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Tamiflu in the Water

Resistance Dynamics of Influenza A Virus in Mallards Exposed to Oseltamivir

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Abstract

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The natural reservoir of influenza A virus (IAV) is wild waterfowl, and all human IAVs have their genetic origins from avian viruses. Neuraminidase inhibitors (NAIs) are currently the best drugs for treatment of human influenza; therefore, the orally available NAI oseltamivir (Tamiflu®) has been stockpiled worldwide as part of pandemic preparedness planning. Re-sistance to NAIs is related to worse clinical outcomes and if a new pandemic influenza virus would be oseltamivir-resistant its public health impact would be substantially worsened.

The active metabolite oseltamivir carboxylate (OC) is not removed by sewage treatment and ends up in river water, where OC-concentrations up to 0.86µg/L have been detected.

We hypothesize that occasional OC exposure of wild waterfowl carrying IAVs may result in circulation of resistant variants that may potentially evolve to become human-pathogenic.

We tested the hypothesis in an in vivo Mallard (*Anas platyrhynchos*) model in which birds were infected with avian IAVs and exposed to OC. Excreted viruses were analyzed regarding genotypic and phenotypic resistance by neuraminidase (NA) sequencing and a functional NA inhibition assay.

Two viruses with NAs of the phylogenetic N2-group, H6N2 and H7N9, acquired the NA substitutions R292K and I222T when host ducks were exposed to 12µg/L and 2.5µg/L of OC, respectively. Drug susceptibilities were at previously described levels for the substitutions. To test persistence of resistance, an OC resistant avian H1N1/H274Y virus (with a group N1 NA-protein) from a previous study, and three resistant H6N2/R292K variants were allowed to replicate in Mallards without drug pressure. Resistance was entirely maintained in the H1N1/H274Y virus, but the H6N2/R292K variants were outcompeted by wild type virus, indicating retained fitness of the resistant H1N1 but not the H6N2 variants.

We conclude that OC in the environment may generate resistant IAVs in wild birds. Resistant avian IAVs may become a problem to humans, should the resistance trait become part of a new human pathogenic virus. It implies a need for prudent use of available NAIs, optimized sewage treatment and resistance surveillance of avian IAVs of wild birds.

Keywords: Influenza A virus, avian influenza, oseltamivir, neuraminidase inhibitors, resistance, environmental, Mallard, waterfowl

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To Gölin, Sigrid and Märta

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Gillman A, Muradrasoli S, Söderström H, Nordh J, Bröjer C, Lindberg RH, Latorre-Margalef N, Waldenström J, Olsen B, Järhult JD. (2013) Resistance mutation R292K is induced in influenza A(H6N2) virus by exposure of infected mallards to low levels of oseltamivir. *PLoS One* 8:e71230.

II. Gillman A, Nykvist M, Muradrasoli S, Söderström H, Wille M, Daggfeldt A, Bröjer C, Waldenström J, Olsen B, Järhult JD. (2015) Influenza A(H7N9) Virus Acquires Resistance-Related Neuraminidase I222T Substitution When Infected Mallards Are Exposed to Low Levels of Oseltamivir in Water. *Antimicrobial agents and chemotherapy* 59:5196-5202.

III. Gillman A, Muradrasoli S, Söderström H, Holmberg F, Latorre-Margalef N, Tolf C, Waldenström J, Gunnarsson G, Olsen B, Järhult JD. (2015) Oseltamivir-Resistant Influenza A (H1N1) Virus Strain with an H274Y Mutation in Neuraminidase Persists without Drug Pressure in Infected Mallards. *Applied and environmental microbiology* 81:2378-2383.

IV. Gillman A, Muradrasoli S, Mårdnäs A, Söderström H, Fedorova G, Löwenthal M, Wille M, Daggfeldt A, Järhult JD. (2015) Oseltamivir Resistance in Influenza A(H6N2) Caused by an R292K Substitution in Neuraminidase Is Not Maintained in Mallards without Drug Pressure. *PLoS One*. 10(9):e0139415.

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Contents

Abbreviations

AVWG	Antiviral susceptibility working group
CT	Cycle threshold
ddNTP	Dideoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ECE	Embryonated chicken egg
EID_{50}	50% egg infectious dose
GISRS	Global influenza surveillance and response system
HA	Hemagglutinin
HPAI	Highly pathogenic avian influenza
IAV	Influenza A virus
IC_{50}	50% inhibitory concentration
IHC	Immunohistochemistry
IRAT	Influenza risk assessment tool
LC-MS	Liquid chromatography - Mass spectrometry
LPAI	Low pathogenic avian influenza
MUNANA	4-methylumbelliferyl-α-D-N-neuraminate
NA	Neuraminidase
NAI	Neuraminidase inhibitor
NCBI	National center for biotechnology information
NGS	Next generation sequencing
OC	Oseltamivir carboxylate
OP	Oseltamivir phosphate
PCR	Polymerase chain reaction
pdm09	Pandemic strain of 2009
RNA	Ribonucleic acid
RRT-PCR	Real-time reverse transcriptase PCR
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SEM	Standard error of the mean
SPE	Solid phase extraction
SPF	Specific pathogen free
STP	Sewage treatment plant

TCAD	Triple combination antiviral drug
vRNA	viral RNA
vRNP	viral ribonucleoprotein
WHO	World health organization
ZA	Zanamivir

Introduction

Influenza Viruses

Influenza A, B and C viruses belong to the Ortomyxoviridae family and are enveloped, negative sense single stranded RNA viruses with a segmented genome (Fields, Knipe, and Howley 2007). Influenza B viruses mainly infect humans and cause seasonal influenza epidemics, but can also be infectious to seals (Osterhaus et al. 2000). Influenza C viruses infect swine and humans, typically with mild airway disease, but do not cause epidemics (Fields, Knipe, and Howley 2007). Influenza A viruses (IAVs) can infect many animal species, including humans, but the major natural reservoir hosts are wild waterfowl (Webster et al. 1992, Olsen et al. 2006). IAVs are the most pathogenic and variable influenza viruses and undergo genetic changes both by point mutations (antigenic drift) and by reassortment of gene segments (antigenic shift). IAVs are the only influenza viruses that cause human pandemics (Webster et al. 1992).

Human disease by Influenza A Virus

In humans, IAVs cause airway disease with highly varying severity. Seasonal influenza viruses (currently A(H1N1)pdm09, A(H3N2) and influenza B) can cause yearly epidemics due to continuous antigenic drift, which generates new viral variants that partly escape the herd immunity of the population (Simonsen et al. 1998, Webster et al. 1992, Folkhälsomyndigheten 2015b). Seasonal IAV infections typically generate tracheobronchitis and fever, normally with an uncomplicated clinical course and negligible mortality in young and healthy individuals (Fields, Knipe, and Howley 2007). In medical risk groups, in elderly and in pregnant women seasonal influenza may cause severe disease with secondary complications and an increased mortality (Simonsen et al. 1998, Folkhälsomyndigheten 2015b). Although the strategy has been questioned, the increased over-all mortality in elderly during the winter season is the reason why yearly influenza vaccination is recommended for people over 65 years of age, in addition to medical risk groups (Simonsen et al. 2009, Folkhälsomyndigheten 2015a).

