, periodicity of infection, reassortment, and epidemic initiations and persistence. Several modelling efforts have concluded that AIV requires an environmental component of indirect transmission (Breban et al., 2009; Lebarbenchon et al., 2010; Rohani et al., 2009), and mounting circumstantial evidence supports this hypothesis. In one particularly interesting case, turkeys in Minnesota and their water sources were monitored for AIV. The H13N2 virus, typically associated with gulls, was detected in a turkey for the first time only after it was detected in the pond water two weeks prior (Sivanandan et al., 1991). This led the authors to conclude that gulls likely shed virus into the pond where it persisted and ultimately infected turkeys. Another study monitored ducks and turkeys that shared a water habitat and found in nearly all cases, viruses detected in turkeys were first detected in the ducks (Halvorson et al., 1983). A recent report describes an H9N2 isolated from an egret in Dongting Lake (Wang et al., 2012a), the same subtype typically found in ducks and chickens and isolated several years prior from the water of that same lake (Zhang et al., 2011b). On the heels of the 2004 H5N1 epidemic, an extensive survey of H5N1 seropositive individuals in Cambodia found that swimming or bathing in pond water were strong risk factors for seropositive status (Vong et al., 2009). Supporting this correlation, a Vietnam woman and a Cambodian child each contracted H5N1 with no identifiable risk factors other than swimming in contaminated water (WHO, 2007). Further H5N1 epidemiological analyses showed a strong correlation between minimal distance to the nearest lake or wetland and the likelihood of an outbreak, as well as an inverse relationship between outbreaks, precipitation and bird density (Fang et al., 2008).

Given the logistics of collecting and screening large volumes of water for virus, sampling methodologies have not yet been refined. When large enough quantities of virus are present, unconcentrated water can be tested with some success (Halvorson et al., 1983; Hinshaw et al., 1979, 1980; Leung et al., 2007; Stallknecht et al., 2010). For a much more sensitive screening, large volumes of water need to be concentrated through a number of possible mechanisms (Heijnen and Medema, 2009; Roepke et al., 1989; Sivanandan et al., 1991) and there is concern that the handling may destroy the influenza virus. Although the concentration of natural water samples needs further optimization, the erythrocyte binding assay described by Roepke et al. (1989) has become a broadly accepted means for detecting virus from water. This assay capitalizes on HA binding to SA and uses SA expressing chicken erythrocytes to precipitate virus out of solution. The recovered fraction can then be screened for viral RNA using RTPCR or live virus via egg or tissue inoculation. Unfortunately, several PCR inhibitors reside in environmental samples and therefore limit the efficiency of RTPCR, and concentration methods can damage virus particles and therefore limit the efficiency of egg inoculations. Coupled with the difficulties of obtaining, filtering and concentrating large volumes of water, environmental surveillance is understandably in its infancy.

2.3.2 Virus is lurking in water and dirt—While screening has been limited, 13 of the 16 HA subtypes known to circulate in wild birds have been detected in a wide range of freshwater, including river water, lakes and ponds, standing puddles near farms, and drinking water from poultry cages (see Table 2 and Fig. 2). While only a small number of viruses have been recovered in total, the geographic distribution is surprisingly large given these low numbers.

Environmental recovery of virus has spanned multiple countries, flyways, and biomes, even including warm, temperate regions considered to be inhospitable to virus persistence (Henaux et al., 2012) (Fig. 3). In many cases, only viral RNA was detected (Heijnen and Medema, 2009; Henaux et al., 2012; Lang et al., 2008), either because the virus failed to grow in eggs or the studies were not designed to test for live virus. Nonetheless, a wide representation of live virus has been recovered from egg inoculations using both concentrated and unconcentrated natural water sources (Halvorson et al., 1983; Hinshaw et al., 1979, 1980; Ito et al., 1995; Leung et al., 2007; Markwell and Shortridge, 1982; Sivanandan et al., 1991; Stallknecht et al., 2010; Zhang et al., 2011a). H2 and H8 are the only subtypes present in ducks that have not yet been recovered from water, however both of these subtypes are rare even within the duck population (0.9% and 0.3%, respectively) (Krauss et al., 2004), and each has been recovered from soil. Interestingly, despite the nearly exclusive prevalence of H9–H13 in shorebirds with only rare occurrences in ducks (Krauss et al., 2004), all of these subtypes have been recovered from freshwater sources. The only subtypes that have not yet been isolated from any environmental source are H14–H16, typically associated with marine birds and detected at low prevalence even in the natural reservoir. There are currently no reports attempting AIV detection from a marine environment.

Even fewer studies have looked at persistence and/or detection of virus in environmental reservoirs beyond water, such as lake sediment, soil, flora and fauna. The analyses that have been done yielded a remarkably high prevalence of virus (Fig. 2). Although Horm et al. (2012) only looked for H5N1, a high prevalence of viral RNA was still found in environmental samples spanning straw, dust, mud, and aquatic plants from five households in Cambodia. An analysis of lake sediment from small Alaskan ponds heavily utilized by migratory birds found AIV RNA in 55.6% of the samples tested (Lang et al., 2008), with a large diversity in the subtypes identified. There has been one report of H5N1 recovered from a small fish in Cambodia (Horm et al., 2012) and another of H6N8 recovered from freshwater clams (Huyvaert et al., 2012).

Only three studies to date have compared concurrent data from waterfowl and either water (Halvorson et al., 1983; Hinshaw et al., 1980) or lake sediment (Lang et al., 2008). As might be expected, one of these studies (Hinshaw et al., 1980) found only a subset of the high diversity of viruses detected in ducks were recovered from the water and in two subsequent years, reflected the most prevalent duck subtypes. The question remains as to whether the subtypes were the most prevalent in ducks because they persist the best in water, or if their detection was purely stochastic and reflected higher viral shedding. In contrast to a strictly stochastic model, a similar study design found twenty-one subtypes in ducks over a two year period, with only four that could be recovered from pond water (Halvorson et al., 1983). In this instance, the water associated AIV did not reflect the most common subtypes found in ducks. Likewise, a study of lake sediment recovered numerous AIV which largely reflected those present in ducks the prior season, however there were subtypes prevalent in ducks not found in water and a subtype found in water that was not seen in the ducks (Lang et al., 2008). These discrepancies highlight the current inability to predict what might be found in the environment purely based on what is found in the bird population and underscores our need to better understand environmental dynamics.

Viral pathogenicity may also differ between viruses persisting in water and those found by surveillance of birds. One study recovered several H9N2 isolates from both surface water in the Dongting Lake wetland, and feces deposited along the shore. An assessment of key amino acid changes known to impact pathogenicity revealed no apparent differences amongst the isolates, yet those derived from the water were more pathogenic in mice than those derived from feces (Zhang et al., 2011b). Similarly, H13N2 recovered concurrently from pond water and turkey also showed differences in pathogenicity, with the water derived virus being more

pathogenic in mice (Laudert et al., 1993). It is unclear if viruses are under selective pressure for pathogenicity in the environment, or whether this selection would impact the severity of disease in subsequently infected wild birds. Alternatively, adaptation towards stability in the environment may inadvertently result in unrecognized amino acid changes associated with increased pathogenicity in mammals. Both explanations highlight the need to identify the molecular basis for environmental stability of AIV and consequences for transmission in the natural reservoir.

2.3.3 Lessons learned from experimental studies—A handful of experimental studies have begun to define the dynamics of AIV stability outside of the host. The majority of these studies have inoculated distilled water with a known quantity of virus isolated from amniotic allantoic fluid and manipulated the model for temperature, pH, and salinity. Inoculated virus is followed over time and typically assayed by either egg inoculation or titration in a tissue culture system to quantify the presence of live virus. Under these carefully controlled laboratory conditions, general trends have emerged. As a broad statement, clean filtered water maintained at a low temperature (below 17C), low salinity (freshwater, < 0.5 ppt), and neutral pH (6.8–7.4) provides the virus with the greatest longevity. Under these ideal conditions, it is not uncommon to see virus maintaining infectivity for nearly a year (Lebarbenchon et al., 2012, 2011; Nazir et al., 2011), with one report recovering live virus after 667 days (Brown et al., 2007). These analyses clearly provide the proof-of-principle that influenza can readily persist across wild bird migratory seasons. Unfortunately, while it is convenient to look for general trends in viral persistence under a defined panel of parameters, mounting evidence underscores that the picture is much more complex and only beginning to be understood.

Experimental studies have convincingly demonstrated that as temperature increases, viral persistence decreases at an exponential rate (Stallknecht et al., 2010). As a general approximation, most studies have found that virus persists for nearly one year at 0–4C (Lebarbenchon et al., 2012, 2011; Nazir et al., 2010), approximately 6 months at 10–17C (Brown et al., 2007; Lebarbenchon et al., 2012, 2011; Nazir et al., 2011; Stallknecht et al., 1990a, 1990b), 1 month at 20–23C (Lebarbenchon et al., 2012; 2011), and a week or less at 28C or higher (Lebarbenchon et al., 2012; Nazir et al., 2010, 2011; Stallknecht et al., 1990a, 1990b). Despite these temperature generalities, a great deal is still unknown about virus circulation in the natural setting. Natural water sources are not maintained at constant temperatures, and while modest fluctuations (17C/23C) have little effect on virus stability (Lebarbenchon et al., 2012), more extreme fluctuations could be more damaging. One study showed that cycling between −20C and 4C dramatically reduced virus longevity (Lebarbenchon et al., 2011), while another (Shoham et al., 2012) found only a 25% decline in infectivity after multiple freeze/thaw cycles or a year of frozen storage. Virus isolates further differ in thermostability, particularly at colder temperatures (Brown et al., 2009; Nazir et al., 2011) and irrespective of HA subtype (Scholtissek, 1985). Interestingly, even within the same virus strain, differences in stability are found. An H7N3 recovered from a mallard remained infectious for 6 months, while an H7N3 recovered from a laughing gull retained infectiousness for 7.5 months (Stallknecht et al., 2010). In an analysis of five closely related reassortant of low pathogenic avian influenza (LPAI), all viruses showed decreased persistence with higher temperatures, but the rate of that decline differed between viruses and the rank order for virus stability differed across temperatures. For example, H3N8 was the shortest lived of the five viruses at 4C, but was one of the longest lived at 17C (Lebarbenchon et al., 2012). Likewise H6N1 declined rapidly with increased temperature, while H4N8 was relatively robust at all temperatures tested. These five viruses were reassortants isolated from wild ducks in Minnesota from the same region at the same time, and since they showed no difference in viral shedding, these modest differences in viral persistence could conceivably impact which strain is propagated in the host population. These sorts of studies further illuminate the incomplete

picture that is obtained when surveillance efforts focus exclusively on the viruses present in the bird population without consideration to the environmental component.

