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SEROLOGICAL AND MOLECULAR METHODS FOR SURVEILLANCE OF INFLUENZA A VIRUS

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Front page: electron microscopy image of pseudoparticles with A/Puerto Rico/8/1934 hemagglutinin on the surface. Kindly provided by Sofia Helgesson, the Public Health Agency of Sweden.

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I learned long ago, never to wrestle with a pig.
You get dirty, and besides, the pig likes it.

George Bernard Shaw

ABSTRACT

The natural reservoir for influenza A viruses is birds, and numerous outbreaks of highly pathogenic avian influenza viruses have been documented. There is a risk of novel subtypes originating from birds infecting humans, and the question of migratory birds as long-distance vectors for highly pathogenic avian influenza viruses has also been raised. Areas where migratory flyways meet and birds nest have been suggested as hot spots for influenza A viruses to mix. In our study we found no evidence of recent genetic mixing at Point Barrow in Alaska. In order to know which subtypes currently circulate, influenza surveillance in domestic and wild birds is crucial. Detection of viral RNA from bird faeces is commonly used. However, detection of antibodies against influenza A virus provides useful information after birds have ceased to shed virus. We evaluated a pseudoparticle neutralization test, based on highly pathogenic avian influenza virus hemagglutinin. Our results show that the test can be used for detection of H5 and H7 specific antibodies, which offers an alternative to using standard neutralization tests where live virus is required.

Influenza virus with new genetic material from birds has caused several human pandemics during the 20th century. In 2009, the A(H1N1)pdm09 virus emerged. The receptor binding structure of the virus, the hemagglutinin, was phylogenetically closely related to the virus of the 1918 Spanish influenza. During the 2009 pandemic the elderly population was only mildly affected, possibly due to pre-existing cross-reactive neutralizing antibodies. Using a pseudoparticle neutralization assay we were able to investigate neutralizing antibody cross-reactivity patterns in different age groups against H1 influenza viruses from 1918, 1934, 1999, and 2007. A significant difference between age groups in antibody titers against the 1918 and 1934 viruses was observed. Individuals over the age of 90 had the highest levels of neutralizing antibodies against the 1918 virus, while those aged 71-90 had the highest levels against the 1934 strain. The 1918 virus is antigenically similar to the 2009 virus and antibodies against the 1918 virus may have protected against the 2009 virus. We also tested the sera for presence of neuraminidase inhibiting (NI) antibodies against the A(H1N1)pdm09 virus. The results revealed a strong correlation between NI antibodies and age. NI antibodies did, however, not appear to significantly influence the neutralizing titers in a long-incubation neutralization assay. Antibodies targeting the neuraminidase may prevent severe illness and could together with pre-existing cross-reactive neutralizing antibodies have contributed to the mild outcome in the elderly during the 2009 pandemic.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following original publications, which will be referred to in the text by their roman numerals:

I. **Gene segment reassortment between American and Asian lineages of avian influenza virus from waterfowl in the Beringia area.**
Wahlgren J, Waldenström J, Sahlin S, Haemig PD, Fouchier RA, Osterhaus AD, Pinhassi J, Bonnedahl J, Pisareva M, Grudinin M, Kiselev O, Hernandez J, Falk KI, Lundkvist Å, Olsen B.
Vector Borne and Zoonotic Diseases 2008, 8(6) 783-790

II. **Detection of antibodies against H5 and H7 strains in birds: evaluation of influenza pseudovirus particle neutralization tests.**
Wallerström S, Lagerqvist N, Temperton NJ, Cassmer M, Moreno A, Karlsson M, Leijon M, Lundkvist Å, Falk KI.
Infection Ecology and Epidemiology 2014 15;4. doi: 10.3402/iee.v4.23011

III. **Age-related prepandemic influenza A(H1N1) neutralizing antibody responses measured by a pseudoparticle neutralization test.**
Wallerström S, Temperton NJ, Ferrara F, Mörner A, Linde A, Falk KI.
Submitted

IV. **Age-related anti-neuraminidase and neutralizing antibodies against influenza A(H1N1)pdm09 in Sweden before the pandemic in 2009.**
Wallerström S, Aktas T, Linde A, Falk KI, Mörner A.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

CTL	Cytotoxic T lymphocytes
ELISA	Enzyme-linked immunosorbent assay
ELLA	Enzyme-linked lectin assay
ER	Endoplasmatic reticulum
HA	Hemagglutinin
HA assay	Hemagglutination assay
HAT	Human airway trypsin-like
HI	Hemagglutination inhibition
HPAI	Highly pathogenic avian influenza
HRP	Horseradish peroxidase
IAV	Influenza A virus
IFN	Interferon
IIV	Inactivated influenza vaccine
LPAI	Low pathogenic avian influenza
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NI	Neuraminidase inhibiting
NLRP3	NOD-like receptor family pyrin domain containing 3
NP	Nucleoprotein
PCR	Polymerase chain reaction
PNA	Peanut agglutinin
pp-NT	Pseudoparticle neutralization test
RIG-I	Retinoic acid-inducible gene-I
RNA	Ribonucleic acid
SA	Sialic acid
TLR	Toll-like receptor
TMPRSS2	Trans-membrane protease serine S1 member 2
vRNA	Viral ribonucleic acid
vRNP	Viral ribonucleoprotein
WHO	World Health Organization

1 INTRODUCTION

Influenza A virus (IAV) causes significant human illness and death each year. The fact that around one billion cases of seasonal influenza occur each year is concerning, and it is also a burden for society from an economic perspective. A pandemic is an epidemic of an infectious disease that spreads across several continents. During the 20th century at least three severe pandemics have been caused by influenza viruses. It is evident from the annual epidemics and several pandemics that influenza is among the most serious infectious threats to humans. In the past, we have been ill-prepared for pandemics that strike fast and without warning.

Birds are the natural hosts of influenza viruses. The pandemic influenza viruses have avoided the pre-existing influenza immunity by the introduction of new surface molecules from bird viruses. Since the detection and continued reports of human cases of highly pathogenic avian influenza (HPAI) A(H5N1) in 1997 [1,2], the interest for research that focus on influenza as part of an ecosystem has dramatically increased. Research and surveillance increase our chances to be prepared when the next influenza pandemic strikes. The 2009 pandemic was the first pandemic where we could fully utilize the benefits of molecular techniques and instant information, and it was a first trial of a well-organized prevention and control of influenza pandemics.

1.1 BACKGROUND

Influenza virus is an enveloped ribonucleic acid (RNA) virus belonging to the *Orthomyxoviridae* family. *Influenzavirus A* is a genus within this family, together with four other generas; *Influenzavirus B*, *Influenzavirus C*, *Isavirus*, and *Thogotovirus*. Influenza B virus is epidemiologically important and has been found primarily in humans, and influenza C virus only cause a mild and rarely diagnosed disease in humans and pigs [3,4]. Isavirus causes disease in fish [5] and Thogotovirus has been shown to infect both animals and humans [6]. Only IAV will be further discussed in this thesis.

The first mammalian IAV strain was isolated from swine in 1931 [7]. In 1933, a human strain was isolated in the laboratory of Wilson Smith [8]. Throat-washings from patients with influenza symptoms were filtered and administered to different animal species. The ferret was the only animal that became infected. On the third day after inoculation, the ferrets became ill with catarrhal signs and a fever [8]. This was the first isolation of human IAV, although the disease was recognized and documented hundreds of years before. It is difficult to know when

the IAV was first introduced into humans but there are epidemics in the past that are believed to have been influenza outbreaks. One of the first pandemics clearly verified by epidemiology was the Russian Flu in 1889-1892 [9], and since then data have been more reliable. After 1933, when viruses were available for analysis, the existence of pandemics is not questioned.

It is widely accepted that influenza is an infection originating from aquatic birds [10,11]. In their natural bird reservoir, the viruses mainly cause enteric infections with no clinical signs [12]. Classification of IAV is based on two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [13]. To date, there are 16 well characterized types of HA and nine types of NA [10,14-17], yielding a theoretical possibility of 144 unique combinations [17]. The majority of these combinations have been found in birds [18], and all 16 HA subtypes can replicate in domestic ducks and/or quail [19,20]. Recently, a 17th and 18th HA and a 10th and 11th NA combined into H17N10 and H18N11 were discovered, but these two new subtypes have so far only been found in bats [21,22].

It is likely that the use of an intermediate host, such as swine, facilitates the adaptation of viruses to other mammalian hosts, including man. Only IAV, and to the present only subtypes H1N1, H2N2, and H3N2, has been identified as causes of pandemics in humans, but the identity of viruses causing pandemics before 1918 is unknown. Although other IAV subtypes such as H5N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7, and H10N8 have been shown to infect humans occasionally, there is no evidence of sustained human-to-human transmission [23]. Of 16 HA and nine NA subtypes there are 13 HA and seven NA subtypes of IAV that the majority of the world's population is immunologically naïve to.

1.1.1 Classification and nomenclature

The nomenclature system that is in use for influenza virus strains include type of influenza (A, B, or C), geographic location of isolation, strain number, and year of isolation. If the virus is isolated from a different species than human, this is specified between type of influenza and geographic location. The HA and NA subtypes are specified last within parentheses. Examples: A/South Carolina/1/18 (H1N1) and A/Mallard/Sweden/7206/2004 (H7N7).

1.1.2 Influenza A virus structure and function

The shape of IAV can vary, but it is roughly spherical (although somewhat pleomorphic) or filamentous. The spherical virion is 80-120 nm in diameter, but the filamentous forms are often more than 300 nm in length. The genome consists of single stranded negative sense RNA divided into eight segments of various lengths that together code for a minimum of 10 proteins. The segments are numbered (1-8) in order of decreasing length (Table 1). Segments 4 and 6 encode two surface glycoproteins, HA and NA, which project out like spikes from the virion envelope (Figure 1).

The virion (Figure 1) is made up by the interior matrix protein M1 and the nucleocapsid, which consists of viral RNA (vRNA) and nucleoprotein (NP). Three segments (1, 2, and 3) encode for the polymerase subunits PB2, PB1, and PA. Together they form a heterotrimeric RNA-dependent RNA polymerase found in the viral ribonucleoprotein (vRNP) complex. The envelope surrounding the nucleocapsid is derived from the host cell, and it contains the virus encoded transmembrane matrix protein M2, that in a tetrameric fashion forms an ion-channel. The NA is found as a tetramer, and HA as a trimer. HA is about four to five times more abundant than NA, but the ratio varies from strain to strain [24].