Pandemic influenza occurs when an IAV with new antigenic properties is introduced to humans and there is very little or no immunity in the population. Consequently, the virus can transmit rapidly and extensively worldwide and

cause severe disease. The clinical picture is typically more severe than during seasonal influenza epidemics with viral pneumonitis, acute respiratory distress syndrome and secondary bacterial pneumonia, and often the mortality is increased also in young age groups (Webster et al. 1992, Simonsen et al. 1998, Monto and Webster 2013). Case fatality rates of influenza pandemics vary considerably, from estimated 2.5% of the H1N1 Spanish flu 1918-1919, to approximately 0.1% of later pandemics (Taubenberger and Morens 2006, Cheng et al. 2012).

Avian influenza infections may occur in humans if avian IAVs are directly transmitted from birds to humans and generate disease. Serological studies confirm that transmission of low pathogenic avian influenza (LPAI) viruses to humans is common, but that they normally generates no or very limited symptoms (Fouchier and Guan 2013, To et al. 2013). There are however avian poultry adapted viruses that can be highly pathogenic to humans; these include subtypes that may be either asymptomatic or highly pathogenic to poultry (HPAI viruses). Avian influenza viruses that are highly pathogenic to humans typically cause severe progressive pulmonary disease with respiratory failure and generalized disease with multi organ failure, leading to a high mortality. Currently, HPAI H5N1 and H7N9 viruses give rise to occasional human cases with fatality rates of laboratory confirmed cases of approximately 40 to 60% (To et al. 2013, Abdel-Ghafar et al. 2008, Tanner, Toth, and Gundlapalli 2015).

Structure and Genome of Influenza A Virus

Structurally the IAV particle is composed of a lipid bilayer envelope, an underlying matrix and a ribonucleoprotein core. It is pleomorphic and may be spherical, then measuring approximately 100nM in diameter, or filamentous, then >1 µm long (Figure 1) (Nayak et al. 2013). As there is no latent form of infection with IAVs, the survival and evolution of viruses is maintained by continuous transmission to new susceptible hosts (Nayak et al. 2013, Webster et al. 1992).

The IAV genome has eight separate segments that code for a number of proteins; 10 of them have been described since the 1970s. In addition truncated versions or proteins coded by alternative splicing or reading frames, whereof several are related to the pathogenesis of the virus, continue to be discovered (Vasin et al. 2014).

Hemagglutinin (HA) and neuraminidase (NA), coded by segment 4 and 6 respectively, are antigenic surface glycoproteins that span the envelope and that primarily have receptor binding and release functions (Krug and Fodor 2013). The M2 protein is a transmembrane ion channel involved in uncoating of endocytosed virions, in neutralizing the golgi pH and in budding of new viruses. The M1 matrix protein is anchored underneath the envelope

and interacts both with the cytoplasmic parts of HA, NA and M2 and with the viral RNA (vRNA) and nucleoprotein, and is involved in many stages of the replication process (Chunlong, Pinto, and Lamb 2013). Both M1 and M2 are coded by segment 7. The basic polymerase protein 1 (BP1), the basic polymerase protein 2 (BP2) and the acidic polymerase protein (PA), encoded by segments 2, 1 and 3 respectively, compose the heterotrimeric 3P-complex. The 3P-complex is assembled with the vRNA wrapped nucleoprotein (NP), coded by segment 5, to form the higher order ribonucleoprotein (vRNP) complex that mediates transcription and replication (Nayak et al. 2013, Mehle and McCullers 2013). PB1-N40 is a shortened version of PB1. PB1-F2 is produced from the +1 reading frame and has ion channel and protein binding functions with multiple modifying effects related to virulence and host immune response (Mehle and McCullers 2013). The nonstructural protein NS1 is also multifunctional and, for example, inhibits host interferon production that otherwise prevents viral replication (Krug and Garcia-Sastre 2013). The nuclear export protein NEP/NS2 mediates vRNP export from the nucleus to the cytoplasm (Vasin et al. 2014). Both the NS1 and the NEP/NS2 proteins are coded by segment 8 (Krug and Garcia-Sastre 2013).
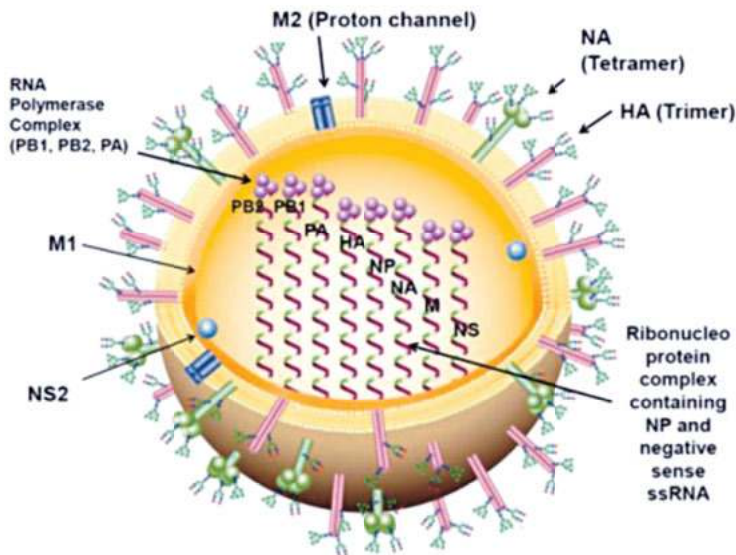
Figure 1. Schematic structure of influenza A virus particle. The pleomorphic particle, ~100nM in diameter in its spherical form, has an envelope derived from the host cell membrane, with the underlying matrix (M1) protein and an 8-segmented genome. The membrane spanning proteins hemagglutinin (HA) and neuraminidase (NA), with glycans synthesized by the host cell, have sialic acid receptor binding and releasing functions. The M2 protein is a proton channel. The ribonucleoprotein complex comprises viral RNA associated with the nucleoprotein (NP) and the three RNA polymerase proteins (PA, PB1 and PB2). (From (Suzuki 2013), reproduced with permission from Glycoforum:http://www.glycoforum.gr.jp.)

Classification and Grouping of Influenza A Viruses

IAVs are classified in subtypes by the antigenic surface glycoproteins HA and NA and are termed by the H and N numbers. Currently 18 HA and 11 NA proteins in multiple combinations have been described. The H17N10 and H18N11 subtypes have only been found in bats (Wu et al. 2014), while all other HAs and NAs can be combined in avian IAV subtypes (Olsen et al. 2006, Webster et al. 1992). In humans only the H1N1, H2N2 and H3N2 subtypes have caused widespread disease (Cox and Subbarao 2000, Brockwell-Staats, Webster, and Webby 2009, Webster et al. 1992).

Based on primary sequences, both HA and NA are phylogenetically grouped, and HAs are further separated in clades and subclades (WHO 2015b, Fields, Knipe, and Howley 2007). Group H1 include clade H1 (subtypes H1, H2, H5, H6), clade H9 (subtypes H8, H9, H12), and clade H11 (subtypes H11, H13 and H16). Group H2 include clade H3 (subtypes H3, H4, H14), and clade 7 (subtypes H7, H10 and H15).