Since influenza is an enveloped virus, the inverse correlation between viral persistence and temperature may in part be explained by the effect of temperature on lipid fluidity and membrane stability. At 4C the lipids that comprise the influenza outer envelope are ordered, rigid, and therefore environmentally stable. This state changes as temperature increases, with the lipids becoming completely disordered and therefore fluid at 41C (Polozov et al., 2008). The disordered fraction facilitates the ability of the virus to fuse, uncoat, and establish infection. Temperature has been demonstrated as a key determinant for aerosolization (Lowen et al., 2007, 2008), and lipid composition of influenza has been shown to impact fusability, infectivity (Sun and Whittaker, 2003; Takeda et al., 2003) and the potential for aerosolization (Polozov et al., 2008). The balance between ordered (cold tolerant) and disordered (fusion competent) states of the virus likely impacts tolerance for indirect transmission and pathogenicity and is probably determined by lipid composition. As lipid composition is acquired from the host cell and at least partially directed by viral components (Rossman and Lamb, 2011; Veit and Thaa, 2011), lipid composition would be expected to vary between different viruses and host species. These differences may therefore help to explain the variation in temperature stability across influenza isolates and merits further study.

While most viruses are labile in high salinity associated with ocean water, there is considerable strain variability in the continuum between fresh and brackish water (Brown et al., 2009; Stallknecht et al., 2010). A close analysis of several LPAI H5 and H7 documented that H7 decrease less rapidly at a high salinity than H5 (Brown et al., 2007). Even between two different isolates of high pathogenic avian influenza (HPAI) H5N1, one isolate persisted longer with low salinity and the other at high salinity (Brown et al., 2007). AIV is typically the most stable at a neutral to slightly basic pH (7.4–8.2) (Brown et al., 2009), with extreme highs and lows detrimental to the virus and resulting in a nearly 7-fold drop in persistence (Irwin et al., 2011). There are however exceptions, since H6N4 shows the greatest stability at pH 8.6 (Brown et al., 2009), and there is considerable variability in the pH threshold isolates can tolerate before being rendered non-infectious (Scholtissek, 1985; Stallknecht et al., 2010). H2 and H11 maintain viability down to 4.8 and 4.6 respectively, while H8 and several H1, H5, and H7s are rendered non-infectious at a pH of approximately 6.0 (Scholtissek, 1985). Finally, there is a general trend for AIV to be more robust than human influenza at low pH (Webster et al., 1978).

Several studies have also found interactions in how temperature, salinity, and pH impact viral persistence. Viruses in brackish conditions (0.5 – 20 ppt) persist the longest at pH 6.2, while the same virus in freshwater (> 0.5 ppt) persist the longest at a pH 8.2 (Stallknecht et al., 1990a). This same trend for higher salinity to overcome low pH was also seen for at least some virus isolates by Keeler et al. (2012). For a given virus, temperature can influence both ideal salinity (Brown et al., 2007) and pH tolerance (Stegmann et al., 1987). The HA fusion mechanism is irreversibly triggered at pH 5 and in physiological conditions, triggering outside of the endosomal compartment renders the virus nonviable. However the HA conformational change at low pH does not occur when the virus is at 0C (Stegmann et al., 1987) and therefore lower temperatures are protective against low pH.

Differences in aquatic compositions might influence which subtypes can persist in different habitats, thereby impacting species specificity of AIV. Several environmental parameters of interest have previously been proposed, including presence of or adherence to metals and organic compounds, sunlight, bacteria, biofilms and bivalves (Stallknecht et al., 2010). The handful of studies that have begun to address these additional features of the biotope and its role in viral stability have already begun to uncover intriguing results. Survival of virus is

favoured in filtered water compared to unfiltered water (Domanska-Blicharz et al., 2010; Irwin et al., 2011). This was nicely demonstrated by inoculating virus into Baltic Sea water. Untreated water resulted in a rapid loss of infectiousness, while pre-filtering the sample of water prior to inoculation resulted in prolonged virus survival (Domanska-Blicharz et al., 2010). This suggests that at least some microorganisms may be detrimental to environmental persistence of virus. Likewise, clams may also limit virus persistence. When placed into H3N8 inoculated water, freshwater clams reduced the virus concentration to just above the detection limit within 24 hours and the virus laden clams did not infect ducks upon consumption (Faust et al., 2009). Differences in susceptibility across subtypes and how that may relate to host specificity have not yet been examined. Microorganisms or freshwater clams may limit indirect virus transmission in specific water habitats and thereby influence which hosts become infected. While some aquatic life forms may hinder the persistence of virus in water, a small number of studies have suggested such individuals may act as a potential reservoir for virus. Following experimental inoculation, live virus (H5N1) can be recovered from tadpoles and fighting fish one day after the animals are submerged into infected water (Horm et al., 2012). High concentrations of viral RNA were detected in water fleas placed into H4N6 or H5N1 inoculated water (Abbas et al., 2012), and virus was recovered from mussels for up to six days after placement into contaminated water (Horm et al., 2012). Further work is needed to know if these potential reservoirs either release infectious virus back into the environment, or serve as a viral delivery vehicle to birds that include them in their diet.

Environmental contributions to the indirect transmission of AIV are complex, multifaceted, and barely beginning to be understood. A more nuanced understanding of how environmental parameters impact virus persistence can help to identify habitats where transmission, and especially interspecies transmission, is most likely to occur. While the current methodological limitations render environmental surveillance a particularly challenging task, the critical nature of the results in understanding AIV ecology and potential human epidemics necessitates that such surveillance efforts are pursued.

3. Genomic signatures of potentially pandemic viruses

Transmission of IAV to humans from wild animals or environmental sources caused four major pandemics in the last two centuries: H1N1 (Spanish flu) in 1918, H2N2 in 1957, H3N2 in 1968 and H1N1 in 2009 (Cheng et al., 2012; Taubenberger and Morens, 2006; Wright et al., 2007). The past pandemics and the threat of H5 and H9 emergence in humans highlight the public health concern. Unfortunately, frequent genetic drift and shifts are driving influenza virus evolution at a very high pace (Chen and Holmes 2006; Taubenberger and Morens 2009), making predicting features of the next pandemic virus difficult. Pandemic preparedness involves three main approaches: analysing viruses that caused the previous pandemics, understanding virus/host ecology, and monitoring viral evolution by continuous surveillance. Analysing the sequences of previous pandemic viruses is instrumental in understanding how they evolved and identifying the molecular signatures associated with host switching and enhanced virulence to humans. Studying the virus ecology, which is tightly bound to the host ecology, is essential for understating the patterns of AIV global spread. Early detection of pandemic virus precursors via continuous surveillance of wild animals is a pre-emptive strike against the aftermath of a pandemic because it provides us a golden opportunity to prepare vaccine seed strains before the pandemic even begins (Monto et al., 2006). The numerous studies of previous pandemic viruses revealed that they all carried at least one or more avian genomic segments and arose by one of two mechanisms; either via gradual adaptation of a purely avian virus or modification of a human-adapted virus by genetic reassortment (Smith et al., 2009; Taubenberger and Kash, 2010). In this section of our review, we will highlight the known molecular changes (see Fig. 5) associated with the complex multi-step process of host

switching from wild to domestic birds and then to mammalian species and eventually inter-mammalian transmissibility (the pandemic prototype).

3.1 Learning from avian influenza viruses in the wild

Surveillance in wild birds may serve as an early warning system for highly pathogenic or potentially pandemic influenza strains (Hoye et al., 2010b), but isolation of strongly pathogenic strains, including H5N1 has been infrequent. Infection of birds is mostly asymptomatic, where the virus infects epithelial cells lining the intestine and is shed in the feces. Transmission of influenza virus to a non-infected bird is thought to occur mainly via the fecal-oral route (Munster et al., 2007) but only on rare occasions is the highly pathogenic form of IAV reported in wild birds. The first reported case of high mortality in wild birds was due to H5N3 infection in common terns (*Sterna hirundo*) of South Africa in 1961 (Becker, 1966). More recently, in late 2002, an outbreak of HPAI H5N1 was first reported in migratory waterfowl in Hong Kong, and a few months later, an avian H5N1 virus closely related to one of these viruses was isolated from two human cases (Sturm-Ramirez et al., 2004).

Direct transmission from wild birds to humans, however, is extremely rare and usually has involved an intermediate species such as domestic poultry (Reperant et al., 2012). Serologic evidence suggests that some bird hunters and banders are exposed to IAV strains that are found mainly in wild ducks such as H11N9 (Gill et al., 2006; Gray et al., 2011), but clinical infection is not well documented. For H5N1, the only report of suspected direct transmission of H5N1 from wild birds to humans was in Azerbaijan as a result of de-feathering of infected swans (Gilsdorf et al., 2006).

Phylogenetic analysis has revealed that some gene segments belonging to previous human pandemic strains are still circulating in wild bird reservoirs. For instance, the NA genes of some H9N2 viruses isolated from migratory ducks in Hokkaido, Japan, clustered with those of H3N2 viruses that caused the human pandemic of 1968 (Liu et al., 2003). Moreover, it has been speculated that the 3 parents of the triple reassortant virus that caused the 2009 H1N1 pandemic may have been assembled in one place by migratory birds (Gibbs et al., 2009). With the exponential rise in sequence information in public databases, a critical need is to phenotypically characterize individual strains which appear to be unique or to carry characteristic mutations of highly pathogenic virus.

3.1.1 Intercontinental mixing—Mixed infection and reassortment have been shown to be extremely common in wild birds (Wang et al., 2008). After analyzing 167 complete genomes recovered from cloacal swabs of 14 bird species sampled across North America, (Dugan et al., 2008) proposed that, in the absence of strong mammalian selective pressure that favours the spread of only a limited number of stable subtypes, IAV exist in wild birds as a large pool of transient genome constellations that are continuously reshuffled by reassortment. The impact of these reassortment events on the evolution of LPAI viruses is not clearly understood. A similar assessment of oral-pharyngeal samples has yet to be attempted, possibly owing to the poor recovery of virus from the trachea of dabbling ducks (Munster et al., 2009; Webster et al., 1978). Gulls and passerines fit the description of an intermediate host more closely than mallards, but oral-pharyngeal samples are lacking because of difficulties implicit with capture of these non-game species. Collection of droppings or fecal samples is a more popular strategy for sampling Laridae - fecal samples comprised 28% of total samples collected globally, compared to the 5% collected from Anatidae (Fig. 2). Collection of fresh droppings from the ground is easier and less involved than live capture and allows a larger number of samples to be collected (Hoye et al., 2010b). However, this strategy is likely to be biased when used to assess prevalence for Laridae that shed IAV primarily from the respiratory tract based on experimental studies (Brown et al., 2006; Costa et al., 2011). Moreover, Strum-Ramirez et al.