Table 1. Influenza A virus segments, gene products, and their function

Segment	Length (bp)	Protein	Main function
1	2341	PB2	Polymerase subunit, binds the 5' cap-1 structure of host pre-mRNA
2	2341	PB1	Polymerase subunit, responsible for elongation in template RNA and vRNA synthesis
		N40	Unknown function
		PB1-F2	Pro-apoptotic, virulence factor
3	2233	PA	Polymerase subunit, endonuclease activity
		PA-X	Modulates the host immune responses
4	1778	HA	Surface glycoprotein, receptor binding and membrane fusion
5	1565	NP	Binds to and encapsidates vRNA
6	1413	NA	Surface glycoprotein, prevents aggregation by cleaving terminal sialic acid from glycoproteins and glycolipids
7	1027	M1	Matrix protein, forms a shell underneath the virion envelope
		M2	Ion channel protein, uncoating of virus and control of pH in Golgi
8	890	NS1	Nonstructural protein, interferon antagonist and post-transcriptional modulation
		NS2/NEP	Nonstructural protein, nuclear export protein, mediates nuclear export of vRNA

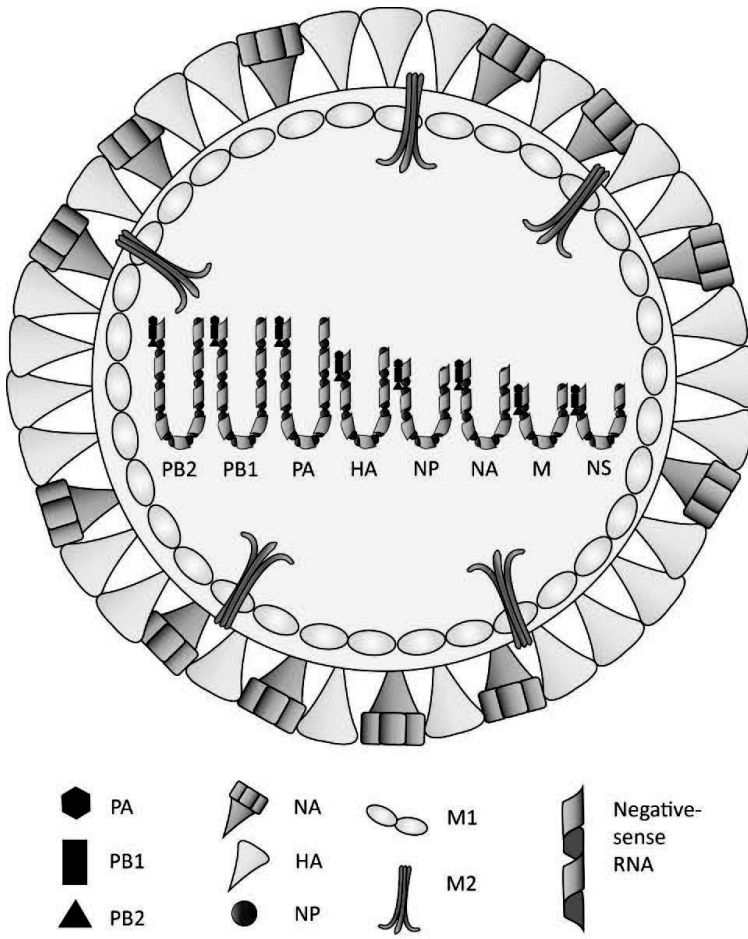

Figure 1. Influenza A virion. Embedded in the viral envelope are the two surface proteins, HA and NA, and the transmembrane M2 ion-channel. The negative single-stranded RNA segments together with NP and the polymerase complex (PA, PB1, and PB2) form the vRNP complex. M1 is associated with both the envelope and the vRNP.

1.1.3 Virus life cycle

1.1.3.1 Entry

When the HA protein of IAV attaches to its designated sialic acid (SA) receptor, the dynamic process of replication begins (Figure 2). IAV recognizes oligosaccharides containing a terminal SA. Different SA receptors and the affinity of different HAs to them have a critical role in determining species specificity, zoonoses transmission, and pathogenesis, as well as pandemic behavior of influenza virus strains. Human IAV bind with stronger affinity to α2,6-linked SA which predominate in the human upper respiratory tract [25], while avian IAV preferentially binds to α2,3-linked SA which is present on epithelial cells in the intestine of birds [26]. This is one of the barriers for transmission between birds and humans, although in the lower part of the human respiratory tract both of these receptors can be found [27].

Endocytosis is initiated after binding of ligand to receptor (Figure 2) [28,29]. Here, HA plays a critical role. The uncoating of the virus is a process where two criteria must be fulfilled: the HA must be cleaved into HA1 and HA2 by a protease [30,31] and the pH must be lowered to ~5. Low pH triggers an irreversible conformational change in the cleaved HA that allows the virion membrane to fuse with the endosomal membrane (Figure 2). The M1-vRNP interactions are interrupted, leading to the release of the vRNPs into the cytoplasm. The vRNP complexes are then transported into the host cell nucleus for transcription [32].

1.1.3.2 Transcription

Once inside the host cell nucleus, vRNA is transcribed to messenger RNA (mRNA) (Figure 2). The heterotrimeric RNA-dependent RNA-polymerase complex consists of subunit PB1, PB2, and PA [33-37] and catalyzes RNA polymerization, polyadenylation of mRNA, and cleavage of host mRNAs to generate capped RNA fragments. IAV uses a “cap-snatching” mechanism, meaning that transcription is initiated with an RNA primer that is excised from cellular mRNA [38,39]. For IAV, the genomic vRNA is initially transcribed to complementary RNA and thereafter complementary RNA is transcribed to vRNA.

1.1.3.3 Translation, assembly, and budding

The host cell translation machinery translates IAV mRNA to proteins in the cytoplasm (Figure 2). The newly synthesized viral polymerase subunits and NP are imported into the nucleus where they assemble with newly transcribed vRNAs [40,41]. Export of vRNPs into the cytoplasm is mediated by the viral NEP/NS2 and M1 proteins. Newly synthesized HA, NA, and M2 proteins enter the endoplasmic reticulum (ER) where they are post-translationally processed [42-45]. Recent studies have identified human serine proteases located in the human airways to be involved in cleaving of HA into HA1 and HA2. Human airway trypsin-like (HAT) protease and transmembrane protease serine S1 member 2 (TMPRSS2) both cleave HA proteins containing a single arginine at the cleavage site [46]. HAT has been shown to cleave newly synthesized HA before or during the release of newly produced viruses as well as the HA of incoming viruses. TMPRSS2 cleaves HA that is present within the cell, and cannot support the proteolytic activation of HA of incoming viruses [47]. The HA of HPAI virus contains a polybasic cleavage site and is processed in the *trans*-Golgi network, most likely by cellular furin-like proteases [48]. The cleavage of HA is critical for the virulence of IAV.

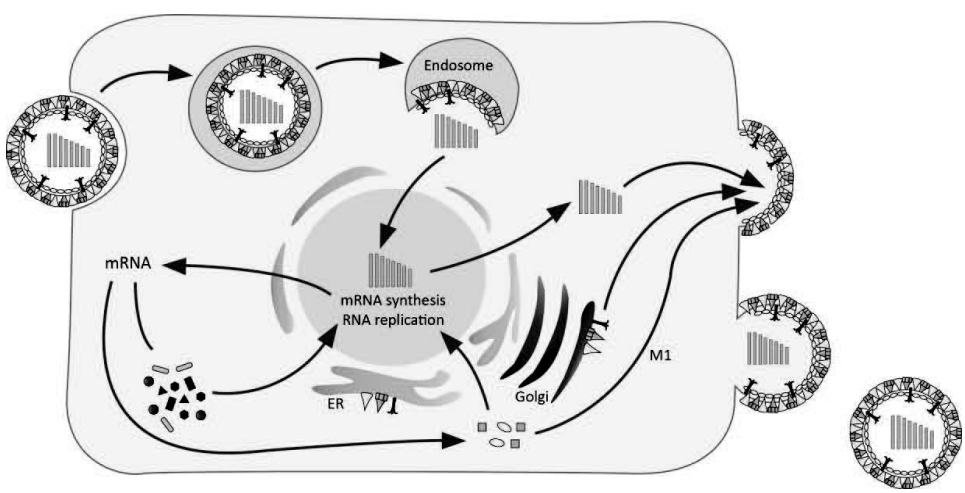

Figure 2. Replication cycle of IAV. The virus enters the cell through receptor-mediated endocytosis. After the viral envelope fuses with the endosome, vRNP complexes are released into the cytoplasm and subsequently transported to the nucleus where replication and transcription take place. The new virions are assembled at, and buds off, the host cell plasma membrane.

The folded and processed proteins and vRNPs are then transported to the cell membrane for virus particle formation and budding (Figure 2). The different vRNPs are thought to have specific inter-vRNP interactions to maintain a conformation that results in eight different vRNPs being packed into an influenza particle [49,50]. Recent research suggests that the vRNPs are held together by direct base-pairing between packaging signals present in the vRNAs [51]. The virus buds from lipid rafts in the plasma membrane. The NA protein is responsible for removing SA from both the host cell membrane and from viral glycoproteins, thereby preventing virion aggregation [52-54].

1.2 EVOLUTION AND ECOLOGY

1.2.1 Antigenic drift and shift

The IAV strategy for survival is to efficiently evade the immune system. After infection, viral load usually peak within 48-72 hours [55]. The peak coincides with the time for the first wave of specific antibodies. At this point, transmission to the next susceptible host has already occurred, ensuring the survival of the virus. Eventually, however, the majority of the population will have developed HA specific antibodies, and transmission among others than very young children will end. However, IAV uses two evasion strategies, the first being antigenic drift (Figure 3). The RNA-dependent RNA polymerase lacks proofreading mechanisms, thus allowing for mutations during transcription at a rate of approximately $1.5x10^{-5}$ mutations per nucleotide per infectious cycle [10,56]. Due to the high selective pressure on HA and NA, new variants that can evade neutralizing antibodies and replication inhibiting antibodies are favored. The influenza HA remains surprisingly functional despite profound antigenic changes. This is the explanation to long term persistence of a virus subtype and the reason why annual influenza epidemics occur. It is not due to a lack of immunity from infection or vaccination. Analyses of strains isolated from the natural reservoir of IAV, birds, have revealed that antigenic drift does not occur to the same extent in birds [10]. Instead, the strains existing in wild birds appear to be in evolutionary stasis. Sequences obtained from birds captured more than 80 years apart have been shown to be very similar [57].

The second evasion strategy is called antigenic shift (Figure 3), involving instant major antigenic changes in the IAV genetic material. To cross species barriers, the virus needs to either accumulate changes that make adaption to new host cells possible, or gain changes through gene segment reassortment. Antigenic shift requires that one cell is co-infected with multiple influenza subtypes. The reassortment can result in the creation of a novel combination of HA and sometimes NA, and such an event poses a pandemic threat if the subtype is naïve to the population. A common example of a "mixing vessel" is the pig, a species that can be infected with avian IAV as well as human IAV. As described, most avian and human IAV preferentially bind to specific receptor types, α2,3-linked SA (avian) or α2,6-linked SA (human) [58]. In the trachea of pigs, both these receptors are present [59], making it an ideal "mixing vessel". If genetic material from both human and avian viruses were mixed, resulting in a novel subtype that has retained its ability of human to human transmission, the virus would have pandemic potential. Pigs are not the only possible "mixing vessel", there is also a risk that the same thing can happen in humans, as IAVs circulating in bird populations can infect

humans. An antigenic shift is devastating since it leaves the pre-existing antibodies and T cells ineffective. Antigenic drift and shift are what enables the virus to efficiently evade antibody mediated immune responses at population level.