The NAs are phylogenetically divided in group N1, including subtypes N1, N4, N5, N8, and group N2, including subtypes N2, N3, N6, N7 and N9 (Fields, Knipe, and Howley 2007, Russel, Gamblin, and Skehel 2013). The bat H17 and H18, cluster to group H1, whereas the N10 and N11 are suggested to a separate IAV-like NA group 3 (Figure 2) (Wu et al. 2014).
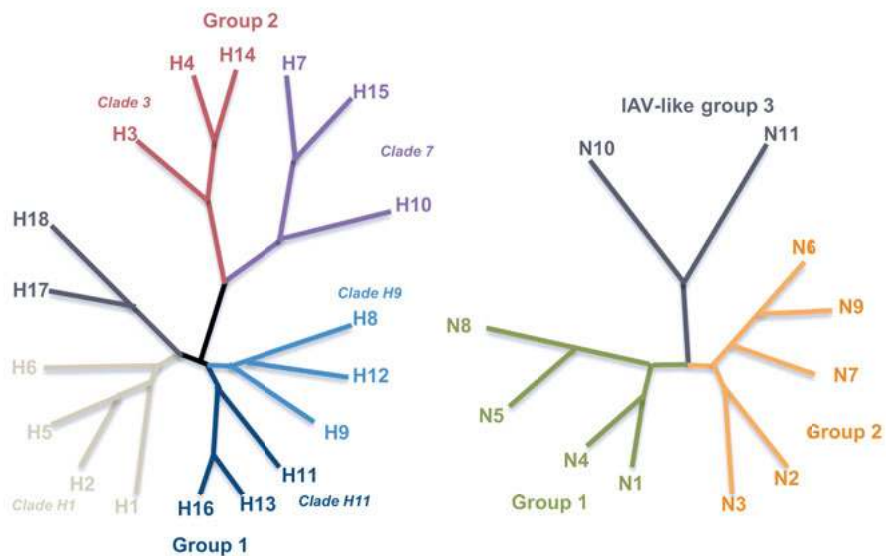
Figure 2. Phylogenetic grouping of hemagglutinin (H) and neuraminidase (N), including suggested bat IAV grouping.

Structure and Functions of Hemagglutinin

The binding and endocytosis of IAVs to target cells are receptor mediated and species specific. The IAV receptor structures at the surface of epithelial cells are glycoproteins or glycolipids with an oligosaccharide side chain that is terminated with a sialic acid (N-acetyl neuraminic acid / Neu5Ac) (Wiley and Skehel 1987). HA is the viral structure that binds to the sialic acid cell surface receptor.

HA is synthesized as a single 550 amino acid polypeptide chain, HA0, which is cleaved by host cellular proteases to two chains, HA1 and HA2. HA1 and HA2 are covalently linked to form a two-chain monomer. Three HA monomers associate to form a trimer, which is glycosylated by five to seven oligosaccharides and attached to the surface membrane by its C-terminal anchor sequences (Russel, Gamblin, and Skehel 2013, Wiley and Skehel 1987). On a spherical virus approximately 500 HA glycoproteins are distributed around the membrane; they project 13nM from the surface and constitute ~80% of the transmembrane proteins (Figure 1) (Nayak et al. 2013, Russel, Gamblin, and Skehel 2013).

When a new host is infected, the HA protein is: (i) the antigenic structure to which neutralizing antibodies are directed, (ii) the viral ligand that binds to the target cell receptor, and (iii) the structure that generates membrane fusion with endosomes.

Antigenicity

The antigenic sites that give rise to neutralizing antibodies are located at the globular parts of the HA structure. Viral selection under antibody pressure leads to reduced affinity of existing antibodies, i.e. to antigenic drift, either by amino acid substitutions or by incorporation of new oligosaccharides at the antigenic sites. (Knossow and Skehel 2006). Antibodies produced to-wards IAVs are subtype and strain specific and have varying affinity de-pending on the degree of antigenic drift, which is relevant for example in human seasonal vaccine production (WHO 2014).

HA-antibodies towards a specific strain efficiently prevent reinfection with the same strain. On the other hand, if an IAV with entirely new antigen-ic properties enters the human population, pre-existing antibodies cannot recognize the virus, and if the virus is human-to-human transmissible, the spread may become pandemic (Taubenberger and Morens 2008). In compar-ison, avian IAV infections in wild Mallards generate both homo-subtypic and hetero-subtypic immunity. Thereby infection with one viral subtype generates immunity not only to the infective strains but also in part towards other subtypes within the HA clade (Latorre-Margalef et al. 2013).

Receptor Binding

HA binding to sialic acid receptors at the cell surface of host cells is the essential first step to initialize viral infection (Figure 3) (Wiley and Skehel 1987). Sialic acids are also present in mucins secreted by endothelial cells, and HA-binding to these prevent viral spread, as viruses become stuck in the mucus.

The receptor binding structure of HA is in a pocket at the membrane distal tip of each subunit of the trimer. The pocket consists of a base of conserved amino acids (Y98, W153, H183, Y195) (H3-numbering, used hereafter), surrounded by the secondary structures of the 130-loop, the 190-α-helix and the 220-loop (Russel, Gamblin, and Skehel 2013). To obtain a high binding avidity and specificity, receptor binding of approximately four HA molecules is required (Russel, Gamblin, and Skehel 2013).

Binding of HA to its target receptor is species specific and represents one of the barriers for species crossing of IAVs. HAs of avian IAVs prefer binding to sialic acids in α2,3 linkage to galactose (Neu5Acα2,3Gal), while HAs of human IAVs prefer binding to sialic acids in α2,6 linkage. Swine IAVs can bind to sialic acids in both α2,3 and α2,6 linkages (Russel, Gamblin, and Skehel 2013).

The receptor specificity of HAs corresponds to the presence of α2,3 linked sialic acids in birds and to α2,6 linked sialic acids in human upper respiratory airways (in lower human respiratory airways both α2,3 and α2,6 linkages are present). Swine, accordingly, express both types of sialic acid-galactose linkages and are therefore suggested to be mixing vessels for avian and human IAVs (Brockwell-Staats, Webster, and Webby 2009).

Five subtype specific amino acid substitutions of the HA binding site (S138A, E190D, G225D in H1 and Q226L, G228S in H2 and H3) can change the binding specificity from α2,3 to α2,6 linkage. These substitutions are suggested to have been important in the evolution of the 1918 (H1), 1957 (H2) and 1968 (H3) pandemic strains of avian origin (Russel, Gamblin, and Skehel 2013, Matrosovich et al. 2000, Webster et al. 1992, Zhang et al. 2013).

In addition to preferring α2,3 linkage to galactose, avian IAVs adapted to different bird species differ in the fine receptor affinity, such that waterfowl viruses and poultry viruses have slightly different HA binding properties. These differences mainly depend on the interaction of saccharides of the inner part of the receptor carbohydrate chain with amino acid residues at the edges of the HA binding site (Gambaryan et al. 2005, Russel, Gamblin, and Skehel 2013).