(2005) observed that viruses replicated to higher levels in the trachea than in the cloaca of both inoculated and contact birds, suggesting that the digestive tract might not be the main site of H5N1 influenza virus replication in ducks and that the fecal-oral route might not be the only transmission route. Thorough assessment of potential intermediate hosts in nature will require increased collection of oral-pharyngeal swabs from avian species including those that have traditionally not been a focus of surveillance.

Global phylogenetic analysis splits IAV into two main lineages: Eurasian and American, which reflect the ecological and geographic separation of avian hosts. However, they are not completely separated because of the intercontinental movements of some long-distance migrant ducks, gulls, and shorebirds (Liu et al., 2004; Makarova et al., 1999). A majority of these intercontinental gene-mixing events in the U.S. are found in viruses isolated from Alaska, which is located at the crossroads of Eurasian and North American migratory flyways and receives about 1.5–2.9 million birds from Asia every year (Winker and Gibson, 2010). Although still a rare event overall, viruses carrying a mix of Eurasian and American genes have been isolated at a high frequency from gulls (Dugan et al., 2008; Widjaja et al., 2004). A thorough sequence analysis of gull influenza isolates from the U.S. revealed segments with a mosaic phylogeographic pattern; with at least one segment in the majority of those viruses originating from Eurasian lineages (Wille et al., 2011a). No study to date has detected movement of a complete virus derived from either clade into the other continent (Krauss et al., 2007).

3.1.2 Molecular determinants of host specificity within wild birds—Wild birds play an important role in the ecology of IAV, however, the factors governing interspecies transmission or host-subtype associations are largely unknown. Studying the patterns of attachment of a human (H3N2) and an avian (H6N1) virus to the colon and trachea sections from 12 wild bird species using histochemistry techniques revealed significant variations between closely related avian species, suggesting that the ability of wild birds to serve as hosts for AIV strongly varies among species (Jourdain et al., 2011). Some gull species, in particular, may be important to IAV reassortment due to their frequent intercontinental movements. Although H13 and H16 subtypes are believed to be gull-specific, gulls as a group have been shown to host many other viral subtypes (Munster et al., 2007; Olsen et al., 2006; Wille et al., 2011a). Phylogenetic analysis of the HA and other internal genes of H7N3 viruses isolated from gulls and shorebirds in the Delaware Bay area showed that they are closely related to HPAI H7N3 viruses that caused the 2004 outbreak in chickens in British Columbia (Hirst et al., 2004; Krauss et al., 2007). The Delaware Bay H7N3 viruses replicated well in chickens and killed chicken embryos, suggesting that they might have high potential to evolve into HPAI if transmitted to chickens.

The majority of viruses isolated from gulls though are unable to infect experimentally inoculated ducks, suggesting there are host barriers between wild bird species (Kawaoka et al., 1988). The exact mechanisms controlling these observed species preferences are not clearly understood, but will most likely involve differences in receptor specificities between viruses isolated from ducks and gulls as a result of host adaptation (Matrosovich et al., 2009, 2008). Some receptor-binding site substitutions that are unique for gull-specific subtypes (such as Y98F, A138S and E190T in H16, G228S and R229W in both H13 and H16) could be playing a role in fine-tuning the interaction with non-identical receptors in these hosts. The substitution of G to S at position 228 is of particular importance because it has been shown to affect receptor-binding preference of human H2 and H3 viruses (see further discussion in section 3.3.1 below). A switch from P to L at position 215 is capable of changing the configuration of the receptor binding domain (RBD). Furthermore, at position 222, all viruses isolated from ducks carry a bulky amino acid (K, P, R, L, Q or W), which is substituted by a small one (G) in the HA of H13 viruses.

It is often overlooked that avian viruses may not be uniform in their binding ability to α-2,3 linked SA receptors. However, by comparing the patterns of viral binding to a panel of synthetic sialylglycopolymers (SGPs) having the same terminal α-2,3 linked SA fragment and differing only in the structure of the inner parts of the carbohydrate chain, Yamnikova et al. (2003) and Gambaryan (2005) were able to demonstrate significant differences between chicken, duck, and gull viruses. These findings raised questions about the impact of these differences on the transmissibility of IAV.

A better understanding of the exact chemical nature of glycan receptors and their distribution on tissues from different species of wild birds would greatly enhance our knowledge of the virus/host interactions in the wild. Although several investigators have studied the receptor expression patterns in chickens, ducks and other species of domestic and wild birds (Costa et al., 2012; Kuchipudi et al., 2009; Pillai and Lee, 2010; Yu et al., 2011), these studies have probed only for the two main glycosidic linkage types using lectin histochemistry staining. Therefore, a comprehensive understanding of glycan receptor distribution on tissues of different species of birds is still lacking. Mass spectrometry is an important tool that provides a systematic analysis of the total glycan content of tissues because of its ability to detect glycans in complex mixtures with high sensitivity. It provides an insight into the fine structural details of glycans such as length and branching, beyond the simple description of the glycosidic linkage (Nicholls et al., 2012; Viswanathan, 2010).

3.1.3 HPAI H5N1 in wild birds—H5N1 viruses are a particularly high priority due to their frequent emergence in poultry as HPAI and to documented human disease. Surveillance studies of wild birds in northern Europe provided evidence that they harbor the LPAI ancestral viruses of HPAI H5 and H7 strains found in poultry. For each of the HPAI outbreaks that occurred in Europe since 1997, closely related LPAI relatives were found in mallards (Munster et al., 2005). In countries where H5N1 infections of wild birds have been documented, such as China, there was little evidence that HPAI H5N1 strains were perpetuated early on (Ellis et al., 2004). Therefore, the role of wild birds in the geographic spread of HPAI H5N1, particularly to the US, is strongly debated (Flint, 2007). It has been speculated that infected Asian wild birds can't transport H5N1 for long distances because infection would negatively affect their health and hinder or significantly delay migration (Normile, 2005). Although surveys conducted in the U.S. during the period 2006–2008 showed that wild birds were free of HPAI H5N1 (Deliberto et al., 2009), globally, H5N1 disease clusters along several flyways were found to be associated with the seasonal migration of wild birds, spreading from endemic poultry sources in southern China to other regions (Si et al., 2009). Wild birds have also been implicated in the spread of H5N1 to countries in the Middle East such as Egypt (Saad et al., 2007). Since H5N1 viruses were present in apparently healthy migratory birds just before their migration in this region, it was proposed that wild birds in synergism with poultry trade play an important role in the spread of H5N1 over long distances (Chen et al., 2006c; Kilpatrick et al., 2006). The question of whether wild birds can be silent carriers may rely on the documentation of infection in birds that are healthy enough to migrate on both ends of the flyways. Indeed, some experimental infection studies, showed that HPAV H5N1 infection is not fatal for certain species of waterfowl and shorebirds (Brown et al., 2006; Kalthoff et al., 2008; Keawcharoen et al., 2008; Perkins and Swayne, 2002), suggesting that wild bird species, particularly mallards, can potentially be long-distance vectors of highly pathogenic avian influenza virus (H5N1).

3.1.3.1 Molecular markers of H5N1 pathogenicity in wild birds: As mentioned, several H5N1 outbreaks in Asia have resulted in mortalities in waterfowl since 2002. The molecular determinants of pathogenicity in ducks are poorly understood. A few studies have pointed to the PA and PB1 subunits of the polymerase complex as major contributors to virulence in ducks. Introducing two mutations into the PB1 (Y436H) and PA (T515A) genes reduced the

virulence of a small-plaque phenotype of A/Vietnam/1203/04 (H5N1), which is known to be highly virulent for ferrets, mice and mallards (Hulse-Post et al., 2007). Two amino acid substitutions in the PA (S224P and N383D) of the A/duck/Hubei/49/05 virus were associated with a highly virulent phenotype (Song et al., 2011). Additional work on H5N1 pathogenicity in wild birds is needed to clarify the constraints on H5N1 evolution and transmission in reservoir species.

3.1.3.2 Spillover from domestic to wild birds: Many of the wild bird mortalities due to H5N1 have coincided with outbreaks in poultry (Kwon et al., 2005; Lee et al., 2005). Molecular analysis of these viruses indicated that the majority of them are spillover events from domestic poultry outbreaks. H5N1 viruses that caused an outbreak in Qinghai Lake in western China in 2005, which resulted in high mortalities in bar-headed geese (*Anser indicus*) and gulls had multi-basic insertions at the cleavage site of HA and a deletion of 20 amino acids in the NA, which indicate previous adaptation to domestic chickens (see section 3.2.1 below) (Chen et al., 2006b, 2005). In addition, surveys on IAV in wild black-billed magpies (*Pica hudsonia*) in Guangxi, China, have identified some interesting H9N2 reassortants carrying H5N1-like PB1 genes, which presumably derived from the co-circulating H9N2 and H5N1 viruses. These reassortants had similar motifs at the HA cleavage site to LPAI H9 chicken isolates and also NA stalk deletions similar to current prevailing chicken isolates, suggesting that these viruses were transmitted from domestic chickens (Dong et al. 2011b). The repeated transmission from poultry to wild birds has raised some concerns about the possibility of H5N1 adapting to and becoming endemic in wild bird populations. As of 2011 the United Nations Food and Agriculture Organization considers H5N1 virus to be endemic in China, Bangeladesh, Vietnam, Indonesia, India, and Egypt (http://www.cdc.gov/flu/avianflu/h5n1-animals.htm). The establishment of silent H5N1 infections in wild birds would pose a serious threat, especially if they retain pathogenicity to other species (Boyce et al., 2009).

3.2 Host switching - wild to domestic birds

IAV are usually introduced to domestic poultry either directly via shared aquatic habitats and drinking water sources or indirectly via contaminated farming equipment (Alexander, 2007; Reperant et al., 2012). However, adaptive changes are commonly seen to establish infection in domestic poultry with wild bird derived IAV. In fact, since human isolates from domestic poultry outbreaks frequently resemble them, it was proposed that adaptation to land-based poultry facilitates transmission of novel IAV to humans (Wright et al., 2007). The additional finding that α-2,6 linked SA receptors were found with great abundance in chicken tracheal sections strongly suggests that chickens can be important intermediate hosts for generating zoonotic IAVs (Kuchipudi et al., 2009).

The HA protein is synthesized as a single polypeptide precursor (HA0), which is matured by proteolytic cleavage via trypsin-like cellular enzymes, producing the HA1 and HA2 proteins (Skehel and Wiley, 2000). Many H5 and H7 viruses evolve into HPAI in the chicken, usually through the acquisition of polybasic amino acid insertions (R and K residues) at the HA0 cleavage site. This change facilitates systemic virus spread by rendering the HA0 cleavable by ubiquitous proteases available in many body tissues. However, it was shown that acquisition of a polybasic cleavage site by itself was not sufficient for converting virus into HPAI for chickens, and other changes involving additional viral proteins are required (Stech et al., 2009).