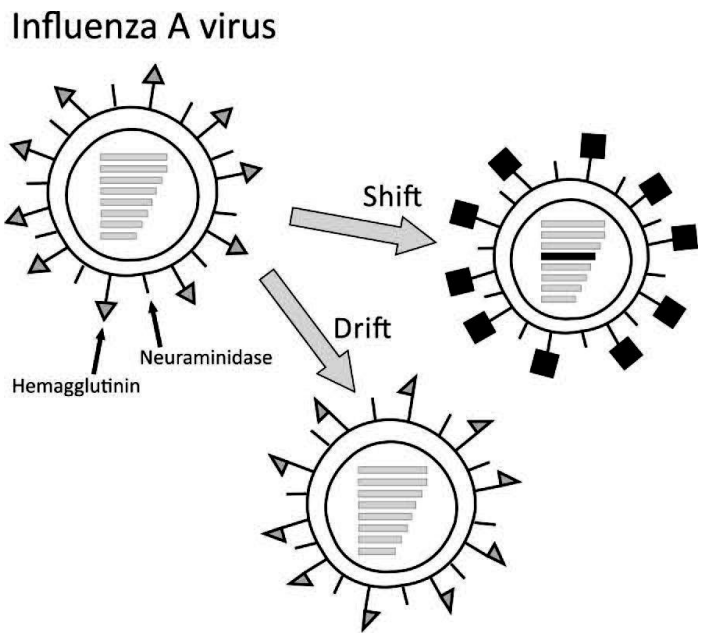

Figure 3. Antigenic shift and drift. The virus can change antigenically in two ways. Either existing antigens are subtly altered, called antigenic drift, or two or more virus strains in the same host may give rise to antigenically shifted progeny virus, where one (or several) segment/s is/are replaced by a segment/s from another strain. The two glycoproteins, HA and NA, as well as the internal gene segments are subject to antigenic shift.

1.2.2 Persistence and modes of transmission

The incidence of influenza in birds exhibits strong seasonal fluctuations and raises questions concerning where the virus is perpetuated between outbreaks. Both biotic and abiotic reservoirs may provide an explanation to the seasonality of avian IAV. A biotic reservoir is constituted by living organisms, such as animals. An abiotic reservoir can be a part of an ecosystem, for example soil, water, or air. The IAV is unable to replicate outside a host cell, and in order to infect new individuals it needs to persist for some time in the environment. IAV is quite well adapted to persist in an aqueous environment, and factors such as temperature, humidity, pH, and salinity have been shown to influence the persistence of the virus. IAV stored at 28°C in distilled water could remain infective for up to 100 days, at 17°C virus was infective after 200 days, and at 4°C virus could remain infective for much longer [60]. It has also been shown that virus infectivity decreases with higher salt content in water [61]. Optimal conditions for virus survival are not common in nature, considering additional factors that may limit virus persistence; pH fluctuations, UV-radiation, the presence of degrading enzymes, bacteria, and other microorganisms. One difference between human IAV and avian IAV is tolerance to low pH. In contrast to human viruses avian IAV strains can persist and remain active at pH 4.0 [62].

Avian IAV circulates in wild bird populations and infected birds shed high titers of virus, both orally and in feces, for several days [12,63]. Infected waterfowl swimming in the same pond or lake as uninfected birds can transmit the virus via feces excreted in the water [64,65]. Excreted virus can persist for long periods of time in water. One study show that virus could be detected after 32 days at 4°C [62]. There is evidence of IAV RNA in frozen lake water even after nesting birds have migrated south [66], and in lakes in Alaska, IAV RNA was found in lake sediment [67]. Further analysis of that RNA revealed a diversity of virus subtypes including a subtype that had not been found from sampled birds the same year [67]. It supports the theory that IAV can persist and reappear after a long period of time, however, infective virus in sediments has not yet been demonstrated.

1.3 INFLUENZA A VIRUS IN DIFFERENT HOSTS

1.3.1 Wild and domestic birds

The idea that all IAV strains originate from wild birds is widely accepted (Figure 4) [10]. Subtypes H1-H16 and N1-N9 have been detected in virus isolates from avian species. Most avian viruses are low pathogenic in poultry, although H5 and H7 IAV have the potential to become highly pathogenic. Disease caused by highly pathogenic avian influenza (HPAI) virus is characterized by severe illness and rapid death [68]. The main difference between a low pathogenic avian influenza (LPAI) virus and an HPAI virus is the HA cleavage site, which in HPAI virus has become a polybasic cleavage site. The elongated cleavage site can be cleaved intracellularly by ubiquitous proteases, which facilitates systemic virus replication in infected birds [69].

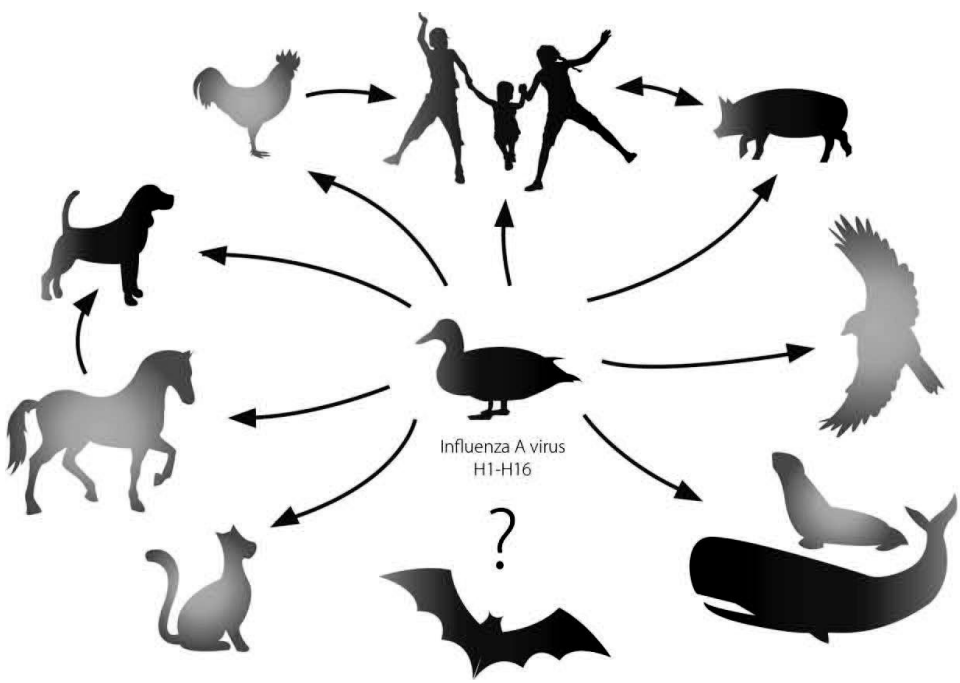

Figure 4. Host range of IAV. The natural reservoir in the middle, and other animals in which IAV have been found. Two HA subtypes, H17 and H18, have been found in bats only

Avian IAV has been isolated from more than 100 avian species [70]. Waterfowl, shorebirds, and gulls are more commonly infected than other wild bird species. The introduction of high-throughput molecular diagnostic methods have led to increased surveillance [71]. Several studies of wild ducks in the Northern hemisphere have revealed a high prevalence of IAV, with a peak in early fall [10,72]. During spring-migration the prevalence is much lower, around 1% [10], although some report prevalence of up to 6,5% [73]. Surveillance of birds at breeding grounds in Alaska and Siberia show an IAV prevalence of 0,03% in the summer [74]. These prevalence patterns raise the possibility that IAV can persist in ducks all year round. The pattern observed corresponds with the immunological status of ducks. When a large proportion of immunologically naïve hatch-year individuals are recruited to the population, a peak of IAV can be observed [72,75,76]. The IAV prevalence in ducks increases at pre-migratory gatherings, and subsequently drops during winter. The drop in prevalence is probably because most individuals have been infected and developed an immune response to IAV [77]. It has been shown that experimental infection of mallards with LPAI virus protects against subsequent challenge with the same virus [12]. Moreover, mallards that were infected with LPAI H5 or H4 virus developed immunity that led to reduced viral shedding after challenge with HPAI H5 virus [78]. At Ottenby bird observatory, located on Öland, Sweden, research has been conducted since the 1940s. A recent publication [79] revealed that since 2002, 74 different combinations of IAV have been found in mallards. All N1-N9 subtypes were identified, and almost all HA subtypes (H1-H12). Some combinations were more frequently observed than others, and LPAI virus of subtypes H5N1 and H7N9 were only found once [79].

In domestic ducks, IAV can be isolated year round. Several factors favor the maintenance of IAV in domestic birds. The virus can remain infective in lake water [64], and for that reason ponds and rice fields are ideal for effectively transmitting the virus via the fecal-oral route [80]. Many duck farms in China are located near lakes which may provide opportunities for IAV exchange among ducks from different populations and between domestic and migratory birds. Ducks can shed virus for several weeks [12,63] and viruses can persist in the population if birds of different ages, with different immunological statuses, when maintained in the same farm [80]. However, only a few subtypes are known to persist in poultry. The endemic strains H6N1 and H9N2 cause mild clinical signs in poultry [80]. Moreover, these viruses have changed their replication site from the intestine to the trachea, which may change the transmission from the fecal-oral route to aerosol [80]. There are studies suggesting that interspecies transmission to humans is not rare in endemic regions [81,82].

So far only HA subtypes H5 and H7 have been found in the highly pathogenic form. LPAI virus of these subtypes may evolve to HPAI virus when introduced into poultry. Outbreaks of HPAI caused by H5N1 virus have been frequently reported from several countries since the 1990s, and numerous genotypes of H5N1 have been detected [83].

1.3.2 Humans

Epidemics of IAV in humans occur every year, both in the Northern and the Southern hemisphere. The epidemics occur during the winter season, roughly October to April in the Northern hemisphere, and May to September in the Southern hemisphere. In tropical regions, IAV can circulate for much longer. Of all respiratory illnesses, influenza has the most dramatic effect on communities.

Influenza is an ancient disease, most likely first described in 412 BC. Historically, there are many records of outbreaks that may have been caused by influenza virus. The outbreaks have occurred at irregular intervals and varied in severity. The pandemic in 1830-1833 began in the winter of 1830 in China, and from there it spread to the Philippines, India, Indonesia, and across Russia into Europe. The pandemic reached Sweden in May 1831 [84]. The pandemic was characterized by a high attack rate but the case mortality rates were low [85]. Thereafter the influenza outbreaks seem to have been scarce until 1889.

The first well-documented pandemic was the Russian flu pandemic of 1889-1893 (Figure 5). Seroarcheological research has suggested that this pandemic was caused by an IAV of subtype H3N8 [86,87]. It has also been speculated that the virus that caused the Russian flu was an H2 subtype and that the H3 subtype emerged somewhere around year 1900 [86,88]. Later in the 20th century, when H3N2 IAV caused the Hong Kong pandemic in 1968, the low mortality observed in individuals of age 70 or older indicated that the IAV strain of their childhood may have been subtype H3 [89,90]. The Russian flu pandemic appeares to have started in the spring in Russia and it was confined to that area until late summer and from October, it emerged globally in repeated waves.

It has been suggested that this particular H3N8 strain continued to circulate until 1918, when the H1N1 Spanish flu emerged. However, seroarcheological data suggests that only half of those born in 1893 were primed with H3 [90], and among individuals born after 1900 H3 antibodies were rare or absent [86]. This indicates that another subtype was circulating prior to the 1918 pandemic. Although there is no clear evidence that H1 emerged around 1900, there are indications of prior adaptation of the H1 HA from the Spanish flu to humans before 1918 [87].