Endosomal Membrane Fusion

Following receptor binding and endocytosis of bound virus, HA mediates fusion of endosomal and viral membranes, which releases the vRNP to the

cytoplasm and allows further replication (Figure 3). The membrane fusion process is initiated at pH 5.0 to 6.0, achieved by endosomal membrane H+ pumps, and involves extensive structural rearrangements of HA with disso- ciation of the trimer conformation and bridge formation between endosomal and viral membranes (Wiley and Skehel 1987, Russel, Gamblin, and Skehel 2013). Changes in the pH optimum for the HA conformational changes is suggested to be a host restricting factor, as the pH of endosomes in target membranes differ between species, and as the pH optimum of HA changes during avian to swine host adaptation (Baumann et al. 2015).
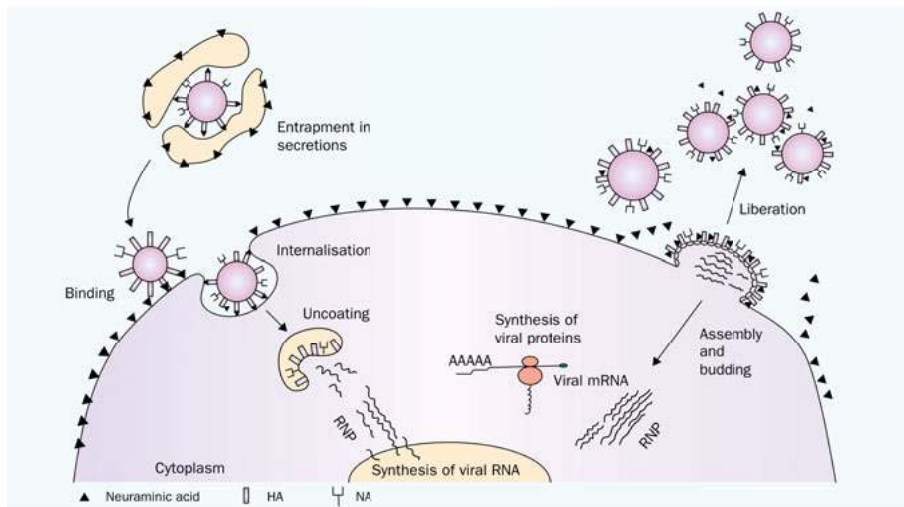
Figure 3. Replication cycle of influenza A virus. The IAV is attached to the surface of target cells by hemagglutinin (HA) binding to receptor structures with terminal sialic acid residues. HA attachment is followed by endocytosis and fusion of viral and endosomal membranes, which releases the viral ribonucleoprotein (RNP) com- plex and allows translation and replication of viral RNA. New proteins are synthe- sized, and together with viral RNA, assembled and budded as new viral particles at the cell membrane. Finally new virions are released from the cell and from each other by neuraminidase (NA) removal of sialic acid structures. (Reprinted from The Lancet: (Gubareva, Kaiser, and Hayden 2000), reproduced with permission from Elsevier.)

Structure and Functions of Neuraminidase

The IAV membrane glycoprotein NA is synthesized as a 470 amino acid monomer. These are assembled to tetramers that are glycosylated at seven sites and anchored to the membrane by an uncharged signaling region close to the N-terminus of each subunit (Russel, Gamblin, and Skehel 2013). From a spherical virus particle approximately 100 NA structures project from the surface. They are clustered at sites that are involved in membrane fusion and

the release of virions and compose ~17% of the membrane spanning proteins (Nayak et al. 2013).

NA is an enzyme that evolves in parallel with HA and primarily effects the opposite action to the HA receptor binding by enzymatic hydrolysis of the sialic acid receptors. The functions of NA are: (i) release of viral particles from entrapment in mucins, (ii) release of newly formed viral particles from the cell surface, and (iii) prevention of viral particles clotting to each other (Figure 3). In addition, NA is antigenic and gives rise to an antibody response by the host; but although the produced antibodies block the NA activity, they do not - in contrast to HA antibodies - neutralize viral infectivity (Nayak et al. 2013). The balance of binding to and release from sialic acid structures is essential for the viral fitness, and changes in either the NA or HA function can be compensated by adaptive changes in the other protein (Wagner 2002).

Neuraminidase Enzyme Activity

The active substrate binding enzymatic site of NA is located at the tip of each NA subunit and is composed of 8 amino acids (R118, D151, R152, R224, E276, R292, R371, and Y406), surrounded by eleven supportive framework residues (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294, and E425) (N2 numbering, used hereafter) (Colman, Hoyne, and Lawrence 1993).

The catalytic mechanism of NA begins by the binding of sialic acid, in which the three arginine residues 118, 292 and 371 are essential. The substrate binding is followed by numerous catalytic steps that result in the removal of sialic acid from cellular glycoconjugates, from HA and NA, and from mucins. The enzymatic destruction of the sialic acid receptors result in the release of newly made viral particles from the infected cell, but also in prevention of clotting together and from entrapment in mucins (Russel, Gamblin, and Skehel 2013).

NA can catalyze sialic acid bound both by the α2,3 and the α2,6 linkage to an oligosaccharide, but often with a preference for the α2,3 linkage, also in human IAVs (Russel, Gamblin, and Skehel 2013). This feature is probably a result of the avian origin of NA, but also of the α2,3 linkage being the most common sialic acid-galactose linkage of mucins (Wagner 2002).

NA Group Differences

The two phylogenetic groups of NA (group N1 and group N2) (Figure 2) have different structural features at the active binding site. The most prominent difference is the conformation of the 150-loop adjacent to the active site; in group N1 but not in group N2 NAs the 150-loop structure gives rise to a large hydrophobic cavity accessible from the active site (Figure 8) (Russell et al. 2006). Though the reason for the differences between the phylogenetic groups is unknown, the structures are highly conserved within the

NA groups and have implications for drug binding and for resistance mutations, as discussed below (Russell et al. 2006).

Ecology and Evolution of Avian Influenza A Viruses

Influenza A Viruses in the Natural Reservoir of Wild Waterfowl

Many mammal and bird species can be infected by IAVs, but wild waterfowl are the natural reservoir hosts; primarily Charadriiformes (in particular gulls, terns and waders) and Anseriformes (in particular ducks, geese and swans) (Figure 4) (Webster et al. 1992, Olsen et al. 2006). Viral prevalence in reservoir birds is highest in dabbling ducks, though subtype diversity is more pronounced in shorebirds (Munster et al. 2007, Krauss et al. 2004). The Mallard (*Anas platyrhynchos*) is considered to be the most common IAV host species and is the one most studied (Fouchier and Guan 2013).