Surveillance of IAV circulating in domestic poultry is considered a high priority for eliminating potential human epidemics resulting from zoonotic transmission from poultry. The H5N1 viruses that spread in Hong Kong in late 1997 crossed the species barrier to humans, causing respiratory infection in 18 patients and death in 6 after close contact with poultry. Fortunately

the virus didn't spread from person to person and a culling of over 1.5 million chickens is largely credited with averting further human infection and possibly pandemic spread (Shortridge et al., 2000). A comprehensive map of the various determinants involved in adaptation to domestic birds is still lacking. The currently known molecular features that reflect viral adaptation to poultry include: in-frame deletion in the NA stalk region and substitutions in HA and NS.

3.2.1 NA stalk deletions—HA binds to SA linked to cellular membrane glycoproteins, whereas the sialidase activity of the NA facilitates the release of progeny virions as a receptor-destroying enzyme, essential for release of viral particles from the host cell after budding. The NA stalk is a structure that separates the globular, enzymatically active head of the tetrameric NA from the hydrophobic transmembrane domain (Russell et al., 2006). Stalk deletions associated with adaptation to poultry have been reported in many subtypes and usually range from 20–30 amino acid residues (Campitelli et al., 2004; Giannecchini et al., 2010; Li et al., 2010; Mundt et al., 2009; Sorrell et al., 2010). IAV with a short stalk have not been isolated from waterfowl except in cases where HPAI H5N1 spilled over to wild birds (Chen et al., 2006b; Liu et al., 2005), strongly suggesting that this variant does not have a selective advantage in wild birds. A comprehensive analysis of thousands of NA sequences by Li et al. (2011) revealed that these deletions were often accompanied by changes in the HA such as addition of glycosylation sites, presumably to maintain functional balance between HA and NA, which is necessary for viral infectivity (Lu et al., 2005; Matrosovich et al., 1999; Mitnaul et al., 2000; Wagner et al., 2000).

Experimentally, it was shown that NA stalk deletions enhanced IAV replication in chickens; however, the molecular mechanism behind this growth advantage is still unclear. The sialidase activity of NA orchestrates the release of progeny virions. Although these deletions are expected to negatively affect the function of NA, the release of a recombinant LPAI H1N1 virus carrying an engineered NA stalk deletion was not affected (Munier et al., 2010).

Previous studies have shown that Japanese quails (*Coturnix japonica*) can play an important role as an intermediate host in the adaptation of IAV to land-based birds. Japanese quail are highly susceptible to wild-bird derived IAV and have been implicated in the transmission of IAV subtypes that have crossed the species barrier to humans, including H5N1 and H9N2 (Guan et al., 1999; Makarova et al., 2003; Wan and Perez, 2006). Sequence analysis of a quail-adapted mallard strain of H2N2 (A/Mallard/Potsdam/178-4/83) identified 6 mutations in 4 genes, PB2 (A588V), PB1 (Q268R, D398E, S654I), NP (A234T) and HA (N155D), suggesting that the internal genes also play a role in host adaptation (Sorrell and Perez, 2007). However, adaption of the quail-adapted virus to chickens was accompanied by an additional mutation in the HA (K303Q) and a deletion in the NA stalk region. These adaptive changes altered viral behaviour from intestinal shedding to shedding and transmission via the respiratory tract, indicating that the NA stalk deletion is a major determinant of respiratory tropism of IAV (Sorrell et al., 2010).

3.2.2 HA acid stability—After virus uptake by receptor-mediated endocytosis, the virus is exposed to the acidic pH of the endosome, which triggers fusion between the viral and endosomal membrane and release of the viral nucleocapsids into the cytoplasm (Palese and Shaw, 2007). The acid stability of HA is another factor affecting IAV pathogenicity and ecology. Mutations that modulate HA acid stability have been associated with changes in viral pathogenicity and environmental persistence. An increase in H5N1 pathogenicity in chickens was correlated with an increase in the pH of HA activation, which was linked to variations at residues 104 and 115 located in the N- and C-termini of helix-110 of HA1 (DuBois et al., 2011). On the other hand, an H5N1 virus carrying an H24Q mutation, which decreased the pH of HA activation, was shed more extensively from infected mallards into drinking water and

persisted for a longer period in the environment (Reed et al., 2010). The molecular determinants of viral persistence in the environment deserve further investigation as described above. Although a few studies have revealed some intriguing differences in the environmental persistence of viral isolates belonging to the same HA subtype in particular, decoding these determinants has been hampered by the lack of HA sequences (Brown et al., 2007; Stallknecht et al., 2010).

3.2.3 HA and NS substitutions—Other HA amino acid substitutions associated with IAV adaptation to chickens are A198V and S274F (Li and Cardona, 2010). The NS genes of H5N1 viruses, which re-emerged from geese in Hong Kong's chickens in 2001, carried a unique 5-aa deletion (position 80–84) in the middle of the NS1 protein (Guan et al., 2002). Whether these substitutions are associated with the interspecies transmission from aquatic birds to domestic birds is still unknown. More experiments are needed to delineate the biological role of these mutations.

3.3 Host switching – Mammalian jumps

IAV circulating in birds may also acquire certain changes that render them transmissible to mammals, including humans, pigs, horses, dogs and seals. Despite the debate in the literature about the origin of the 1918 H1N1 pandemic virus and whether it originated from a purely adapted avian virus or as a result of reassortment (Smith et al., 2009; Taubenberger and Kash, 2010), H5N1 and H9N2 viruses represent elegant examples of mammalian adaptation. H9N2 are low-pathogenic IAVs that were firstly isolated in 1966 in the US from turkeys (Homme and Easterday, 1970). Since their isolation in North America, H9N2 IAV on the North American continent have been found mainly in shorebirds and wild ducks, with no evidence of permanent lineages of these viruses in land-based poultry. In 1988, the isolation of an H9N2 virus from Japanese quail in Southern China was the first recorded land-based poultry case of H9N2 in Asia (Perez et al., 2003). H9N2 viruses continued to disseminate and became endemic in domestic poultry outside of North America (Bi et al., 2010; Dong et al., 2011a; Fusaro et al., 2011; Hossain et al., 2008; Xu et al., 2007). Since 1997, there have been several reports of transmission of H9N2 IAV from land-based poultry to mammals, including humans and pigs (Cong et al., 2007; Lin et al., 2000). Experimentally, it was shown that H9N2 IAV acquired affinity to bind efficiently to α-2,6 linked SA receptors (Matrosovich et al., 2001), considered one of the key elements for human infectivity. Given the potential of H9N2 to transmit to humans, this group of viruses is currently on the list of the WHO as a potentially pandemic virus (Alexander et al., 2009; Li et al., 2003).

In section 3.3, we will highlight the main genetic markers of such IAV species jumps to mammalian hosts, particularly humans, where research efforts have focused.

3.3.1 HA receptor binding domain—The RBD of HA is a critical determinant of IAV host tropism and transmissibility because it mediates the initial interaction between the virus and the SA receptor (Chandrasekaran et al., 2008). Structurally, it is composed of 3 main elements: helix-190 (residues 188–194), loop-220 (residues 221–228) and loop-130 (residues 134–138). Other highly conserved residues, such as Tyr98, Trp153, His183 and Tyr195, form the base of the receptor-binding pocket (Skehel and Wiley, 2000). Amino acid substitutions affecting the conformation of the RBD usually result in changes in the receptor-binding affinity of HA and a consequent switch in host species specificity (Medina and Garcia-Sastre, 2011). HA recognizes host glycans with terminal SA residues, which represent a diverse family of sugars with a 9-carbon backbone that vary in structure among different species. SA are the outermost unit on glycan chains with two main types of linkage to the underlying galactose (Gal) arising from carbon-2. SA can either be linked to carbon-3 of Gal to form an α-2, 3 glycosidic linkage or to carbon-6 of Gal to form an α-2,6 glycosidic linkage (Nicholls, 2008;

Wilks, 2012). It is generally believed that avian viruses preferentially bind to SA receptors with an α-2,3 linkage, whereas human viruses prefer an α-2,6 linked SA; and a switch from α-2,3 to α-2,6 is a prerequisite for the adaptation of avian viruses to the human host (Wright et al., 2007). Therefore, identifying RBD mutations that would enable this switch might be of great value to preparing for the emergence of pandemic strains. Additional studies also suggest that HA-receptor interactions are more complex than the simple α-2,3 versus α-2,6 dichotomy. In addition to the type of the linkage, the terminal SA itself and the overall glycan size and topology are also important determinants of binding affinity (Chandrasekaran et al., 2008; Gambaryan et al., 2003; Imai and Kawaoka, 2012; Ito et al., 2000; Suzuki et al, 2000).

The effects of point mutations and topology of the RBD on the receptor binding affinity of IAV have been extensively studied in viruses that caused pandemics in humans (H1, H2 and H3) and viruses considered to be potentially pandemic (H5 and H9). In particular, the recent advances in glycan microarray technologies have revolutionized our understanding of the interaction between influenza viruses and their host cell receptors. This technology enables investigators to pinpoint, with a high degree of accuracy, the differences between HA binding to hundreds of different glycans attached to a single chip (Stevens et al., 2006c). Comparative sequence analysis revealed that human adapted H2N2 and H3N2 viruses, which caused the 1957 and 1968 pandemics, required as few as two amino acid substitutions near the RBD (Q226L and G228S) to switch their receptor binding affinity from the avian α-2,3 to the human α-2,6 type (Connor et al., 1994). On the other hand, two different amino acid substitutions (E190D and G225D) within the RBD of H1N1 viruses, which caused the 1918 Spanish flu pandemic, mediated the direct avian-to-human switch (Matrosovich et al., 2000; Stevens et al., 2006a). Despite the structural similarities between the HA proteins of H5N1 and 1918 H1N1, introducing the E190D/G225D mutations didn't enhance the binding of the HA of a HPAI H5N1 virus (A/Vietnam/1203/2004) to α-2-6 linked SA on a glycan array chip. Surprisingly, however, introducing the G226L/G228S double mutation (typical of H2 and H3), did not fully convert the H5N1's HA to α-2,6 linked SA specificity; although it reduced its binding affinity to α-2,3 linked SA (Stevens et al., 2006b). Other HA mutations, such as N182K and Q192R, have been reported to enhance the binding of H5 to the human-type receptor (Yamada et al., 2006). In H9N2 viruses, a frequently detected mutation, Q226L, was shown to increase the affinity of virus to bind to human-type α-2,6 linked SA receptors, replicate better in human airway epithelial cells, and transmit more efficiently to direct contacts in a ferret model (Matrosovich et al., 2001; Wan and Perez, 2007; Wan et al., 2008). However, amino acid substitutions within the RBD do not always correlate with enhanced virus transmissibility. An example is the D222G mutation in 2009 pandemic H1N1 virus (Belser et al., 2011). Therefore, changes in the RBD that are associated with IAV adaptation to humans seem to be very complex and subtype-dependent. More work will be needed to determine if patterns are apparent in the repertoire of potential sequence changes.