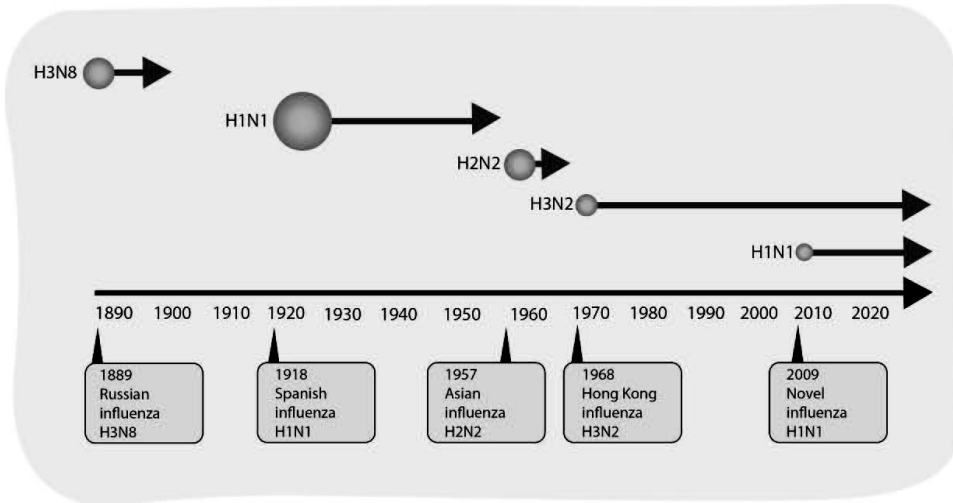

Figure 5. Major influenza A pandemics. Several pandemics have occurred during the past century. The Spanish flu in 1918 caused an estimated 50 millions deaths. The three pandemics that followed were relatively mild compared to the Spanish flu. Length of arrows show how long the subtype circulated in the human population.

In March 1918, several severe cases of influenza were reported in the United States (Figure 5). The virus spread throughout the United States, Europe, and probably Asia. During this first wave of the Spanish flu, mortality rates were in the normal range. However, when the second wave came in the fall of 1918, the mortality rates had increased dramatically. The third wave came in the winter of 1918-1919 and it has been estimated that 30% of the population in the world were clinically affected by the 1918 pandemic [91]. Globally, it is thought to have killed up to 50 million people, unexpectedly targeting young adults. Influenza and pneumonia death rates were 20 times higher in 1918 compared to the previous year [91]. Most deaths were caused by secondary bacterial pneumonia, but the 1918 virus could also kill quickly and directly with a viral pneumonia [91]. During the second and third wave, there was a case-fatality rate of 1-2% in 20-39 year-olds, which is an age group not usually severely affected by influenza.

With modern technology, the genome of the causative agent of the Spanish flu has been sequenced [92,93]. The material used was formalin-fixed, paraffin-embedded autopsy tissues from an American soldier and tissue from an Alaskan Inuit woman who had been buried in permafrost [92,93]. The successful re-creation of the Spanish flu H1N1 virus has enabled

researchers to further study the genetic composition of this virus. Seven of the eight gene segments of the Spanish flu show signs of avian origin. Usually, the uracil content increases after transmission to mammals. The seven segments exhibit avian-like uracil content, while the segment encoding for the HA shows signs of being introduced to humans earlier [87,94].

The next pandemic occurred in 1957 and is called the Asian flu (Figure 5). It started with a major outbreak in Hong Kong. Three of the gene segments, HA, NA, and PB1, from an avian IAV had combined with the previously circulating A(H1N1) strain [95]. The antigenically shifted virus, now of subtype H2N2, spread throughout the world. It started in central China and spread worldwide. It is estimated that the pandemic affected 40-50% of people, of which 25-30% experienced clinical illness [88]. The viral seed needed to develop a vaccine was ready in early May, and about six months later a vaccine was available for use [96].

Eleven years later, in 1968, the Hong Kong flu caused a pandemic originating in China (Figure 5). The subtype was determined as an H3N2 IAV, this time a reassortant with HA and PB1 from an avian source [95]. The virus that caused the Hong Kong pandemic was substantially less pathogenic compared to its predecessors, which partly may be because the NA of the virus remained the same as for the previously circulating virus.

In 1976, there was an episode of swine influenza outbreaks among military recruits in the United States. The causative agent was identified as an A(H1N1) virus of swine origin. It was thought to be the beginning of a new pandemic and vaccine production was started. The virus never returned, and there have been other reports of abortive spread of swine viruses in humans since this episode. The year after, in 1977, the H1N1 virus re-emerged. In an outbreak in Siberia, the causative agent was identified. However, it is known that the virus circulated in China prior to the Siberian outbreak [97]. The virus was similar to H1N1 viruses circulating in 1950 [98], and it rarely affected individuals > 25 years of age [99]. Outbreaks of mainly mild influenza occurred in younger individuals. The reintroduction of H1N1 did not result in the disappearance of the H3N2. After this, influenza vaccines were made trivalent, containing both IAV subtypes H3N2 and H1N1 as well as influenza B.

Influenza pandemics are expected to occur at irregular intervals. The 1957 and 1968 pandemics took place 11 years apart, so the outbreak in 1977, 11 years after the 1968 pandemic, was alarming. In the years after the 1977 outbreak it was feared that a pandemic was imminent. After 1997, much effort was made to ensure that the outbreaks of HPAI H5N1 were under

control, but no one suspected that the next pandemic would be caused by a subtype that had already circulated in the population (Figure 5). Starting in Mexico, several countries across the globe began to report laboratory confirmed cases of a novel H1N1 IAV to the World Health Organization (WHO). On April 25th 2009, the WHO declared a public health emergency of international concern. In June 2009, 73 countries had reported more than 26 000 laboratory confirmed cases and the WHO declared that the pandemic had reached phase 6. This phase is characterized by community level outbreaks in at least two countries in one WHO region plus one country in a different WHO region [100]. During the course of the 2009 pandemic, mortality among children, young adults, and pregnant women was higher compared to seasonal epidemics. Generally, elderly fared relatively well since few were infected, but the lethality among the few elderly was high in comparison to other groups. Data from 19 countries was analyzed for prepandemic seroprevalence, and these results showed the highest rates of cross-reactive prepandemic antibodies in persons >65 years [101]. However, this was not found in all studies. In Sweden the prevalence of pre-existing cross-reactive antibodies in individuals >65 years of age was not significantly higher than in other age groups [102] but the same disease pattern, that elderly were relatively spared, was observed. In typical epidemics, 90% of mortality occurs in this age group [103]. On august 10th 2010 the WHO declared that the H1N1 influenza virus had moved into the post-pandemic period.

Apart from the pandemics and seasonal epidemics of influenza, there have been reports of spillover from the natural reservoir. In 1997, the first case of HPAI H5N1 virus in humans was reported [1]. Since then, H5N1 IAV has caused nearly 700 human cases, with a case-fatality rate of 60% [2]. There are also reports of H7 IAV causing disease in humans, with symptoms ranging from conjunctivitis to pneumonia to death [104]. Recently, the emergence of H7N9 [105] has once again proven that influenza surveillance in birds is of utmost importance. This particular strain had neither been seen in animals nor in humans until march 2013 [106]. Since then, it has caused infections in both birds and humans. H7N9 is epidemic and considered low pathogenic in poultry, but most infected humans have become severely ill. So far no human-to-human transmission indicating air-borne transmission has been observed. The H7N9 virus has gene segments from H9N2 [107], which has been shown to infect numerous avian and mammalian hosts [108]. Reports of H10N8 human cases have also been made [109]. It is evident that IAV from birds pose a threat to human health, and it is crucial to be on guard and prepared for the next pandemic.

1.3.3 Other mammals

1.3.3.1 Swine

As with human influenza, swine influenza can be detected year-round, although seasonal peaks occur in temperate regions. Typically it is a mild disease with clinical signs, including fever, cough, respiratory distress, nasal discharge, weakness and dyspnea. More severe clinical manifestations can occur in co-infections with other respiratory pathogens. In swine, viruses differ according to geographical regions, and new epidemic strains do not necessarily replace existing endemic strains. The classical swine H1N1 virus has been suggested to be derived from the 1918 human pandemic H1N1 virus, and there are reports from the 1918 period of humans and pigs developing respiratory illness simultaneously [99]. This suggests the establishment of H1N1 in North American swine around the same time as the 1918 pandemic.

In 1998, outbreaks of swine respiratory disease revealed a triple reassortant H3N2 lineage, comprised of gene segments from classical swine influenza, human influenza and avian influenza. Since then, many different reassortants have been detected between triple reassortant swine IAV and classical swine [110,111], human H1N1 [112], human H3N2 [113], and avian H2N3 [114] lineages, respectively. Some reassortants have continued to circulate, and some have been transient. In Europe, classical swine IAV was not detected until 1976. Early variants of human H3N2 virus were detected in European and Asian swine, but it was not until 1979 that another IAV lineage could be detected in European swine. This was an avian-like H1N1. There are further reports of avian virus strains in swine; more specifically subtypes H1N1, H3N3 [115] and H4N6 [116] which were isolated in Canadian swine. In the 1970s, classical swine H1N1 was isolated in South-East Asia.

1.3.3.2 Horses

One of the most common respiratory pathogen in horses is equine influenza virus. Throughout history, only Iceland and New Zealand have remained free from equine influenza. Two subtypes of equine influenza virus are recognized today: equine-1 (H7N7) and equine-2 (H3N8). Both are believed to have originated from avian IAV ancestors [117], and an outbreak of H3N8 in horses in the Chinese provinces of Jilin and Heilonjiang showed that horses are susceptible to avian IAV [118].

1.3.3.3 Felines

Studies from the 70s have provided evidence that cats are susceptible to experimental infection with human IAV variants H2N2 and H3N2, as well as influenza B virus [119,120]. The animals shed virus for up to six days, and it was shown that virus inoculated cats could transmit virus to susceptible animals. Interspecies transmission has been observed from an infected human to a cat [119]. In South-East Asia, captive tigers and leopards became ill and died after feeding on H5N1 contaminated chicken carcasses [121] and it was shown that there was direct transmission from the contaminated food, and also probable transmission between tigers [122]. When surveillance was increased due to the emergence of the 2009 pandemic virus, feline cases were identified [123]. These cases of infected domestic cats are likely due to transmission from infected humans. In sentinel-based studies of cats, seroconversions were detected [124], and the pattern seen in cats experimentally infected with the 2009 pandemic virus resembled a milder form of the H5N1 induced disease [125].

1.3.3.4 Canines

In 2004, an IAV was isolated from a greyhound in Florida [126]. It was a unique lineage of H3N8, and all eight gene segments were of equine origin. Since then, cases of canine influenza have been reported from the US, from England, and Australia [127,128]. Along with the emergence of H5N1 in South-East Asia, it became evident that companion animals were susceptible to H5N1 infection [129]. In 2004, a dog in Thailand died after eating duck carcasses [129]. IAV was isolated from both lungs and extrapulmonary tissue, and genetic analyses revealed that it was essentially identical to the H5N1 virus infecting birds, tigers, and humans in Thailand. In a study in Thailand, antibodies to H5N1 were found in 160 out of 629 village dogs tested [130].