Genetically LPAI viruses are divided in two lineages, Eurasian and American, which is probably the result long time geographical and ecological separation of hosts. The lineages principally follow the migratory routes of wild birds (Olsen et al. 2006) (Figure 5). However, neither bird migration nor LPAI genetics are entirely separated between hemispheres, and occasionally intercontinental transmission of virus occurs, which primarily is detected as reassortant variants in shorebirds and gulls (Dugan et al. 2008, Wallensten et al. 2005, Wille et al. 2011). The Bering Strait, but also Iceland, are suggested to be important geographical locations for mixing of Eurasian and North American birds and of IAV genes (Olsen et al. 2006, Hall et al. 2014). In wild bird avian IAVs in North America, 6% of the genomes were found to contain sequences of Eurasian origin, suggesting substantial intercontinental gene flow (Dugan et al. 2008). When new genes that can compete for susceptible hosts are introduced on a continent the evolutionary dynamics are changed, which may lead to complete replacement of endemic lineages, as observed by H6 replacement in aquatic host birds in North America (Bahl et al. 2009).
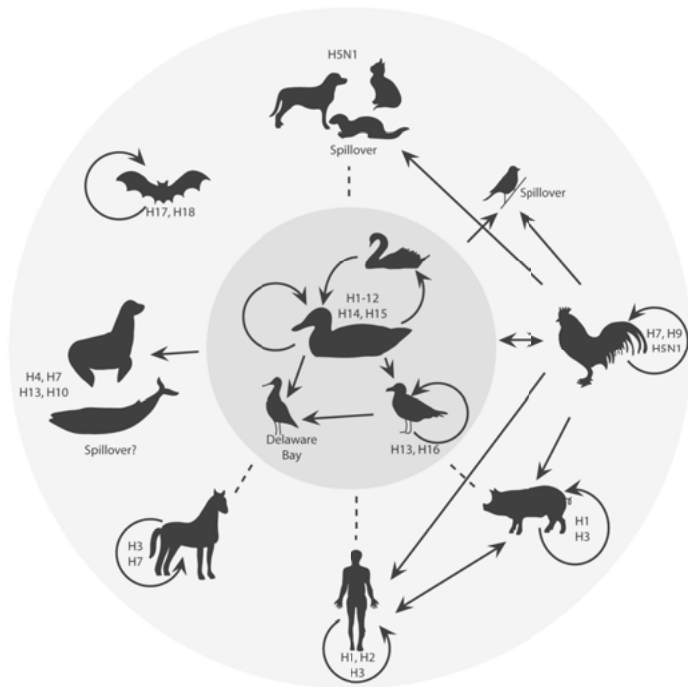
Figure 4. Host range and interspecies transmission of IAVs. The natural wild water-fowl reservoir is indicated in dark grey. Established subtypes with persisting transmission within the host are indicated by circular arrows. Occasional and infrequent transmissions to other hosts are indicated by solid arrows and dashed lines, respectively. (From (Wille 2015), reprinted with permission from Linnaeus University Press.)

Viral Subtypes and Prevalence

Most subtype combinations can be hosted by both Anseriformes and Charadriiformes. A few subtypes are however more common in particular species suggesting different main reservoirs, as observed for H13 and H16 in gulls (Munster et al. 2007, Krauss et al. 2004, Fouchier et al. 2005). As demonstrated by long term IAV surveillance mainly in North America and in Eurasia, the prevalence of different subtypes in various host birds differs over time and in part geographically.

The IAV prevalence in wild ducks typically peaks in the autumn, while in shorebirds (in the Delaware Bay) the prevalence is highest in the springtime; in both cases the predominant subtypes change between years (Wallensten et al. 2007, Hinshaw et al. 1985, Munster et al. 2007, Krauss et al. 2004, Latorre-Margalef et al. 2014). Common subtype combinations both in North America and in Europe in both Anseriformes and Charadriiformes are H3N8, H4N6, H11N9 and H6N2 (Munster et al. 2007, Krauss et al. 2004, Latorre-Margalef et al. 2014).

Globally, the Delaware Bay on the North American east coast appears to be a special case regarding IAV prevalence in shorebirds, a phenomenon not only related to being one of the most studied areas. There, the prevalence of IAVs in shorebirds (waders), predominantly in Ruddy turnstones, is much higher than elsewhere (>10% at spring migration compared to ~0.5% globally) (Olsen et al. 2006, Munster et al. 2007, Gaidet et al. 2012, Krauss et al. 2004, Krauss et al. 2010). The high IAV prevalence coincides with the enormous congregation of waders and gulls foraging horseshoe crab eggs in May. If the exceptionally high prevalence represents spillover of virus from the gull and duck reservoir or is related to a unique Ruddy turnstone - horseshoe crab association remains unclear (Hanson et al. 2008, Krauss et al. 2010). The north migrating shorebirds of the Delaware Bay are suggested to play a role in transmission of IAVs to northern breeding grounds that are shared with ducks (Munster et al. 2007, Krauss et al. 2004).

Perpetuation of IAVs in Wild Waterfowl

In wild waterfowl, LPAI viruses cause an intestinal tract infection, and although large amounts of virus are shed in feces the infection is relatively asymptomatic (Jourdain et al. 2010, Latorre-Margalef et al. 2009, Webster et al. 1992).

The temporal and spatial dynamics, as well as the evolution of IAVs in the natural hosts, are closely related to the ecology, immunology and migration of the birds (Olsen et al. 2006). As dabbling ducks switch breeding grounds between years, there are opportunities for viral transmission to different subpopulations over wide geographical areas (Wallensten et al. 2006, Fouchier and Guan 2013, Olsen et al. 2006). The autumn peak in IAV prevalence (up to 60% compared to 0.4 – 2% at wintering grounds) of dabbling ducks coincides with the autumn migration from breeding areas to more southern winter habitats (Figure 5B), when large amounts of birds from different breeding locations congregate (Latorre-Margalef et al. 2014, Olsen et al. 2006). Additionally, at that time of the year there are high numbers of immunologically naïve juveniles, accounting for a large proportion of the infected birds (Webster et al. 1992).