3.3.2 Polymerase—Receptor binding is only one part of a successful viral life cycle and a productive infection in the host. The polymerase complex (PB2, PB1 and PA) is essential for transcribing and replicating the negative-sense viral genomic RNA. Polymerase genes appear to be critical for adaptation of AIV to the human host (Boivin et al., 2010). Replacing the polymerase gene complex of A/Vietnam/1203/04, a fatal human case H5N1 isolate, with that of a non-lethal strain completely attenuated it, highlighting the importance of the polymerase complex for viral virulence (Salomon et al., 2006). Using the wealth of sequences available for thousands of IAV isolates, several investigators used a suite of computational tools to identify markers that discriminate human from avian viruses, in an attempt to understand how avian viruses adapt to humans and cause pandemics. Only a subset of these markers was conserved in all human pandemic influenza virus sequences, such as the A199S, E627K and K702R substitutions in the PB2 protein. Although these markers were distributed among all

genes, the majority of them were found in the three proteins of the viral polymerase complex, particularly at the domains where these proteins interact (Allen et al., 2009; Chen et al., 2006a; Finkelstein et al., 2007; Tamuri et al., 2009). Moreover, several adaptive evolution experiments in mouse models have linked lethal mutations to the viral polymerase genes (Gabriel et al., 2007, 2005; Ping et al., 2011). Together, these studies suggest that the polymerase complex is highly influenced by the host environment.

3.3.2.1 PB2: Among the 3 proteins (PB2, PB1 and PA) of the viral polymerase complex, PB2 appears to play the most profound role in viral adaptation to mammalian hosts, particularly humans. An E627K mutation is one of the most important determinants that confer the ability to infect humans because it allows the virus, which normally grows at 40C in the avian intestinal tract, to grow at the lower temperature of the human upper respiratory tract (33C) (Gabriel et al., 2005; Subbarao et al., 1993). The E627K change has been correlated with the enhanced virulence of many HPAI H5N1 strains and was shown to be essential for optimal interaction of PB2 with NP and other cellular proteins involved in transcription and replication (Labadie et al., 2007; Ng et al., 2012). D701N is another PB2 mutation that has been shown to be implicated in adaptation to growth in human cells (Li et al., 2005; Steel et al., 2009). These mutations were not found in the H1N1 2009 pandemic virus (pdmH1N1) and introducing them by reverse genetics did not increase polymerase activity or have an impact on virus replication *in vitro* or *in vivo* (Herfst et al., 2010; Jagger et al., 2010). These findings sparked interest in finding other PB2 residues that might contribute to enhanced pdmH1N1 IAV replication in mammalian cells. 590S and 591R mutations have been identified as important residues for polymerase activity and for efficient virus replication (Mehle and Doudna, 2009). Based on a crystal structure of the C-terminal regions of H5N1 and H1N1 PB2, residues 590 and 591 were found to lie very close to residue 627 (Yamada et al., 2010). Therefore, it was concluded that these 2 residues may compensate for the lack of lysine at position 627 and confer efficient replication on pdmH1N1 in mammals. Other PB2 markers of suggested pathogenicity have also been identified in H9N2 viruses, which are another group of high concern for potential pandemic strains. A combination of either D253N/Q591K or M147L/E627K mutations resulted in a polymerase with higher *in vitro* activity and increased viral replication efficiency in human bronchial epithelial cells and mice (Mok et al., 2011; Wang et al., 2012b).

3.3.2.2 PB1: PB1 in the polymerase complex has also been reported to contribute to viral adaptation to the mammalian host. PB1-F2 is a small proapoptotic viral protein (90 amino acids) that is encoded within the PB1 gene by an alternative reading frame (Chen et al., 2001). A single amino acid substitution (N66S), which was found in both Hong Kong 1997 H5N1 and the 1918 pandemic H1N1 virus, was shown to increase virulence in mice (Conenello et al., 2007).

3.3.2.3 PA: Serial passage of the LPAI wild-bird H5N2 in a mouse model identified a T97I in the PA protein to be a key determinant of enhanced virus replication in mice (Song et al., 2009). Moreover, a recent study showed that the exchange of entire PA segments between avian and human viruses (akin to a reassortment event) facilitates viral adaptation to humans. An avian polymerase from A/green-winged teal/Ohio/175/1986(H2N1) carrying a PA subunit from the 2009 pdmH1N1 virus exhibited increased polymerase activity *in vitro* and helped the virus to overcome growth restriction in human cells. Reassortant viruses showed enhanced replication kinetics and pathogenicity to mice. This enhancement in replication efficiency was mapped to a single amino acid substitution in the PA (T552S) (Mehle et al., 2012). These and other mutations in the genes encoding the viral polymerase have demonstrated that genetic diversity encoded in these segments may play a very important role in viral adaptation and pathogenicity in a new host. Many more such changes in wild viruses await analysis.

3.3.3 Genetic reassortments—In addition to adaptational mutation, reassortment is an important mechanism for the generation of potentially pandemic influenza strains. Sequence analysis revealed that the pandemics of 1957 and 1968 were caused by avian-human reassortants that acquired human receptor binding properties (Taubenberger and Kash, 2010). Phylogenetic analysis has suggested that the H5N1 influenza viruses that caused 1997's outbreak in Hong Kong were reassortants that obtained their internal gene segments from a quail H9N2 virus (Qa/HK/G1/97) (Guan et al., 1999). Several studies have been conducted to investigate the possibility of pandemic strain emergence via reassortment between human and avian or swine viruses. A human H3N2 reassortant virus carrying the internal genes of avian H5N1 exhibited reduced replication and transmission in a ferret model, suggesting that the genetic basis of mammalian transmissibility is complex (Maines et al., 2006). Coinfection with H9N2 and 2009 pdmH1N1 influenza viruses in the same host (e.g., pigs and humans) could provide the opportunity for reassortment between these viruses. Experimentally, 127 hybrid viruses derived from these two subtypes by reverse genetics showed high genetic compatibility and more than half replicated to a high titre *in vitro. In vivo* studies of 73 of 127 reassortants revealed that all viruses were able to infect mice without prior adaptation and 8 reassortants exhibited higher pathogenicity than both parental viruses (Sun et al., 2011). Moreover, it was also shown that H9N2/H3N2 (seasonal influenza) and H9N2/pdmH1N1 (swine flu) reassortant viruses have been shown to infect and transmit by respiratory droplet transmission in ferrets after adaptation by serial passage and incorporation of amino acid changes on the surface and internal genes (Kimble et al., 2011; Sorrell et al., 2009). In addition, H9N2/pdmH1N1 reassortants replicated and transmitted more efficiently in pigs than the parental H9N2 virus (Qiao et al., 2012). The sum of this work demonstrates the possibility of novel pandemic strains being generated from reassortment between avian H9N2 and other IAV subtypes.

3.3.4 HA-NA balance—During viral budding, progeny virions remain attached to the cell surface via HA until the enzymatic activity of NA destroys the receptors and releases those cell-bound viruses (Palese and Shaw, 2007). A functional balance between the activities of HA and NA has been suggested to play a role in establishing and sustaining efficient human transmissibility (Xu et al., 2012; Yen et al., 2011). Therefore, mutations that alter this delicate balance might be used as indicators for pandemic potential This concept was best demonstrated with pdm2009 H1N1, which resulted from reassortment of several swine lineage viruses. The NA and M segments of this virus came from Eurasian avianlike swine H1N1, and the other 6 segments came from North American swine H1N2, which itself was a triple reassortant of classical swine H1N1 virus (providing the HA, NP, and NS segments), a North American avian H1N1 virus (providing the PB2 and PA segments), and a human H3N2 virus (providing the PB1 segment) (Garten et al., 2009). Survey of the swine progenitors of pdmH1N1 has identified a swine H1N2 virus from Hong Kong, which differed from the pdmH1N1 by only the NA segment. This H1N2 virus had similar receptor specificity to the pdmH1N1, but grew at lower titres. Introducing the NA segment from pdmH1N1 did not improve viral replication efficiency; however, it did increase the respiratory droplet transmissibility in a ferret model, suggesting that a functional match among the 8 gene segments is required for efficient mammalian/human transmission (Yen et al., 2011).

3.3.5 Codon usage bias—Codon-usage bias is another consideration that may be involved in IAV adaptation to the mammalian host. The degenerate genetic code means that synonymous codons can code for the same amino acid. Although synonymous mutations are not expected to cause any change in the amino acid sequence, it is observed that these codons are not used in equal measure during translation. Furthermore, this bias differs between different species and is generally attributed to differences in the availability of tRNA during protein translation. Accumulating evidence suggests this bias is subject to selective pressure that varies according to the host species and is not due to random mutations (Plotkin and Kudla, 2011). Since viruses

are completely dependent on host cellular machinery for their protein synthesis, it is logical to assume that the codon usage features of the host will most likely influence viral evolution and adaptation. Some studies have proposed that IAV have inherited an evolutionary advantage that allowed them to transmit from a non-human to a human host due to their low codon usage bias (Ahn et al., 2006; Liu et al., 2010). However, it was also shown that the codon usage patterns of human IAV were distinct from those of avian viruses. Generally, human viral genes had a lower GC content and the nucleotide G was used frequently as the 3rd codon position in the viral genome (Wong et al., 2010), suggesting a certain degree of mammalian adaptation is required before sustained transmission in humans.