1.3.3.5 Minks & ferrets

As a disease model, influenza in ferrets mimics human disease best. When infected, ferrets display clinical signs of disease such as sneezing and coughing, but also decreased appetite followed by weight loss, lethargy, and fever [8,131]. Natural infection in minks with subtype H10N4 has been detected in a Swedish outbreak [132]. Further investigation of this particular strain revealed a probable wild bird origin. The virus was similar to avian virus strains but was adapted to transmission between minks [133,134].

1.3.3.6 Seals & whales

It is well established that seals and whales are susceptible to IAV [135-142]. The first virus to be isolated was H1N3 which was found in lung tissue from a South Pacific striped whale in 1976 [135]. Subtypes H13N2 and H13N9 were isolated from a stranded pilot whale in 1984 [138]. In seals, several subtypes have been found, H7N7 caused an outbreak of severe respiratory infection and high mortality in seals along the New England coast in 1979-1980 [136]. In the same geographic area in 1983 there was another outbreak, this time IAV of subtype H4N5 [137]. Follow-up surveillance in this area resulted in the isolation of H4N6 and H3N3 viruses [139], and serological surveillance in Japan revealed exposure to HA subtypes H3 and H6 [141]. Genetic analysis showed that all genes from the isolated viruses were of avian origin [137,139]. Receptor specificity preferences of some of the isolates have been studied [140], and found to be avian-like in the aspect that they preferentially bind to α2,3-linked sialic acid (SA). This type of SA is also found in the lungs of whales and seals. A recent study also reported that A(H1N1)pdm09 virus was isolated from Northern elephant seals, and that the strains were proven to have a greater than 99% homology to A/Calilfornia/04/2009 (H1N1) [142]. The fact that IAV can cause disease in marine mammals, and that serological data indicate exposure of various subtypes, suggest that these types of infections occur sporadically.

1.4 INFLUENZA A IMMUNITY IN HUMANS

Infection by IAV in humans is initiated in the respiratory tract, and the infection is generally contained in this organ. In the oral or nasal cavities, the virus encounters mucus that covers the respiratory epithelium. Next, it attaches to and infects the respiratory epithelial cells. The innate immune response is activated early in infection, followed by the adaptive immune response.

1.4.1 The innate immune response

The innate immune system, which is the first line of defense, provides a number of mechanisms that effectively reduces the burden of infection. Pattern recognition receptors (PRRs) recognize the viral RNA that is present in infected cells and initiates signaling cascades that lead to innate immune responses. There are at least three distinct classes of PRRs, i.e. Toll-like receptors (TLR3, TLR7 and TLR8), retinoic acid-inducible gene I (RIG-I) and a NOD-like receptor (NLRP3). The TLRs recognize dsRNA from virus-infected cells (TLR3) and ssRNA in endosomes of sentinel cells (TLR7/8) [143]. RIG-I detects ssRNA bearing 5'-triphosphates, and NRLP3 detects virus that is present in the cytosol of infected cells. Both TLRs and RIG-I induce interferon (IFN) production, while NRLP3 is involved in the secretion of interleukin-1β and interleukin-18. They act to promote adaptive immune responses and prime natural killer cells and cytotoxic T lymphocytes (CTLs) [144]. The secretion of type I IFN stimulates the expression of hundreds of IFN-stimulated genes in neighboring cells, thereby inducing them into an antiviral state. Interferon stimulated gene products act to limit IAV infection and spread by inhibiting replication and by degrading vRNA [144].

1.4.2 The adaptive immune response

The second line of defense against IAV infection is the adaptive immune response. This highly specific response is relatively slow when first encountered by a specific pathogen. However, with memory being the prominent feature of adaptive immunity, the response is fast and strong when encountered the second time.

1.4.2.1 Cellular immune response

The cellular immune response is activated after recognition of viral epitopes, presented on antigen presenting cells. $CD4^+$ T cells differentiate to T helper cells, which in turn can promote the activation and differentiation of B cells. T helper cells are also involved in promoting cytotoxic T lymphocytes. CTLs are $CD8^+$ T cells which have been activated after recognition of viral epitopes on antigen presenting cells. They migrate to the site of infection where they recognize and eliminate IAV infected cells [145]. CTLs are mainly directed against epitopes of the internal viral proteins [146]. Hence, they display a high degree of cross-reactivity with IAV of different subtypes. After infection, a memory T cell pool persists and can undergo rapid reactivation after reinfection.
The main way in which IAV can evade recognition by CTLs is through the high mutation rate during replication. For example, there are more non-synonymous mutations in CTL epitope regions compared to the rest of the protein [147].

1.4.2.2 Humoral immune response

IAV infection induces production of specific antibodies by B cells. Primary infection induces IgM, IgA, and IgG isotypes, while IgM antibodies are not observed in reinfections [148]. The two surface glycoproteins of IAV, HA and NA, are excellent targets for B cell responses. The globular head of the HA is a major target of neutralizing antibodies. Most antibodies targeting the HA globular head are strain specific and cannot neutralize drift variants and viruses of other subtypes. It seems that IAV infection induces a long lasting antibody response. It has been suggested that exposure to A(H1N1) IAV strains that have been circulating in the past may have partially protected the elderly against the 2009 pandemic virus [149,150]. In addition to antibodies targeting the globular head and the receptor-binding pocket, antibodies targeting the stalk region of HA are also generated. This region is highly conserved, and antibodies binding the stalk region provide protection against various IAV strains [151,152]. Compared to HA

targeting antibodies, NA inhibiting (NI) antibodies have received less attention. However, they do play a crucial role in limiting the spread of newly formed viral particles. Antibodies that block the NA act like the body's own NA inhibitor by effectively reducing the replication efficiency. Several studies have shown that NI antibodies are important for reduced viral replication and disease severity [153-155]. In one study from the 1970s, it was indicated that repeated exposure by natural infection was needed to induce anti-NA responses [156]. In addition, it has been indicated that protection against clinical disease during the Hong Kong A(H3N2) epidemic in 1968 was related to prepandemic NI antibodies [157].

1.4.3 Clinical features, pathogenesis, and transmission

Human IAV is highly contagious and is transmitted by the airborn route. Cough, creating aerosols, is a major symptom of influenza and facilitates spread. Circulating human IAV strains mainly cause respiratory disease and preferably infect the epithelium lining the airways. The respiratory tract is the primary target of infection, not only for human IAV, but also for mammalian IAV in general. The virus does not normally spread beyond the respiratory tract. The protease needed to cleave and activate the HA is located in epithelial cells in the airways and lungs. As a result, free virus is rarely found circulating in the blood or in other organs. The incubation period is 1-5 days, with an average of 2 days. Virus replication peaks at 2-3 days after infection. Factors that determine the clinical course and outcome are virulence of the virus, age of the patient, and the presence of chronic illness, immunosuppression, or pregnancy. The characteristic clinical symptoms for influenza are an abrupt onset, with fever being the most prominent symptom. Other symptoms are headache, chills, dry cough, myalgia, malaise, and anorexia. Rhinorrhea, nasal congestion, and sore throat are present, although these symptoms are overshadowed by the systemic symptoms during the first 3 days of illness. Systemic symptoms are due to the body's own immune system being activated and the release of proinflammatory cytokines [158]. Fever and upper respiratory tract symptoms usually resolve within 7-10 days. Cough and weakness may persist 1-2 weeks after onset.

Differences in IAV virulence may cause a more severe disease. For example, the 1918 Spanish influenza virus was more virulent than viruses causing seasonal epidemics. Some mechanisms that determine pathogenicity are tropism, spread of infection, replication efficiency, and escape from or modulation of host immune response. The 1918 Spanish flu virus has, compared to the 2009 pandemic virus, a higher virus yield in human airway cells. It binds stronger to the receptor, has a functional PBI-F2, and was often associated with secondary bacterial infections [103]. The influenza protein NS1 is involved in inhibiting RIG-I, by blocking downstream signaling and thereby attenuating IFN expression [159]. The tropism of avian and human viruses is restricted to the type of receptor. Avian viruses preferentially bind α2,3-linked SA receptors, and human viruses bind α2,6-linked SA receptors. However, in the human lower respiratory tract (in bronchiolar and alveolar tissue) both of these receptor types are present in equal amounts [27]. Avian viruses can therefore replicate in the lower respiratory tract, which increase their pathogenicity in humans. HPAI viruses have an elongated HA cleavage site, resulting in a virus with increased pathogenicity.

The primary mode of transmission of IAV is by aerosol or droplets. The IAV can also be transmitted by direct contact with virus-contaminated hands, or by fomites [160]. Several studies support the theory that IAV survival and transmission is facilitated by low relative and absolute humidity and low temperatures [161,162]. The number of influenza patients increase during the winter period. There are cases during the summer, but epidemic spread does not start until the temperature drops in autumn. The behavior of IAV enables vaccines to be produced and evaluated in time.

1.5 PREVENTION AND TREATMENT OPTIONS

1.5.1 Vaccines

Vaccination against influenza is an important way to control pandemics and seasonally occuring epidemics since the first inactivated influenza vaccine (IIV) was licensed in 1945 [163]. There are different forms of IIVs available; split vaccines, subunit vaccines, and whole virus vaccines. There is also a live-attenuated influenza vaccine. The traditional method for producing IAV vaccine strains is growth of the virus in embryonated hen's eggs. There are several factors, such as limited availability and vulnerability of the chicken population to potential pandemic strains of IAV, which may limit or complicate the production of egg-grown vaccines. Mammalian cell culture systems, insect cells, and plant cells do not have the same drawbacks as growing in eggs, but highly productive systems have been more difficult than expected to establish and maintain, and thus most vaccines are still egg-based. In Sweden annual vaccination is recommended for risk groups [164] e.g. adults ≥65 years, pregnant women, and people with certain underlying medical conditions. The composition of influenza vaccines change when new or antigenically drifted strains emerge. Since 1977, IIV have contained three influenza strains – one A(H1N1), one A(H3N2) and one influenza B. Due to recent co-circulation of two distinct lineages of influenza B virus quadrivalent formulations have been produced and licensed for use.

The only known correlate of protection for IAV is HI titer. A titer of ≥40 is considered 50% protective. However, it is important to know if vaccine induced immunity can be compared to the immunity elicited by natural infection in regards of duration, cross-protection, humoral response, and cell-mediated response. Immune memory is considered to have reached a resting state a year after the first encounter with IAV. A recent study [165] compared three different patient groups. One group received an adjuvanted monovalent A(H1N1)pdm09 vaccine, one group had been mildly ill with natural IAV infection, and one group had been severely ill. Several parameters were measured one year after vaccination/infection. The results confirmed that vaccination induced humoral and cellular immune responses similar to those of a mild to moderate infection. Patients who had been severely ill had higher hemagglutination inhibition (HI) titers in serum, compared to both mildly ill and vaccinated subjects. It is clear that more research on the effects of vaccination versus infection is needed.