Perpetuation of LPAI viruses in wild waterfowl all year round is suggested to be a combination of (i) continuous transmission to juvenile and nonimmune ducks at areas of breeding and of congregation for migration; (ii) spread of virus with migrating birds; and (iii) low prevalence circulation in resident ducks during the winter season in temperate locations (Hill et al. 2012, Webster et al. 1992, Lewis et al. 2015). In addition, IAVs are known to stay infectious for a long time in lake water (Webster et al. 1992), and freezing of viruses in lakes at breeding areas with reinfection of birds the following season is suggested to be another mechanism for viral perpetuation (Fouchier and Guan 2013).
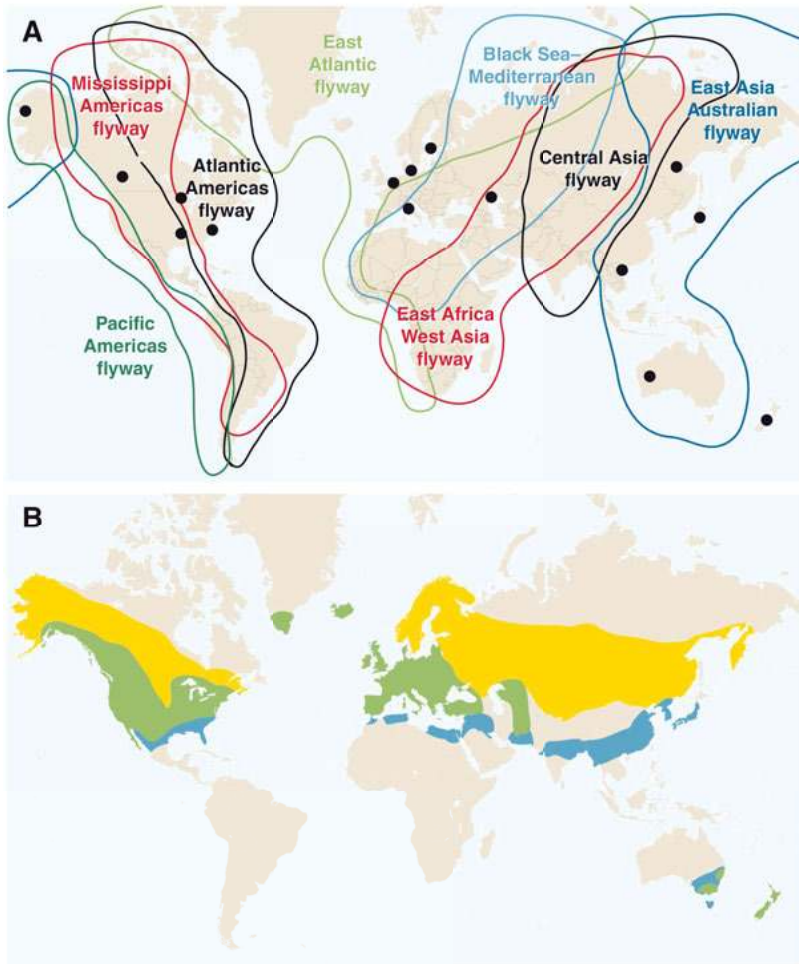
Figure 5. **A.** General migratory flyways of wild bird populations. Black dots indicate influenza virus surveillance sites. **B.** Migration patterns of Mallards. Yellow: summer breeding areas. Green: presence all year round. Blue: Wintering habitats. (From (Olsen et al. 2006). Reprinted with permission from AAAS.)

Genetic Diversity of Wild Bird Influenza A Viruses

The genetic variability of wild waterfowl viruses is much greater than that of IAVs in other hosts, in that most combinations of HA and NA are seen and there is a lack of persisting sub-lineages (Webster et al. 1992). The diversity between both the HA and NA subtypes is high, seen as a low (38-44%) inter-subtype sequence identity, as compared to a high (>89%) intra-subtype identity (Dugan et al. 2008). The NS gene is also highly variable in waterfowl viruses, while the internal gene segments are much more uniform (Dugan et al. 2008, Chen and Holmes 2010).

The genetic diversity and evolution of avian IAVs is a dynamic process with continuous emergence of new variants. These occur by point mutations (genetic drift), reassortment (genetic shift), and rarely by recombination between gene segments (Forrest and Webster 2010).

Point mutations, with or without changes of amino acids, are common in all gene segments as the IAV polymerase complex lacks exonuclease proof-reading activity and errors that occur during transcription remain in the genome (Russell et al. 2012, Chen and Holmes 2006).

Reassortment is a particularly important mechanism for genetic variability in IAVs and occurs when at least two viruses infect the same host cell at the same time and exchange gene segments; thereby new viruses with new gene constellations arise. In IAVs of wild waterfowl reassortment events continuously occur at a high frequency as a result of very frequent co-infections (Dugan et al. 2008, Lewis et al. 2015).

Evolutionary Driving Forces on Wild Bird IAVs

In wild bird populations, there is concomitant circulation of numerous IAV subtypes with similar viral fitness (Lebarbenchon et al. 2012). Which subtypes that circulate at a given time point depend on several not entirely defined mechanisms. Multiple patterns of diversity suggest different evolutionary driving forces.

In general there is no overall species specific pattern for the subtype diversity (Lewis et al. 2015, Dugan et al. 2008, Latorre-Margalef et al. 2014, Wille et al. 2013, Chen and Holmes 2009, Munster et al. 2007), though the selection of H13 and H16 subtypes in gulls appears to be an exception (Fouchier et al. 2005, Munster et al. 2007).

With the need for continuous replication and transmission, escaping host immunity is an evident main driving force for the variability of the immune related genes of HA and NA (and perhaps of NS1) (Worobey, Han, and Rambaut 2014, Chen and Holmes 2006). The immune response by waterfowl is subtype dependent and gives rise to neutralizing antibodies towards the infecting subtype (homo-subtypic immunity), but also generates partial immunity to other subtypes in the same HA clade (hetero-subtypic immunity), which may contribute to the pronounced diversity between avian subtypes (Latorre-Margalef et al. 2013). The variation of subtypes over the season and the periodicity of predominant subtypes every other year, confirm that herd immunity is a main force for the subtype variability (Latorre-Margalef et al. 2014, Krauss et al. 2004, Webster et al. 1992).

Based on the demonstration of common ancestors of internal gene segments to have existed less than 200 years ago, other driving forces than antigenic novelty are suggested to generate occasional selective sweeps that replace all preceding genes (Chen and Holmes 2010, Lewis et al. 2015, Worobey, Han, and Rambaut 2014).

Among the different explanatory models of the evolutionary driving forces of wild bird avian IAVs, one hypothesis on the high level of variability is that viruses exist as a pool of functionally equal gene segments that form transient constellations, without a selective pressure to be maintained as linked genomes (Dugan et al. 2008). The model can however not include viruses adapting to new hosts, which requires specific point mutations in distinct eight-segment genome configurations (Dugan et al. 2008). The circulating subtypes at a specific time point are described as fitness peaks in a host immunity landscape, influenced by all components of the host ecology and migration (Dugan et al. 2008, Lewis et al. 2015).

Interspecies Transmission of Avian Influenza A Viruses

On rare occasions genetic changes of IAVs allow interspecies transmission, and yet more rare an IAV becomes transmissible and established in the new host species (Figure 4) (Webster et al. 1992). The adaptive process required for an avian waterfowl IAV to become infective and pathogenic to humans involves a series of evolutionary events. An essential step is the interspecies transmission to a host that possesses both α2,3 and α2,6 linked sialic acid receptors (Forrest and Webster 2010). Several poultry species have dual sialic acid receptors and especially quail and partridges appears to be permissive to most waterfowl IAV subtypes and have therefore been suggested to be "mixing vessels" for viruses of aquatic and terrestrial birds (Fouchier and Guan 2013). Swine are considered to be suitable intermediate hosts between avian and mammalian viruses; they possess both receptor types and have a lower body temperature of 39°C, which also is relevant for bird to mammal cross-species adaptation (Forrest and Webster 2010).