3.3.6 Glycosylation—Many HA molecules are N-linked glycosylated (NLG) at several sites in the globular head and stem through attachment of a glycan moiety to asparagine at the consensus sequence Asn-X-Ser/Thr; where X is any amino acid except proline (Kim and Park, 2012). Glycosylation of the HAs globular head helps modulate the biological activity and receptor binding specificity of HA, and hence the overall viral virulence in several ways. Glycosylation near antigenic epitopes also shields HA from antibody-mediated neutralization, leading to escape from immune recognition (Das et al., 2010). Therefore, any amino acid changes that would lead to acquisition or loss of glycosylation sites should be carefully scrutinized. An increased number of N-linked glycans attached to the head of HA was shown to attenuate H3N2 in mice (Vigerust et al., 2007). On the other hand, loss of a glycosylation site via a single amino acid substitution (Asn-246-Ser) in the HA of another H3N2 virus was accompanied by increased virulence to mice (Reading et al., 2009). Glycosilation is also a potent regulator of receptor binding affinity. Acquisition of NLG at the globular head can reduce the affinity of HA to its receptors, possibly through simple steric hindrance by the bulky side chain of the oligosaccharide, which blocks access of HA to the SA receptor (Ohuchi et al., 1997; Wagner et al., 2002; Wagner et al., 2000). The presence of NLG on residue N158 of H5N1 was shown to decrease the affinity of HA to bind to α-2,6 linked SA (Chen et al., 2012). On the other hand, loss of a glycosylation site in the lab-adapted strain A/Puerto Rico/8/34 at residue 131, adjacent to the RBD of HA, increased the binding affinity to the α-2,6 linked SA receptor (Das et al., 2011). Introducing a T160A mutation in the HA of A/Vietnam/1203/2004(H5N1) removed a glycosylation site at residue 158, but had no effect on the receptor preference of this virus. However, when compensated with an additional Q226L mutation, which is known to help IAV adapt to the α-2,6 linked SA human receptors, the T160A/Q226L double mutant exhibited altered receptor-binding specificity from α-2,3 linked SA to α-2,6 linked SA (Wang et al., 2010).

Changes in NLG have also been shown to modulate transmissibility. In a guinea pig model, an H5N1 virus that could bind to both α-2,3 linked SA and α-2,6 linked SA lost its affinity for α-2,6 linked SA after introduction of the A160T mutation, which resulted in loss of a glycosylation site at residues 158–160 and a consequent loss of transmissibility of the parental virus (Gao et al., 2009).

3.4 Inter-mammalian airborne transmission

The H5N1 outbreak that occurred in Hong Kong in 1997 provided the first concrete evidence that purely avian viruses can acquire the necessary adaptive changes to be transmitted directly to humans without prior reassortment in a mammalian host and can be fatal (Bender et al., 1999; Shortridge et al., 1998). A fundamental question that influenza virologists are trying to answer is what are the minimal genetic requirements that would render H5N1 potentially pandemic, i.e., airborne transmissible between humans? Two recent experiments were conducted to address this question. These experiments relied on serial passage of H5N1 or H5N1/H1N1 reassortant viruses in ferrets and studied the changes that would allow these viruses to spread via droplet transmission between separately caged animals. Both experiments

have shown that only a handful of changes could convert a virus into one that is efficiently transmissible in mammals (Herfst et al., 2012; Imai et al., 2012). Ferrets are the best mammalian models for avian influenza. Similar to humans, they have a predominance of α-2,6 linked SA in the upper airway and a lesser amount of α-2,3 linked SA on the respiratory epithelia (Matsuoka et al., 2009). Imai et al. (2012) used a reassortant virus containing an H5 HA within the background of pdmH1N1 virus. This virus was capable of droplet transmission in the ferret model after acquiring 4 HA mutations: N158D, N224K, Q226L and T318I. In the sister experiment, Herfst et al. (2012) modified an Indonesian strain of H5N1 by introducing 2 HA mutations (Q222L and G224S) to a priori switch receptor affinity from α-2,3 linked SA to α-2,6 linked SA and the E627K mutation in the PB2 to help the virus grow at the lower temperature of the mammalian upper respiratory tract. This genetically modified virus became airborne transmissible after acquiring 2 more HA mutations (H103Y and T156A) during serial passage in ferrets, suggesting that H5N1 can potentially become airborne transmissible between mammals without reassortment in an intermediate host. Although the starting points in these 2 experiments were different, they selected for viruses with similar phenotypes characterized by altered HA receptor specificity, loss of a glycosylation site and increased pH stability of the HA. A comprehensive analysis of thousands of sequences from surveillance data accumulated over the last 15 years revealed that 2 of these mutations are commonly found in circulating H5N1 strains in some endemic countries, such as Egypt. Therefore, it may be concluded that some viruses might require only 3 additional mutations to become airborne transmissible between mammals in the wild (Russell et al., 2012). These elegant experiments clearly showed that H5N1 pose a serious pandemic threat to humans, and therefore those mutations have to be carefully monitored in all current and future surveillance efforts.

4.0 Concluding remarks

The future of influenza research seems to depend on an ability to marry the persistent efforts of researchers to understand the mechanisms of viral infection and protection, (driven by the need for effective vaccine) with the growing interest in understanding the origin and evolution of new and continuing viral threats in nature. The advent of next generation and third generation sequencing technology and other tools to examine viral and host dynamics in greater detail and in higher throughput is already paying dividends in many areas of research in this field as demonstrated by several experiments referred to in this review. However, even as a new pandemic virus may erupt this season and dictate a need for intense study and vaccine development, we should be mindful of the continuing need for investment in longer-term projects to understand the basic immunology, evolution, and ecology of viruses in the wild. These efforts will aid predictive models and have a major impact on the public health preparedness for future pandemic threats. To push forward, influenza research must continue towards becoming an interdisciplinary effort combining the work of ecologists, ornithologists, epidemiologists, modelers, geneticists and molecular and cellular virologists as well as others. The insights highlighted in this review along with new efforts to fill the gaps in the study of wild virus and to unlock the molecular, ecological, and evolutionary criteria that govern the formation of potential pandemic strains will help to clarify and mitigate the looming threat of influenza to human and animal populations.

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Abbreviations used in the text

HA	hemagglutinin

NA	neuraminidase
IAV	influenza A virus
AIV	avian influenza virus
PB1, PB2, and PA	polymerase complex
NP	nucleoprotein
M	matrix
M2	matrix 2
NS	non-structural
NEP	nuclear export protein
MatAb	maternal antibody
SA	sialic acid
SGPs	sialylglycopolymers
LPAI	low pathogenic avian influenza
HPAI	high, pathogenic avian influenza
RBD	receptor binding domain
NLG	N-linked glycosylated

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Highlights – Influenza review (Runstadler, Hill, Hussein, Puryear, and Keogh)

- We review the literature in influenza research to explore how the study of wild influenza is connected to concerns for human disease.
- We point to what we think are the most important gaps left in studies to date in wild bird, mammal, and environmental influenza surveillance.
- We review work that has defined what the most important adaptations are in the/potential emergence of avian influenza viruses as pandemic viruses.
- Additional emphasis is placed on the molecular determinants that govern interspecies movements of virus and their potential importance in the generation of pandemic strains capable of entering the human population.

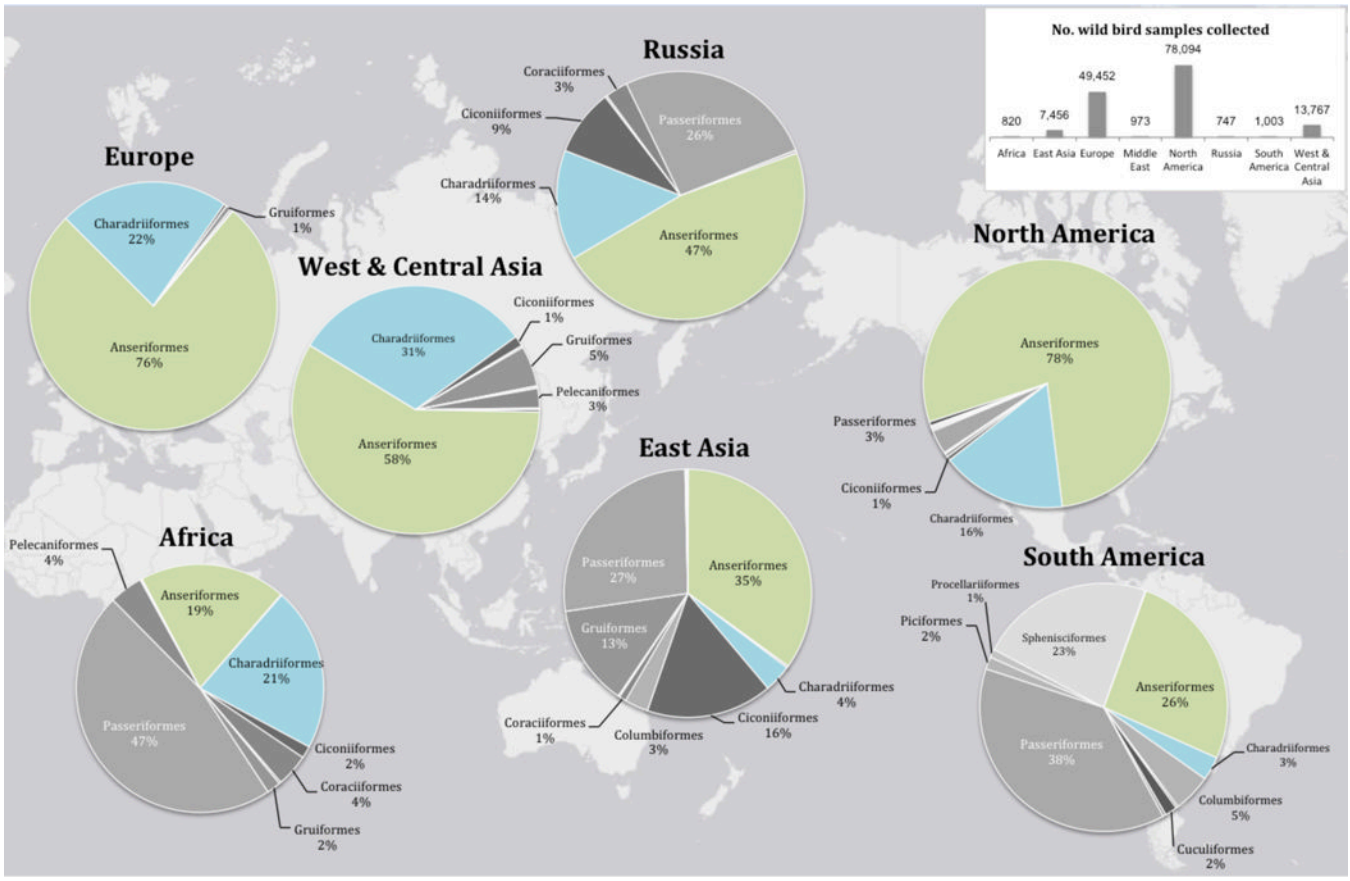

Figure 1.

Surveillance effort targeted towards wild birds. Anseriformes (ducks, geese, swans: green) and Charadriiformes (shorebirds, gulls, auks: blue) account for almost all samples from wild bird orders collected in North America and Europe. Data are based on number of samples (positive, negative & untested) deposited in Influenza Research Database at 20 September, 2012 (n = 152,312). Other sampled wild bird orders include: Ciconiiformes (storks, herons, egrets), Columbiiformes (doves, pigeons), Coraciiformes (kingfishers, bee-eaters, rollers, hornbills), Cuculiformes (cuckoos, roadrunners, Gruiformes (cranes, rails), Sphenisciformes (penguins), Passeriformes (perching birds), Pelicaniformes (pelicans), Piciformes (woodpeckers), Procellariiformes (albatrosses, shearwaters, petrels), Sphenisciformes (penguins).