1.5.2 Antivirals

The use of antivirals against influenza A infection can shorten the duration of disease. Antivirals may also be used as prophylactic strategy to limit the spread in an early pandemic phase. In the case of a new pandemic, the virus can spread rapidly and updated vaccines may not be available in time for a global mass-vaccination. The antivirals commonly used for treatment or prevention of IAV disease are described in the sections below.

1.5.2.1 M2 inhibitors

The M2 protein is a part of the virus envelope, where it functions as a proton channel. During the virus entry process, the M2 proton channel is involved in lowering the pH in the virus, resulting in the dissociation of M1-vRNP interactions. When the virus membrane fuses with the endosomal membrane, the free vRNP is released and transported to the nucleus. There are two antivirals that can inhibit M2 [166], amantadine, and rimantadine. When the M2 channel is blocked, the release of M1-vRNPs into the cytoplasm and further transport of vRNPs into the nucleus is blocked (Figure 6) [167]. These antivirals are not recommended for use due to extensive circulation of amantadine-resistant viruses in the human population [168].

1.5.2.2 Neuraminidase inhibitors

NA is important for promoting release of newly produced virus from infected cells. It prevents aggregation of virions, and it also facilitates infection in the respiratory tract by removal of SA from mucins and by destroying decoy receptors [53]. There are two antiviral agents commonly used to treat influenza A infections: oseltamivir and zanamivir. They inhibit the NA function, thereby hindering the release of newly formed virus (Figure 6) [169]. These compounds are administered orally and inhaled, respectively. There are reports of oseltamivir resistance [170-172], but there are only few reports of zanamivir resistance [173,174]. It has been shown that, if treatment is started within 48 hours after onset of symptoms, these antivirals can reduce the duration of influenza illness with 1-2 days [175].

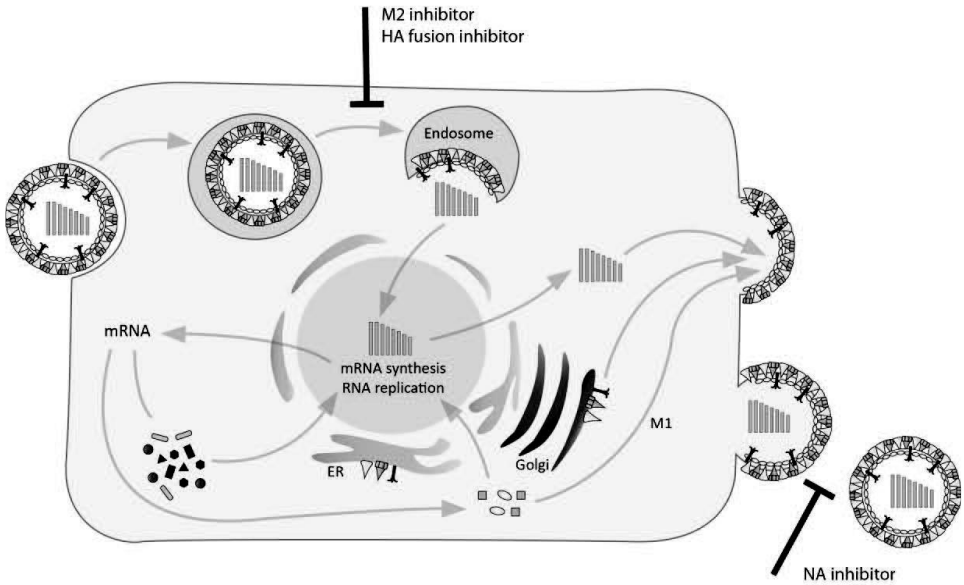

Figure 6. Antivirals and their mechanism of action in the IAV replication cycle. M2 ion-channel inhibitors act by blocking vRNP transport into the nucleus. NA inhibitors target the NA, an interaction that blocks the release of newly formed progeny virus. HA fusion inhibitors targets the HA, more specifically this interaction inhibits the fusion between the viral envelope and the endosomal membrane.

1.5.2.3 Hemagglutinin fusion inhibitiors

A third class of antivirals against IAV targets the fusion activity of the HA (Figure 6). The antiviral, Arbidol, has been shown to inhibit the replication of all IAV subtypes and influenza B viruses [176,177]. The product is only licensed in Russia and China, and its clinical efficacy has not been completely established.

1.5.3 Pandemic preparedness

The WHO has prepared guidelines to aid countries in pandemic preparedness and response planning [100]. A pandemic can be divided into 6 phases, 1 through 6, followed by a post-peak period, a possible new wave period, and the post-pandemic period. Phase 1-3 are primarily aimed at strengthening pandemic preparedness and response, such as developing robust surveillance systems in collaboration with other relevant sectors. In phase 4, there is evidence of human-to-human transmission and ability to sustain community-level outbreaks. This phase emphasizes increased surveillance, and also a first step to limit, or delay, spread by vaccination. During phase 5-6, the actions are no longer focused on preparedness but on response. During the post-peak period, there is time to prepare for a possible future pandemic wave by restocking resources and revising plans. The final post-pandemic phase occurs when levels of influenza activity have returned to normal.

In Sweden, three different antivirals (Tamiflu®, Relenza®, and Symmetrel®) are stock-piled in case of a pandemic [178]. A pandemic vaccine is impossible to stock-pile, since the vaccine cannot be produced until the pandemic IAV strain has been identified.

1.5.3.1 Surveillance in humans

The aim of IAV surveillance is to identify virus with a possible pandemic potential. In Sweden, IAV from clinical samples are collected and characterized during each influenza season. IAV A(H1N1) confirmed cases are subject to notification to the Public Health Agency of Sweden, which issues weekly influenza reports. There is a sentinel program in Sweden, that reports back to WHO and European Center for Disease prevention and Control [179]. Participating health care centres submit nasopharyngeal samples from patients with influenza-like illness, and these samples are screened for IAV. A subset of positive samples are further characterized by sequencing. When a new IAV emerges, it is crucial to establish a polymerase chain reaction (PCR) that targets the new strain.

1.5.3.2 Surveillance in birds

Surveillance of IAV in birds is an important tool to properly assess potential pandemic threats. Much work has been focused on IAV in its natural environment, the aquatic birds. Birds can be sampled by taking cloacal swabs, oropharyngeal swabs or serum. IAV is detected by real time reverse transcriptase PCR, and isolates can be further characterized by sequencing or HI. Sera can also be tested by HI or other serological methods including enzyme-linked immunosorbent assay (ELISA) and microneutralization.

In response to the HPAI H5N1 virus, the European Commission required all member states to increase surveillance for avian influenza. If HPAI virus is detected in wild birds or poultry, or in case LPAI virus of subtype H5 or H7 is detected in poultry, this must be reported to the European Commission and to the World Organisation for Animal Health. The results of the entire surveillance must be reported to the European Commission, which presents an annual report.

2 AIMS OF THE PRESENT STUDIES

In this thesis, focus lies on gaining further, more detailed knowledge about IAV, as it affects animals as well as humans around the world. This thesis helps put a small piece of the puzzle together.

Specific aims

- To investigate if migratory ”hot spots” are places for avian IAV to thrive and to reassort.
- To investigate if a pseudoparticle neutralization test can be used to detect antibodies against H5 and H7 IAV in birds.
- To investigate possible age-related differences in neutralizing antibody patterns against H1N1 IAV before the 2009 pandemic.
- To study the prevalence of anti-neuraminidase (NA) antibodies in Sweden before the 2009 pandemic, and study the influence of anti-NA antibodies in a neutralization assay with prolonged incubation time.

3 METHODOLOGY

There are many different methods used for detection, propagation and characterization of IAV, some are old techniques which have been used for decades and some are recently implemented. The following methods were used in this thesis.

3.1 SCREENING AND CHARACTERIZATION OF INFLUENZA A VIRUS

3.1.1 RNA isolation and virus detection

RNA from fecal samples collected from birds was extracted by using commercially available kits. Subsequently, a reverse transcriptase PCR followed by a real time PCR was carried out. The primers used for detection targeted a conserved region of the M segment. In paper I, fecal samples were screened with two PCR-systems: one SYBR-green system and one TaqMan system.

3.1.2 Virus isolation

There are several ways to isolate influenza virus: either in cell-culture, or in embryonated chicken eggs. In this thesis, egg culture was used to isolate and propagate virus.

All samples that were positive by RT-PCR were inoculated in embryonated chicken eggs. Propagation in eggs has been the standard method for IAV since it was introduced in the 1930's. The World Organisation for Animal Health guidelines [180] state that this is the method of choice for isolation of IAV from birds. Most avian IAVs grow in embryonated eggs while many human and some porcine viruses grow poorly. For the latter, isolation of virus from cell culture (primarily MDCK) is often used. It should however be kept in mind that growth of IAV, both in eggs or cell culture, can induce mutations that may change the virus phenotype. In paper I, the original sample was inoculated in 11-days old embryonated chicken eggs. The allantoic fluid was harvested after two days and growth of IAV was detected by an HA assay with chicken erythrocytes. If no IAV was detected after the first passage, the allantoic fluid was passaged once more. Not all PCR-positive samples could be isolated, indicating that differences in virus content or degradation in the original sample may determine the outcome of an isolation attempt. Another aspect to keep in mind is that if two strains are present in one sample there is no guarantee that they both will grow in embryonated chicken eggs.

3.1.3 Characterization of influenza A virus

3.1.3.1 Hemagglutination assay and hemagglutination inhibition assay

These methods are fast, cheap and reliable tools for confirmation of IAV growth and characterization of IAV. The HA inhibition test was first described in the 1940s [181,182]. Hemagglutination occurs when virus particles cross-bind SA residues on erythrocytes via the receptor-binding site present on the HA, i.e. agglutinated blood cells form a carpet coating the bottom of a test tube or microplate-well, while non-agglutinated erythrocytes form a button. Pre-incubation with anti-HA antibodies inhibits the binding between HA and SA on erythrocytes. A common method to type IAV isolates is to test the virus against a panel of subtype specific antisera (rabbit or ferret).

3.1.3.2 Sequencing

In paper I, PCR products were amplified using specific primers for conserved non-coding regions for all gene segments [183], followed by a gel-purification step prior to sequencing.

3.1.3.3 Genomic analysis and phylogenetic trees

To show the relationship between different subtypes and different strains of IAV (paper I), phylogenetic trees were constructed. The Bioedit 7.0.0 software was used for alignments and subsequently the Mega3 software [184] was used to construct neighbor joining trees for each gene and subtype of the glycoproteins. Representative sequences were selected from each major sub-clade to represent the phylogenetic span in a comprehensible way. The Eurasian and American sequences were then merged and aligned with full length sequences of our own samples. These alignments were used to illustrate phylogenetic and geographic relationships. In paper III, HA nucleotide sequences for phylogenetic analysis were downloaded from http://www.ncbi.nlm.nih.gov/. Alignment was performed using MUSCLE algorithm in MEGA 5.2.2 [185] and Jmodeltest 2.1.2 [186] was used to evaluate the best phylogenetic model. Then, Bayesian evolutionary analysis by sampling trees [187] adding age tips and, resulting from the Jmodeltest analysis, the general time-reversible and invariant sites model was used for phylogenetic analysis. The maximum clade credibility tree was calculated with TreeAnnotator using 10% of the trees as burn-in. The tree was then elaborated and annotated with FigTree [188].