Following introduction of a waterfowl virus to a host with dual sialic acid receptors, subsequent reassortment events and amino acid substitutions that modulate the viral life-cycle are required for crossing the species barrier to humans. Essential alterations of avian virus properties include the ability to replicate at a lower temperature (37°C of human airways instead of 42°C of the intestine of waterfowl); change of tropism from intestinal to respiratory epithelium; change of the HA receptor binding-specificity and pH optimum for membrane fusion; and adaptation of the host dependent polymerases (Forrest and Webster 2010, Baumann et al. 2015). The human host produces the Mx protein, which is highly antiviral to avian but not to human IAVs, and thus resistance to the Mx protein appears to be a contributing factor for crossing of the avian-human species barrier (Riegger et al. 2015).

In order for an avian IAV not only to occasionally infect but to spread between humans, the route of transmission has to change from fecal-oral to respiratory droplet/air-borne. To date, no avian IAVs that are pathogenic to humans can be efficiently transmitted between humans (Webster and Govorkova 2014).

Low Pathogenic Avian Influenza Viruses in Domestic Birds

Domestic ducks, like their wild Mallard duck ancestors (Cherry and Morris 2008), carry IAVs as an asymptomatic intestinal infection and serve as LPAI reservoirs aside the wild waterfowl reservoir (Huang et al. 2010). In Southeast Asia, and particularly in Southeast China where 60% of the world population of domestic duck is farmed, most LPAI subtypes occur (Huang et al. 2010, Weimin 2010, Shortridge 1992, Duan et al. 2011), and transmission of viruses between wild migratory waterfowl and domestic ducks is common (Duan et al. 2011, Huang et al. 2010). Dense populations with continuous new juveniles in close contact with terrestrial poultry and swine, and in proximity to humans, make domestic ducks a perfect intermediate host for genetic reassortment. Novel viral variants containing gene segments from viruses of wild, domestic, aquatic and terrestrial birds and mammals can emerge (Fouchier and Guan 2013, Huang et al. 2010).

Chickens are the most common domestic poultry worldwide and the major type to facilitate the genesis of HPAI H5 and H7 subtypes. Turkeys are also highly permissive influenza hosts, and in Europe and North America where they are intensely farmed, there are regular IAV outbreaks (Fouchier and Guan 2013). In contrast to the genetic variability of IAVs in ducks, the two subtypes H9N2 and H6N1, each with multiple lineages, dominate in terrestrial poultry. In China and Southeast Asia these subtypes have become enzootic since the 1990s (Xu et al. 2007, Cheung et al. 2007). After their introduction to terrestrial poultry, genes of both internal and surface proteins have undergone significant host and immune adaptive changes; for example the primary replication site of the viruses has changed from the intestine to the trachea (Fouchier and Guan 2013). The poultry H9N2 virus has also gained attention as it has served as donor of internal gene segments in reassortment events that have generated the two highly human pathogenic viruses HPAI H5N1 and H7N9/2013 in China (To et al. 2013).

Influenza A Viruses Highly Pathogenic to Birds and Humans

H5 and H7 subtype viruses can evolve from LPAI to HPAI variants following introduction to terrestrial poultry. The HPAI definition refers to a phenotype with high chicken mortality, which is associated to the introduction of multiple basic amino acids at the HA0 cleavage site (To et al. 2013). In contrast to the native HA0 of LPAI poultry viruses, which is cleaved to HA1 and HA2 by proteases only present in the respiratory epithelium, the HPAI HA0 polypeptide can be cleaved and activated by ubiquitous furin endoproteases. This leads to HA activation throughout the organism and accounts for the systemic disease with multi-organ involvement that is seen both in poultry and human HPAI virus infections (Klenk, Garten, and Matrosovich 2013).

HPAI H5N1

In 1997, an HPAI H5N1 strain caused a major outbreak in live poultry markets in Hong Kong, and also generated human cases with a 63% case fatality rate (To et al. 2013). Since then, the HPAI H5N1 lineage has evolved, spread, and become enzootic in poultry throughout Southeast Asia and China (Forrest and Webster 2010). Numerous reassortment events and antigenic drift of the HA continuously generate new HPAI H5N1 variants; these are systematically defined in clades and subclades by the HA phylogeny and in genotypes (B,X,Z,V) with regard to the other gene segments (WHO 2015b, 2011). Repeatedly, there are HPAI H5N1 epizootics in poultry, which especially affect commercial production. In 2003 to 2005 there was a large HPAI H5N1 outbreak in Southeast Asia and subsequent calculations estimated that 50% of the inhabitants derived their income from poultry, and that the economic consequences of the outbreak were 0.5 to 2% of the region's GDP (Chmielewski 2011).

HPAI H5N1 is also prevalent in domestic ducks and since ten years HPAI H5N1 viruses account for 30% of the IAVs carried by domestic ducks throughout Southeast Asia; in fact domestic ducks are suggested to be the source of the HPAI H5N1 enzootic in the region (Fouchier and Guan 2013). HPAI H5N1 transmission from domestic ducks to migrating wild waterfowl probably explains the ongoing spread of the virus to most continents (Fouchier and Guan 2013, Forrest and Webster 2010), recently also to North America (Clifford 2015). Egypt is highly affected by HPAI H5N1 circulating in poultry, and since 2014 there have been numerous human cases with high fatality rates (El-Shesheny et al. 2014, WHO 2015a).

Since 2003, human cases with HPAI H5N1 infections are reported to the WHO; since then through 2015 there were reports of 844 laboratory confirmed cases from 16 countries, whereof 449 (53%) have died. The large majority of cases from the last year were reported from Egypt (WHO 2015c).

H7N9/2013

In March 2013, a human influenza outbreak with high mortality was caused by a novel H7N9 virus in eastern China (Gao, Lu, et al. 2013, Gao, Cao, et al. 2013). It had emerged through multiple reassortment events between viruses of wild waterfowl, domestic waterfowl and poultry, and it is considered to have pandemic potential (Liu et al. 2013, Lam et al. 2013, Kageyama et al. 2013). Since then the number of human H7N9 cases in China has continued to increase with a seasonal incidence pattern; throughout 2015, 683 laboratory confirmed cases, with at least 275 (40%) deaths, had been reported by Chinese authorities (WHO 2015c).

Similarly as for human HPAI H5N1 infections, exposure to poultry at wild bird markets is the main risk factor for human disease (Liu et al. 2014,

Jernigan and Cox 2015). Although highly pathogenic to humans, the H7N9/2013 virus does not confer disease to poultry (Liu et al. 2014, Ge et al. 2014, Pantin-Jackwood et al. 2014, Han et al. 2014), a feature that may be related to the absence of the HPAI typical basic amino acids at the HA0 cleavage site (To et al. 2013).The LPAI phenotype in birds makes the detection of introduced H7N9/2013 virus in a poultry population difficult and requires active surveillance (Han et al. 2014).

Since the human outbreak in eastern China 2013, the H7N9/2013 virus has spread in poultry to several regions of China. It has become persistent in chickens with establishment of distinct lineages with different reassortant genotypes (Lam et al. 2015); a situation that raises concern considering the pandemic potential of the virus (Jernigan and Cox 2015).