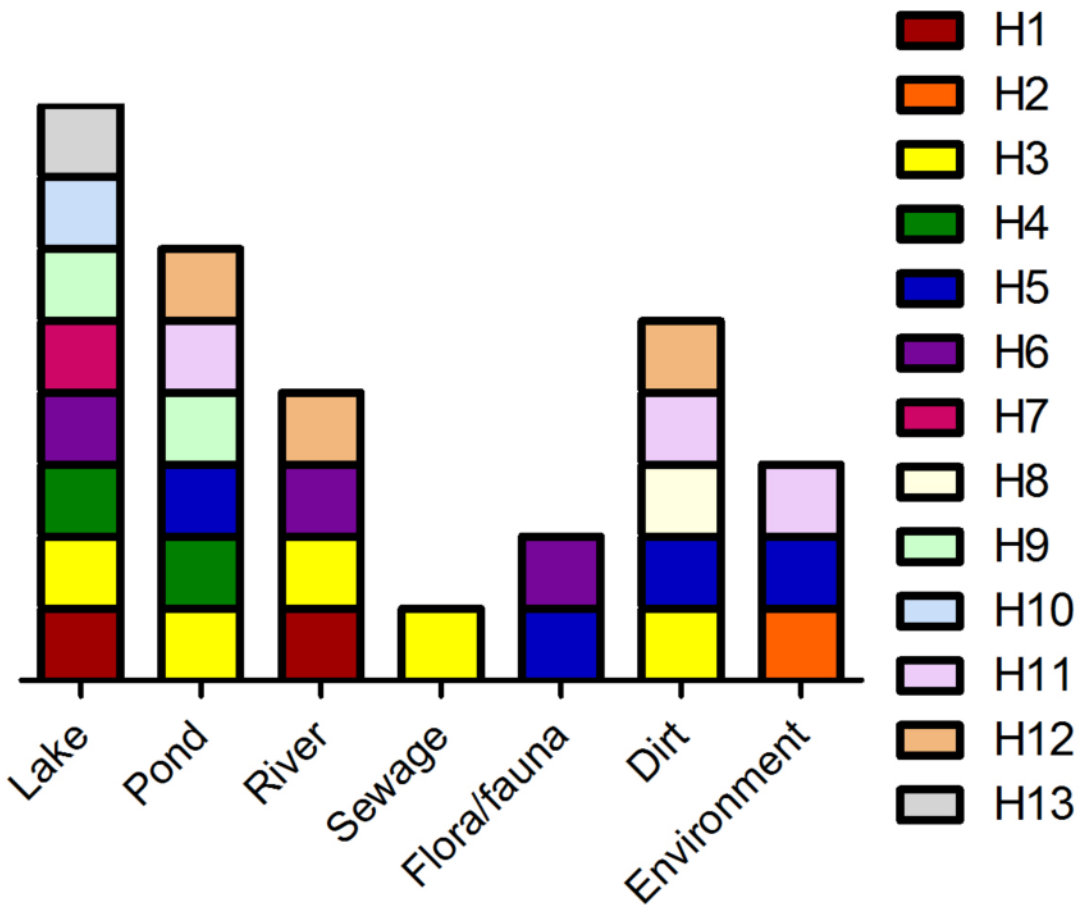

Figure 2.
Detection of IAV from environmental sources. Data represent counts of the thirteen hemagglutinin (HA) subtypes detected. Data are sourced from reports of environmental detection published in the literature and sequences available on Genbank.

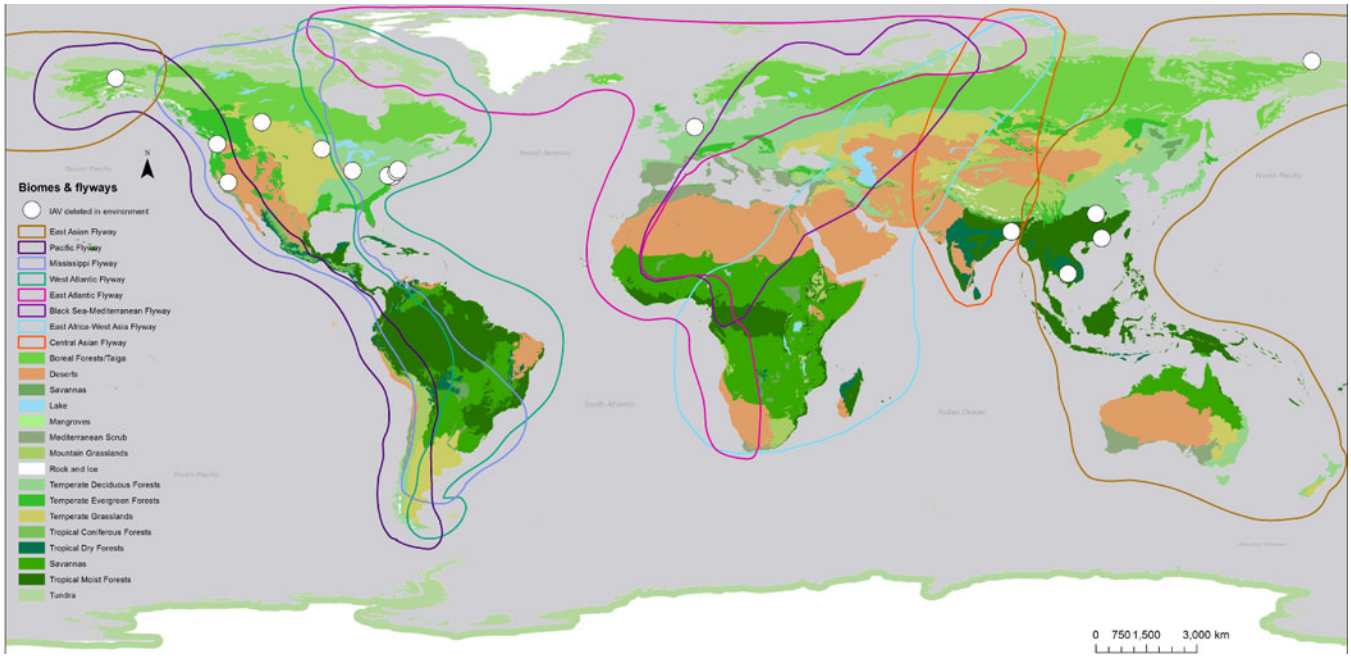

Figure 3.
Geographic distribution of IAV detected from the environment spans biomes and migratory flyways of wild birds. Sampling sites are indicated by a white circle.

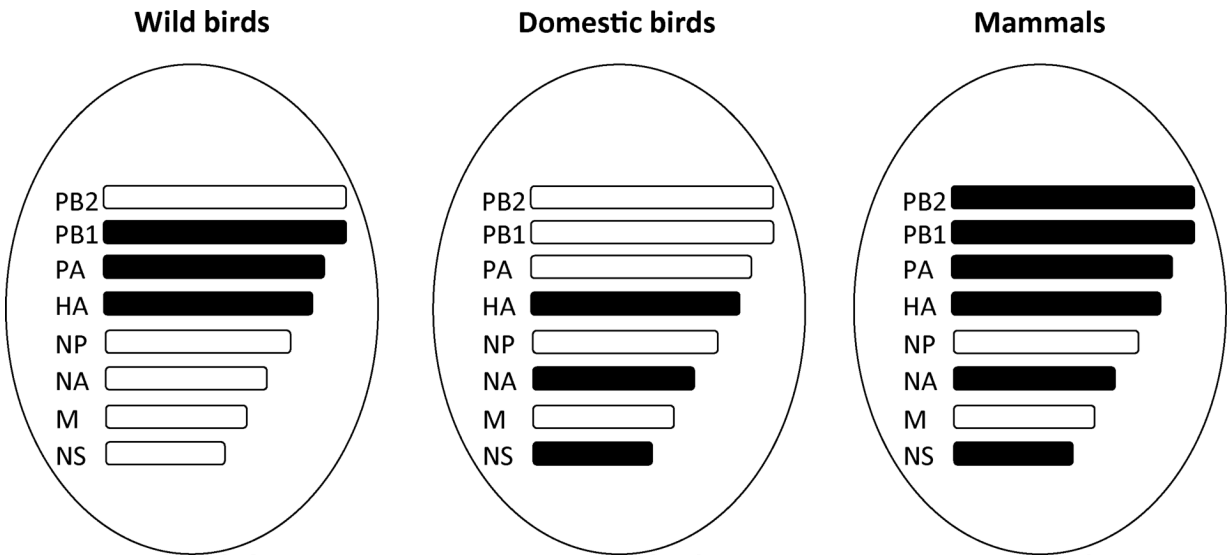

Figure 4.

Genomic segments involved in enhanced virulence and/or host switching. Segments carrying mutations that have been shown experimentally to be involved in such phenotypic changes are highlighted in solid black and discussed within the text. Most of the mutations recorded in viruses isolated from wild birds are related to spill over events of HPAI H5N1 from domestic birds. The fact that 6 segments are involved in mammalian adaptation reflects the complexity of this process and the bias towards studying IAV that switches to mammals.