3.2 ANTIBODY DETECTION

The detection of antibodies against IAV is an important tool when it comes to influenza surveillance, both human and animal. Here, we describe four methods used for the detection of anti-influenza antibodies.

3.2.1 Hemagglutination inhibition

The HI assay is a very useful technique to evaluate influenza antibody levels in a population. The method relies on the hemagglutination of erythrocytes (as described in section 3.1.3.1) and the addition of sera that inhibit this interaction. Although HI antibodies are not the same as neutralizing antibodies, the serum titers obtained with the HI assay do correlate with protection against influenza [189,190]. This approach can also be used to diagnose influenza, if viral shedding has ceased and no virus can be identified. The acute phase and convalescent phase sera can be tested and if there is an increase in antibody titers, four-fold or higher, the result is considered positive.

3.2.2 Microneutralization test

A neutralization test detects protective antibodies, and is thus useful to study immunity after disease or vaccination. It can be carried out in a micro-format, and it does not require any other pre-treatment of samples than heat-inactivation of sera. Serum is mixed with virus and incubated for 1 hour at 37ºC in 96-well plates before target cells are added. Cells may then be monitored daily for cytopathic effect by microscopy, which can be difficult and requires training. Another option is to fix the cells and use an antibody towards influenza NP followed by a secondary peroxidase labelled antibody. The subsequent addition of an appropriate substrate will vizualise the presence of influenza antigen by change of colour in the supernatant. If virus has been neutralized no colour change will be observed. It is possible to measure the optical density of the colour change which reflects the level of neutralization.

3.2.3 Pseudoparticle neutralization test

One alternative to a standard microneutralization assay is to use pseudotype virus particles. The advantages are many: there is no need for live virus, the particles are easy to produce and standardize, and the assay is sensitive. The particles are produced in a packaging cell line, HEK-293T, by transfection with plasmid constructs which create virus like particles (Figure 7). One plasmid contains HIV gag/pol, one contains influenza HA and one is a reporter gene construct. For some subtypes of influenza, a plasmid expressing a protease is also needed for successful cleavage of HA [191]. The NA can be co-transfected if desired. The produced particles carry a reporter gene, in this case luciferase, which makes it possible to detect infection/inhibited infection. The assay is then performed similarly to a standard microneutralization test.

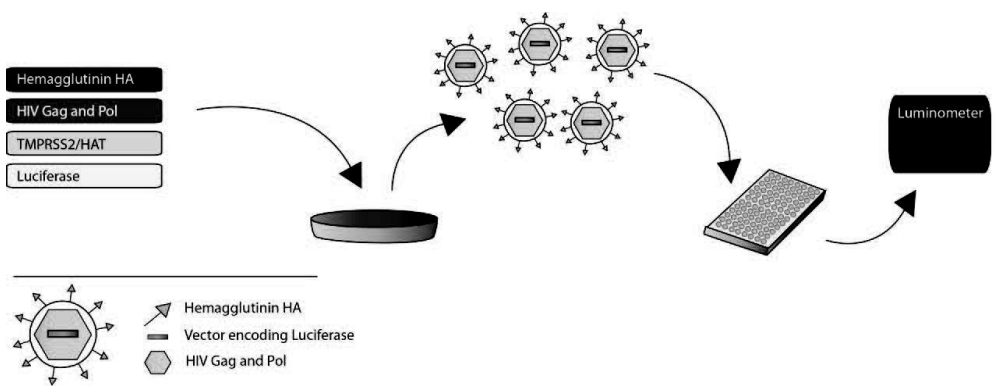

Figure 7. Pseudoparticle neutralization assay. HEK293T cells are transfected with plasmids containing IAV HA, HIV gag and pol, a protease to cleave IAV HA into its active form, and a luciferase reporter gene for later detection. The produced pseudoparticle carries the IAV HA on its surface, and the luciferase reporter gene inside. The produced pseudoparticles are subsequently used in neutralization test, where they are mixed with serum. After 48 hours incubation on HEK293T cells, an appropriate substrate is added and relative luminescence is detected in a luminometer.

3.2.4 Enzyme-linked lectin assay

The surface glycoprotein NA is crucial for the release of newly formed virus particles [46,47]. There are commercial methods available for the measurement of NA activity, but only one method that can measure neuraminidase inhibiting (NI) antibodies [192,193]. In paper IV, the enzyme-linked lectin assay (ELLA, Figure 8) was used to measure NI antibodies in human sera. The method relies on the use of reassortant IAV, i.e. non-human HA, to avoid interference by HA-specific antibodies. A serum-virus mixture is added to plates coated with fetuin. If no antibodies targeting NA are present in the serum, the NA will cleave terminal residues of the fetuin, exposing sites where HRP-conjugated peanut-agglutinin (PNA-HRP), can bind. The bound PNA-HRP can be detected by adding an appropriate substrate and measuring the optical density.

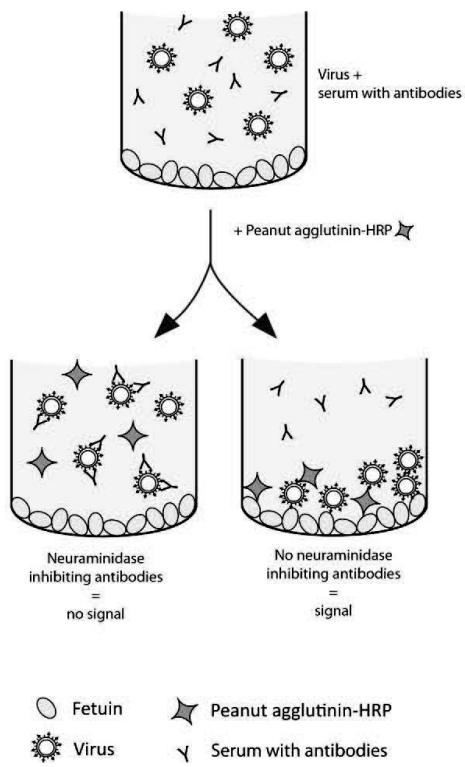

Figure 8. Enzyme-linked lectin assay. Serum and IAV is added to a fetuin coated plate and incubated. PNA-HRP is added to the plate, and if the NA is inhibited the PNA-HRP is not able to bind to the fetuin (= no signal). If the NA is not inhibited, terminal residues on fetuin is cleaved thereby exposing binding sites for PNA (=signal).

4 RESULTS AND DISCUSSION

In the sections below, the results from papers I-IV will be discussed.

4.1 PAPER I

The aim in paper I was to study the presence and characteristics of avian IAV in wild birds in Alaska. The proximity of two continents and major migratory flyways that intersect here could be an ideal geographic area for IAV from Eurasian lineages and North American lineages to mix. Dabbling ducks, geese, shorebirds, and gulls were sampled from the Chukchi Peninsula and Point Barrow. Substantial effort was made to ensure proper handling of the collected samples. The material was treated and stored in -80°C within six hours after being collected.

Our results show similar prevalences (2%) of IAV in avian species from this area, as compared to other parts of the world [72]. Four samples from Point Barrow, Alaska, were positive by PCR, and three of them could be isolated in embryonated chicken eggs. Phylogenetical analysis revealed three distinct subtypes: H6N1 (from a dunlin), H8N4 (from two northern pintails), and H3N8 (from a northern pintail). The analysis of all gene segments showed that the H6 was of Asian lineage origin. All other gene segments were from the American lineage. However, when compared to all Asian and American H6 genes the closest match was an American isolate from Delaware. This finding suggest that the isolated Asian origin H6 virus was an already established introduction in North America. No completely Eurasian viruses have been found in North America [194,195], but chimeric IAV have been found [194,196-198], although they are considered to be the result of rare events of reassortment.

Relevant bird species (e.g. northern pintails) have common Holarctic breeding grounds, and birds banded in Alaska have been recovered in Eurasia. Thus, this is a species that makes trans-hemispheric movements [198]. The possibility of genetic exchange between Eurasian and North American virus lineages is a potential threat, although these events are relatively rare [194,196-198]. In our study, one gene segment was of Asian origin, but the closest match was a virus isolate from North America, retrieved before the current expedition. This indicates that mixing of lineages does occur. Humans may occasionally be infected by avian IAV, including the recent H7N9 human cases [199]. There is a need for better understanding of the dynamics of genetic reassortment and the barrier that it might constitute, and relate it

to bird migration and breeding habitats. Threats of highly pathogenic avian IAV spread via migratory birds should be properly assessed, in order to undertake appropriate measures in the face of a new pandemic.

4.2 PAPER II

In surveillance programs, birds are primarily screened for the presence of IAV genetic material but it may also be valuable to detect antibodies against IAV, which reflect previous IAV infections. Methods such as HI and ELISA are available and used for this purpose. In paper II, we compared a pseudoparticle neutralization test (pp-NT) (based on HPAI H5 and H7 strains) with standard tests such as microneutralization and HI. The pseudotype particles were produced by transfecting cells with plasmids, encoding MLV gag/pol, and IAV HA, respectively, and a luciferase reporter gene construct. The resulting particles carry the IAV HA on the surface, and the reporter gene on the inside. In this paper, we also aimed to investigate the possibilities to use the pp-NT to screen sera from a variety of avian species. The titers observed with the pp-NT were comparable to those observed with microneutralization test and HI. Moreover, when a panel of avian sera was tested, the pp-NT could identify sera containing antibodies against subtypes H5 and H7. However, two of the H7N3 sera were negative when tested with the H7 pp-NT, and whether this was due to low titers in these field-samples, or too large differences in the HA, remains unclear.

IAV can infect many different species [70] and IAV from the natural reservoir (i.e. birds) pose a threat to human health [1,104,105]. HPAI virus of subtypes H5 and H7 are the causative agents of fowl plague in poultry [200], and since 1997 there have been reports of human cases of HPAI H5N1 virus infections [201]. HPAI H7 virus has also been reported in humans, causing conjunctivitis and/or influenza-like illness [104]. Surveillance of IAV in poultry and wild birds is a key factor when it comes to prediction and control of a pandemic. A surveillance study conducted in China [202] in live poultry markets showed that H7N9 was prevalent in samples from the birds as well as in environmental samples from cage floors, drinking water, and slaughter zones. Workers from these poultry markets were also tested. One asymptomatic H7N9 infection was detected by RT-PCR. Additionally, there was serological evidence of H7N9 exposure in 1,6 % of the workers when sera were tested by HI. Thus, a serological method such as the pp-NT, that measures neutralizing antibodies in a safe way, could be an option in both bird and human surveillance studies instead of traditional methods that rely on the use of live virus. We believe that surveillance of birds is absolutely necessary to catch early

emergence of HPAI virus strains, or IAV of subtypes that might evolve to HPAI virus (e.g. H5, and H7). Serological methods may be of great value to further increase the possibility to detect such subtypes.

4.3 PAPER III

The 2009 pandemic was caused by a novel A(H1N1) strain. Initial reports suggested that previously circulating strains and vaccines were ineffective. Usually, the elderly (>65 years of age) are considered a risk group for serious influenza illness, but this particular group was not affected as anticipated. The elderly were shown to have pre-existing, cross-reactive antibodies, which could have protected them from infection [149,150]. However, serological analyses by HI in Sweden did not identify significantly higher cross-reactive antibody titers in the elderly compared to other age groups [102].