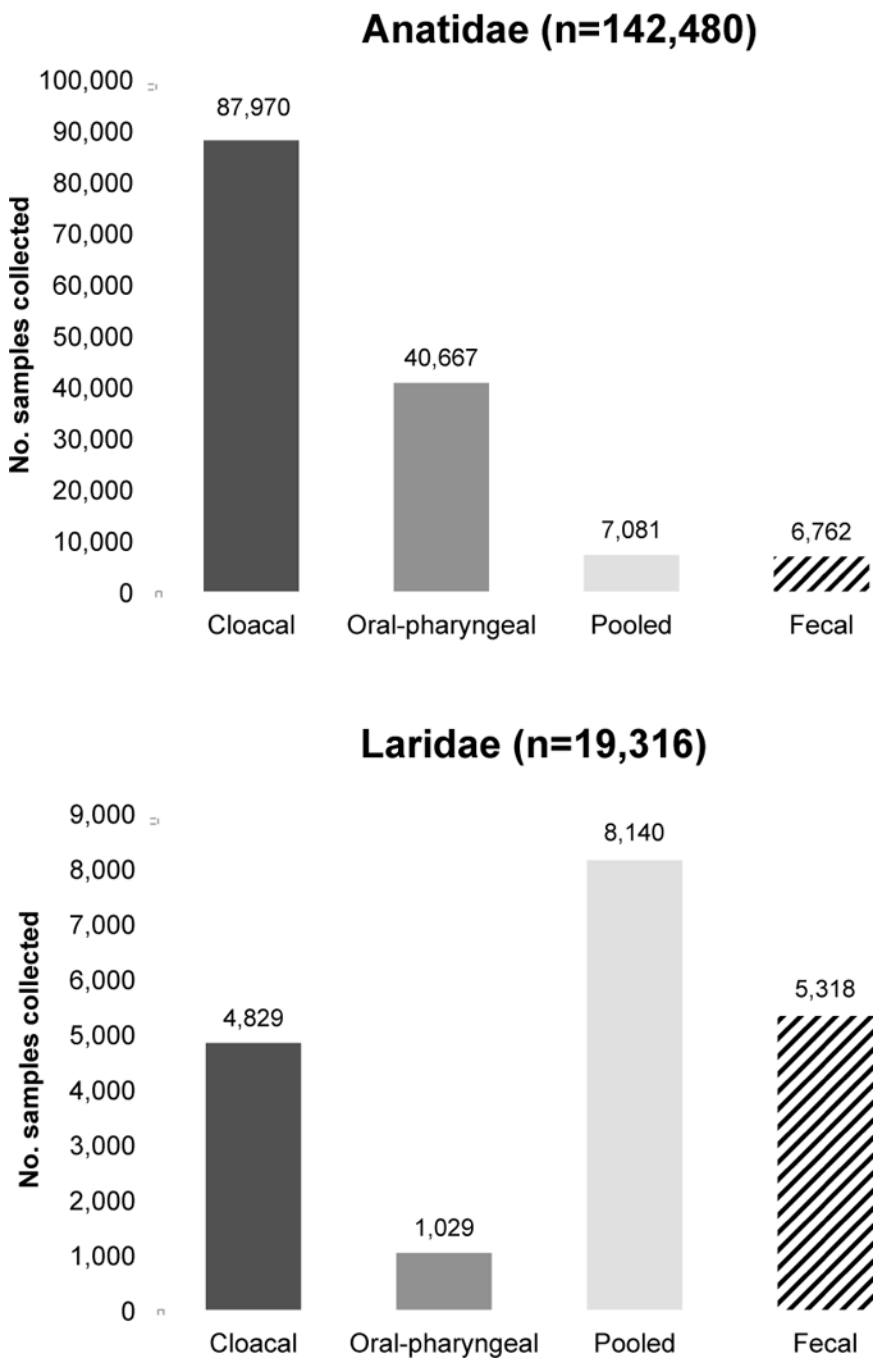

Figure 5.
Sample types from Anatidae (ducks, swans, geese) and Laridae (gulls, terns, kittiwakes) collected during global surveillance. Data are based on number of samples (positive, negative & untested) deposited in the Influenza Research Database at 20 September, 2012.

Table 1

HA	Subtype	Species	Location	Year	Detection method	Ref
	ND[1]	Dall's porpoise	western North Pacific	200–2001	ELISA; Western	Ohishi 2006
		minke whale	western North Pacific	200–2001	ELISA; Western	Ohishi 2006
		beluga	Canadian Arctic	1984–1994	ELISA	Nielson 2001
		ringed seal	Canadian Arctic	1984–1994	ELISA	Nielson 2001
		harbor seal	California	2007		IRD (Dec 2012)
		harbor seal	British Colombia	2007		IRD (Dec 2012)
		northern elephant seal	California	2007		IRD (Dec 2012)
		California sea lion	California	2007		IRD (Dec 2012)
H1	H1	"seals"	Bering Sea	1978–1988	NP-ELISA; HI	de Boer 1990
	H1N1	South American fur seal	Uruguay	Sept. 2004	HI	Blanco 2009
	H1N3	family Balaenopteridae	South Pacific	1974–1976	MDCK cells	Lvov 1978
H3	H3	"seals"	Bering Sea	1978-1988	NP-ELISA; HI	de Boer 1990
	H3	Kuril harbor seal	Hokkaido, Japan	1998-2005	ELISA; HI	Fujii 2007
	H3	ringed seal	Alaska	1978-1995	HI	Danner 1998
	H3N2	Caspian seals	Caspian Sea	1993, 1997, 1998,	ELISA; HI	Ohishi 2002
	H3N2	Baikal seal	Baikal Sea	2000	ELISA; HI	Ohishi 2004
	H3N2	ringed seal	Central Russian Arctic	1998	ELISA; HI	Ohishi 2004
	H3N3	harbor seal	Cape Cod	2002	PCR;HI	Callan 1995
	H3N8	harbor seal	Cape Cod	1991- 1992	RT-PCR	Bogomolni 2008
	H3N8	harbor seal	Cape Cod	2005 -2007; 2011	PCR	Anthony 2012
H4	H4	"seals"	North and Bering Seas	1988; 1978–1988	ELISA	de Boer 1990
	H4N5	Harbor seal	Cape Cod	June 1982-March 1983	HI, NI	Hinshaw 1984
	H4N6	harbor seal	Cape Cod	January 1991	PCR;HI	Callan 1995
H6	H6	Kuril harbor seal	Hokkaido, Japan	1998–2005	ELISA;HI	Fujii 2007
H7	H7	"seals"	Bering Sea	1978–1988	NP-ELISA; HI	de Boer 1990
	H7	ringed seal	Alaska	1978–1995	ELISA; HI	Danner 1998
	H7N7	*harb*or seal	Cape Cod	Dec. 1979		Geraci 1981
	H7N7	ringed seal	Central Russian Arctic	2002		Ohishi 2004
H10	H10 N7 N2 N3	walrus	St. Lawrence Island and Round Island, Alaska	1994–1996	AGID	Calle 2002
H12	H12	"seals"	Bering Sea	1978–1988	NP-ELISA; HI	de Boer 1990
H13	H13N2	pilot whale	New England	1984	Chicken egg	Hinshwa 1986
	H13N9	pilot whale	New England	1984		Hinshwa 1986

Not determined

HI-hemagglutination inhibition assay

NI-neuraminidase inhibition

Agar gel immunodiffusion (AGID)

NP-ELISA nucleoprotein (NP) ELISA

Table 2

HA	Subtype	Source of virus	Location	Season	Isolation method	Detection method	Ref
H1	H1 H1N1 H1N2	Lake water River water Environment[3]	Russia Netherlands Indiana	NR[1] NR NR	NR Ultrafiltration NR	NR RTPCR NR	IRD[2] Heijnen 2009 IRD
H2	H2N2 H2N3	Environment Environment	New Jersey New York	NR NR	NR NR	NR NR	IRD IRD
H3	H3 H3N2 H3N2 H3N3 H3N6 H3N6 H3N8 H3N8 H3N8	Pond sediment Pond water Pond water Pond water River water Sewage Lake water Lake water Pond water	Alaska Minnesota Hong Kong Hong Kong Netherlands Netherlands Minnesota Alaska Minnesota	Fall-Spring Water < 12°C Nov-April Nov-April NR NR Sept Summer/Fall Water < 12°C	Unconcentrated Unconcentrated Concentrated Concentrated Ultrafiltration Ultrafiltration Unconcentrated Erythrocyte assay Unconcentrated	RTPCR Egg inoculation Egg inoculation Egg inoculation RTPCR RTPCR Egg inoculation Egg inoculation Egg inoculation	Lang 2006 Halvorson 1983 Markwell 1982 Markwell 1982 Heijnen 2009 Heijnen 2009 Stallknecht 2010 Ito 1995 Halvorson 1983
H4	H4N1 H4N2 H4N6 H4N6 H4N8	Lake water Lake water Lake water Lake water Pond water	Alberta Alberta Minnesota Alaska Minnesota	Aug Aug Sept Summer/Fall Water < 12°C	Unconcentrated Unconcentrated Unconcentrated Erythrocyte assay Unconcentrated	Egg inoculation Egg inoculation Egg inoculation Egg inoculation Egg inoculation	Hinshaw 1979, ‘80 Hinshaw 1980 Stallknecht 1995 Ito 1995 Halvorson 1983
H5	H5N1 H5N1 H5N1 H5N2 H5N3 H5N4	Pond water, mud, soil Mud Water, plants, soil, mud Environment Environment Environment	Cambodia Cambodia Cambodia Indiana, NY California New York	2007–2010 NR NR NR NR NR	Erythrocyte assay Elution and conc Unconcentrated NR NR NR	RTPCR RTPCR, Egg inoc RTPCR NR NR NR	Horm 2012 Horm 2011 Vong 2008 IRD IRD IRD
H6	H6N2 H6N8 H6N8 H6N8	Lake water River water Freshwater clams New York	Alberta Netherlands NR NR	Aug NR NR NR	Unconcentrated Ultrafiltration NR NR	Egg inoculation RTPCR NR NR	Hinshaw 1980 Heijnen 2009 Huyvaert 2012 IRD
H7	H7N2 H7N3 H7N3	Lake water Sewage Lake water	Alberta Netherlands Alaska	Aug NR Summer/Fall	Unconcentrated Ultrafiltration Erythrocyte assay	Egg inoculation RTPCR Egg inoculation	Hinshaw 1979, ‘80 Heijnen 2009 Ito 1995
H8	H8	Pond sediment	Alaska	Fall-Spring	Unconcentrated	RTPCR	Lang 2006
H9	H9 H9N2 H9N2	Water Lake water Pond water	Bangledash Dongting Lake Hong Kong	NR Spring Nov-April	NR Erythrocyte assay Concentrated	NR Egg inoculation Egg inoculation	IRD Zhang 2011 Markwell 1982
H10	H10N8	Lake water	Dongting Lake	NR	Erythrocyte assay	Egg inoculation	Zhang 2011
H11	H11 H11N6 H11N8 H11N9 H11N9	Pond sediment Environment Environment Pond water Environment	Alaska Delaware Maryland, CA Minnesota Maryland	Fall-Spring NR NR Water < 12°C NR	Unconcentrated NR NR Unconcentrated NR	RTPCR NR NR Egg inoculation NR	Lang 2006 IRD IRD Halvorson 1983 IRD

HA	Subtype	Source of virus	Location	Season	Isolation method	Detection method	Ref
H12	H12 H12N3	Pond sediment River water	Alaska Netherlands	Fall-Spring NR	Unconcentrated Ultrafiltration	RTPCR RTPCR	Lang 2006 Heijnen 2009
H13	H13N2 H13N6	Lake water Pond water	Minnesota Minnesota	NR NR	Erythrocyte assay Erythrocyte assay	Egg inoculation Egg inoculation	Sivandan 1991 Sivandan 1991

[1] Not reported

[2] Influenza Research Database

[3] Reported as "environment" in the Influenza Research Database