From that set of samples collected before the pandemic, 99 samples were randomly chosen, stratified in ten-year age intervals, and tested for neutralizing antibodies against HA from four A(H1N1) strains. The HAs used were derived from A/South Carolina/1/1918, A/Puerto Rico/8/1934, A/New Caledonia/20/1999, and A/Brisbane/59/2007, respectively. The assay was based on pseudovirus particles, with a core of HIV gag/pol, IAV HA on the surface, and a reporter gene expressing luciferase. Our results show that only one age group, the >90 year-olds, had significantly higher neutralizing titers against A/South Carolina/1/1918. This age group had a median neutralizing titer of 320. The 71-80 and the 81-90 year-olds had significantly higher neutralizing titers against A/Puerto Rico/8/1934. The median titers in these two age groups were 3840 and 3200, respectively. There were no significant differences in neutralizing titers between the age groups when A/New Caledonia/20/1999 and A/Brisbane/59/2007 were tested. All samples were positive, with median titers >320. The hypothesis in this study was that the elderly would have dominating cross-reactive neutralizing antibodies towards strains that circulated during their childhood.

A first IAV infection is thought to prime and affect later antibody responses; a phenomenon referred to as the theory of "original antigenic sin" [203]. It is believed that original antigenic sin could have been a reason for the unusal mortality pattern observed during the Spanish flu pandemic in 1918 (caused by A/South Carolina/1/1918-like IAV). During that pandemic, the elderly fared relatively well, while young adults were severely affected [204]. Seroarcheological research suggests that the IAV strain that circulated during the childhood of these young adults was of a different subtype i.e. H3 [87]. The elderly, however were likely to

have been exposed to H1N1 during their childhood, which would have granted them protection against the Spanish Flu. The A/South Carolina/1/1918 strain is phylogenetically closely related to the 2009 pandemic virus A(H1N1)pdm09, and several studies show a cross protection against the two strains [205-208]. Moreover, antibodies against the A/Puerto Rico/8/1934 strain have also been shown to protect against challenge with A(H1N1)pdm09 in mice [209].

4.4 PAPER IV

In paper IV, serum samples collected before the 2009 pandemic were tested for the presence of antibodies against NA. Ninety-nine samples, the same as used in study III, stratified in 10-year age intervals were analyzed by an ELLA and also by a neutralization test. It is believed that anti-NA antibodies are important for mitigating influenza disease [153-155], and we hypothesized that the presence of cross-reactive anti-NA antibodies against the A(H1N1)pdm09 virus could explain why the elderly were so mildly affected by the pandemic. ELLA measures neuraminidase inhibiting (NI) antibodies, i.e. antibodies that inhibit the function of the NA. A neutralization test mainly measures neutralizing antibodies against HA, but it is believed that a prolonged incubation time (up to a week) of the neutralization test can show effects of antibodies targeting other viral proteins, such as NA.

A comparison of ELLA and neutralization test with prolonged incubation time (6 days) were used to evaluate the NI antibody response. We also determined if NI antibody titers were correlated to age. The results showed a strong positive correlation between NI antibodies, as measured by ELLA, and age ($r=0.67$, $p=<0.0001$). The correlation between neutralization and NI titers was however weak and thus a neutralization assay with increased incubation time is not a feasible method to measure NI antibodies. It has been shown that repeated exposure to NA can induce a robust NI antibody response [156]. It is possible that the high titres of NI antibodies found in the elderly, in combination with pre-existing cross-reactive antibodies to HA, contributed to the protection of the elderly during the 2009 pandemic. The NA content is, in contrast to HA, not measured in influenza vaccines. If it is of importance for protection, at least against severe disease, NA should be given more focus in vaccine production. The role of antibodies targeting NA needs to be further evaluated. ELLA was in our hands a robust and practical method to measure NI antibodies, and the method could be extremely helpful in larger studies, evaluating the humoral NA response to various IAV strains.

5 CONCLUDING REMARKS

Influenza epidemics occur every year in all countries across the globe. Influenza viruses infect not only humans, but also animals. The work presented within this thesis aim to increase the knowledge of possible IAV spread, between continents, by wild birds. We have also evaluated and implemented a pp-NT for detection of IAV neutralizing antibodies in both avian and human sera. In addition we evaluated a method for detection of NI antibodies. The titers obtained with the pp-NT and ELLA showed that the elderly in 2009 had higher antibody titers to A(H1N1) strains circulating in 1918 and 1934, and we also found a positive correlation between age and NI antibody titers. These findings may help explain why the elderly were relatively spared during the 2009 pandemic.

Birds are the natural reservoir for IAV and it is of utmost importance to know what types of viruses that circulates among them since virus from this reservoir can spread to humans. In paper I, we investigated if viruses of different genetic lineages (e.g. Eurasian and American) mixed at migratory hot spots, such as Point Barrow in Alaska. Even though we did not find any evidence of recent genetic mixing at this migratory hot spot, the risk of viruses being able to spread by migrating birds could still be a concern.

One way to monitor which viruses are present within this reservoir, besides regularly testing domestic and wild birds for the presence of IAV, is screening for antibodies directed against IAV. There is also a need to monitor humans for the presence of antibodies against avian IAV, since workers at poultry markets and farms have close contact with living and dead poultry. In paper II, we evaluated a pp-NT which was found as sensitive as using the conventional methods microneutralization test and HI assay. When a panel of avian sera was tested, the pp-NT could detect HA subtypes H5 and H7. The pp-NT is based on non-infectious pseudovirus particles and is therefore a safe alternative to other antibody detection assays that are based on live virus.

In humans, IAV cause epidemics every year, and the elderly is considered a risk group. The reason why elderly were only mildly affected by the 2009 pandemic virus is not clear. In paper III, we showed that of all age groups, the >90 year olds had the highest neutralizing titers against the 1918 Spanish flu virus, which is phylogenetically related to the 2009 pandemic virus. In addition, we observed high neutralizing titers against IAV circulating in 1934 in 71-90 year olds. In animal studies, antibodies against this virus has also been shown to offer some protection against the 2009 virus. Our results support the theory that the elderly were protected

during the 2009 pandemic by immune responses induced early in life by IAVs antigenically similar to the 2009 pandemic virus.

In paper IV, we observed a strong correlation between NI antibodies and age. We also investigated if a neutralization test with prolonged incubation time could be an option to measure NI antibodies. Our results do not suggest that such a neutralization test is an alternative to measure NI antibodies. Since IAV vaccines were introduced in the 1940s, the focus has been on the HA part of the virus. However, the mitigating effect of NI antibodies should be considered when vaccines are produced. There is an interest in developing universal IAV vaccines that target the stalk of the HA [210,211], but there could also be of interest to develop vaccines that do not prevent infection but instead lead to a milder disease. However, more research is needed before vaccines that induce broadly neutralizing antibodies targeting the HA stalk and antibodies targeting the NA become a reality.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Influensa orsakas av ett virus och drabbar varje år många människor över hela världen. De vanligaste symtomen är plötsligt insjuknande, feber och ont i kroppen. De allra flesta blir friska inom några veckor, men det finns ett antal riskgrupper där influensa kan orsaka svår sjukdom. När en människa infekteras med influensavirus reagerar kroppen med att aktivera immunförsvaret. Antikroppar bildas, vilket hjälper till att döda viruset. När man blir infekterad av samma virus igen kan kroppen snabbt producera dessa specifika antikroppar och därigenom skydda mot infektion eller svår sjukdom. Antikropparna kan påvisas genom att testa serum i ett så kallat neutralisationstest. Det finns två ytproteiner på influensaviruset, hemagglutinin och neuraminidas, och vid infektion bildas antikroppar mot båda dessa ytproteiner.

Sedan många år tillbaka är det känt att influensavirus från fåglar kan orsaka sjukdom hos människan. Hittills har det handlat om enskilda fall utan vidare spridning mellan människor. Dock finns det en risk att fågelvirus ska smitta människor och börja spridas mellan människor. Därför är det viktigt att ha en god övervakning över vilka virus som sprids, både i människa och i djur. I delarbete II använde vi en neutralisationsmetod för att testa om vi kunde identifiera antikroppar riktade mot de subtyper (H5 och H7) av influensavirus som orsakat svåra utbrott i fågel och även infekterat människor. Vi jämförde denna metod med andra metoder som använts länge för detta ändamål, samt testade om metoden kunde användas för att specifikt detektera antikroppar mot H5 och H7 i serum från olika fåglar. Resultatet visade att metoden var jämförbar med andra metoder, samt att den specifikt kunde detektera H5 och H7 subtyper av influensavirus. Det finns en oro att vilda fåglar ska sprida dessa allvarliga subtyper av influensavirus till olika delar i världen, något som teoretiskt kan ske i och med att många fågelarter förflyttar sig långa sträckor och då kommer i kontakt med andra fåglar som kan bära smittan vidare till andra delar av världen. Ett sådant ställe finns i Alaska, där flera olika flygvägar för flyttfåglar korsar varandra. I delarbete I ville vi undersöka om vi kunde se tecken på att influensavirus från olika världsdelar blandas till nya varianter. Prover togs från fåglar i Alaska, och det genetiska materialet från de virus som hittades analyserades. Resultaten visade inga spår av att influensavirus från olika kontinenter nyligen blandats och gett upphov till nya genetiska varianter.

Under 1900-talet har influensa orsakat flera pandemier. Den mest kända är den så kallade spanska sjukan, som 1918 dödade upp till 50 miljoner människor. År 2009 inträffade den första pandemin detta århundrade. Den drabbade främst yngre människor, och inte de äldre vilka vanligtvis är en riskgrupp för influensa. I den här avhandlingen handlar två arbeten om hur vi identifierat skillnader i antikroppsnivåer mellan olika åldersgrupper. Viruset som orsakade spanska sjukan är likt det virus som gav upphov till pandemin 2009 och man tror att människor som är födda runt 1918 kan ha varit skyddade mot viruset 2009. Eftersom de redan hade antikroppar mot det virusets hemagglutinin, kunde infektion förhindras. I delarbete III visar vi att personer äldre än 90 år i större utsträckning hade antikroppar mot spanska sjukan-viruset än de yngre, något som kan ha skyddat dem mot pandemiviruset 2009. Vi visar även att människor födda runt 1934 hade antikroppar mot ett influensavirus som cirkulerade då, vilket även det är ganska likt viruset som orsakade pandemin 2009. I delarbete IV tittar vi på antikroppar mot det andra ytproteinet, neuraminidas. Vi ser även där att äldre hade antikroppar mot neuraminidas i större utsträckning än de yngre. Det är alltså möjligt att dessa befintliga antikroppar bidrog till att de äldre inte drabbades så hårt år 2009.

Den här avhandlingen har bidragit till att öka kunskapen kring eventuella genetiska utbyten mellan fåglar i Alaska, samt huruvida det finns säkra metoder för att detektera antikroppar mot H5 och H7 influensavirus i serum från fåglar, utan användning av levande influensavirus. Vidare har den ökat förståelsen för hur tidigare influensainfektioner i människa kan påverka senare infektioner, i och med att befintliga antikroppar mot både hemagglutinin och neuraminidas kan skydda mot infektion och svår sjukdom.

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