Adaptations Facilitating Human Infection with Avian IAVs

Both HPAI H5N1 and H7N9/2013 contain several of the traits described for viral adaptation to humans (Tanner, Toth, and Gundlapalli 2015). The HA receptor specificity is changed to α2,6 preference in the H7N9/2013 virus (Gao, Cao, et al. 2013, Chen et al. 2013) and in some HPAI H5N1 variants (Yamada et al. 2006, Imai et al. 2012). PB2 changes that enhance the polymerase activity in mammals and allow for a lower replication temperature is seen both in H7N9/2013 (Gao, Cao, et al. 2013, Chen et al. 2013) and in HPAI H5N1 variants (Hatta et al. 2007). Deletion of amino acids in the NA stalk, which is related to airway tropism, is seen both in HPAI H5N1 and H7N9/2013 viruses (Gao, Cao, et al. 2013). In H7N9/2013, NS1 changes increase virulence and the adaptation to human lung tissue (Gao, Cao, et al. 2013, Knepper et al. 2013), and a specific NP substitution makes it less sensitive to the avian-virus specific immunity mediated by the human Mx protein (Riegger et al. 2015).

To date, no sustained human to human transmission has been observed with the HPAI H5N1 or H7N9/2013 viruses. However, gain of function experiments with HPAI H5N1 in ferrets have demonstrated that only three or four point mutations may be sufficient for mammal to mammal transmission of the virus (Imai et al. 2012, Herfst et al. 2012). This confirms its position as a virus with pandemic potential aside the H7N9/2013 virus (Jernigan and Cox 2015, Tanner, Toth, and Gundlapalli 2015). Ferret studies on the H7N9/2013 virus demonstrated air-borne transmission, but it was accompanied by an overall reduced genetic diversity and decreased viral fitness. Therefore, the change of transmission rout is suggested to represent a genetic bottleneck for further mammal adaptation of the H7N9/2013 virus (Zaraket et al. 2015).

In addition to the HPAI H5N1 and H7N9/2013 viruses, also other avian subtypes, primarily H5N6 and H10N8, have given rise to limited numbers of severe human disease in China during the last years (Xinhua 2016, To et al. 2014, Yang et al. 2015).

Pandemic Influenza

Origins and Pathogenicity of Pandemic Viruses

A dozen influenza pandemics are believed to have occurred since the sixteenth century, based on epidemic descriptions (Wang and Palese 2013), but the H3 subtype Russian flu 1889 is the first pandemic with serologic evidence (Dowdle 1999). All four pandemics of the last 100 years were the result of reassortment events between avian and human or swine IAVs (Cox and Subbarao 2000, Guan et al. 2010).

The genetic origins of the H1N1 virus of 1918, the Spanish flu, has been controversial, as phylogenetic analysis reveals mammalian origin but consensus amino acid comparison are avian like. Dating analysis of the virus suggests that it was generated by reassortment events during a period of years, with introduction of avian virus genes to swine and human strains (Guan et al. 2010).

The H2N2 virus of 1957, the Asian flu, was a reassortant between the circulating human H1N1 virus, with introduction of H2, N2 and PB1 from Eurasian avian IAVs, while the H3N2 virus of 1968, the Hong Kong flu, acquired avian H3 and PB1 segments to the human H2N2 virus (Guan et al. 2010, Webster et al. 1992).

The H1N1 virus of the 2009 pandemic (H1N1/pdm09) was a swine IAV that had undergone multiple reassortment events before being transmitted to humans. Several North American swine triple reassortant variants had circulated in swine since 1998 (Smith et al. 2009). One of them that had derived the PA and PB2 from the North American avian lineage, the M, HA, NP and NS from the classical H1N1 swine lineage (originally a reassortant with avian origin before 1918 (Guan et al. 2010)), and the NA and PB1 from a human H3N2 virus was reassorted by introduction of the NA and M segments from the European swine H1N1 avian-like lineage (Brockwell-Staats, Webster, and Webby 2009, Wang and Palese 2013, Smith et al. 2009). It was thereafter transmitted to humans where it circulated for some months before it was detected and started to spread worldwide (Smith et al. 2009).

Pandemic Influenza Mortality

The H1N1/1918 pandemic virus caused disease in three waves in 1918 and 1919 and was the most devastating one (Cox and Subbarao 2000, Taubenberger and Morens 2006). Though the attack rate has been estimated to a similar level of 25-30% as later pandemics (Nguyen-Van-Tam and Bresee 2013), the overall case fatality rate of the H1N1/1918 virus has been estimated to 2.5% as compared to approximately 0.1% of later pandemic viruses (Taubenberger and Morens 2006). The hospital related mortality, which may better compare to the case fatality rates of today's laboratory confirmed HPAI H5N1 and H7N9/2013 cases, was 17% at a Swedish hospi-

tal (Holtenius and Gillman 2014). The global mortality of the H1N1/1918 pandemic has been estimated to 50 million people or more (Guan et al. 2010, Johnson and Mueller 2002). Aside the ongoing world war with several extreme epidemiologic factors (Erkoreka 2009), the high mortality has been ascribed both to the pathogenicity of the virus and to a high rate of severe secondary bacterial infections; these were primarily caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* (Morens, Taubenberger, and Fauci 2008, HisMajesty'sStationeryOffice 1920).

Also during the H2N2/1957 pandemic, secondary bacterial infections were significant in those with severe disease, then predominantly with *Staphylococcus aureus* (Monto and Webster 2013).

A typical W-shaped pattern of age-mortality curves is described for pandemic influenza, with an exceptionally high peak in mortality among young adults. This pattern differs from the U-shaped age-mortality curves of seasonal influenza, when the highest mortality is seen among infants and elderly (Cox and Subbarao 2000). The pandemic age-mortality pattern was pronounced during the entire 1918-1919 pandemic, with the majority of death cases in young adults (Saglanmak et al. 2011, Taubenberger and Morens 2006, Holtenius and Gillman 2014). Though less pronounced, also following pandemics have followed the pattern (Simonsen et al. 1998). The high mortality among young adults is primarily related to an absence of immunity in the younger age-groups (Reichert, Chowell, and McCullers 2012). As a pandemic virus starts to circulate seasonally the age-mortality curves are gradually restored to a seasonal U-shape, both as a result of acquired immunity in the population and of antigenic drift of the virus (Saglanmak et al. 2011, Simonsen et al. 1998).

Viral Pathogenicity Markers

A number of viral properties are considered to have been critical for the high pathogenicity of the H1N1/1918 virus. These were primarily related to the HA and the polymerase (PB2, PB1 and PA) proteins, but the characteristics of the PB1-F2, NS1 and NA proteins also contributed (Neumann and Kawaoka 2013). Reverse genetics studies on recent human seasonal IAV strains have demonstrated that introduction of gene segments of the H1N1/1918 virus render them much more pathogenic. Introduction of the 1918 HA (+/- NA) generates severe lung damage; the 1918 replication complex (which includes the PB2-K627 variant) generates very high viral titers and severe lung pathology; the NS1 controls host immune response very efficiently; the PB1-F2, with the same S66 variant as in HPAI H5N1, confers high viral titers, pronounced pathogenicity and an increase in bacterial co-infections (Neumann and Kawaoka 2013). In contrast, the H1N1/pdm09 virus does not possess any molecular changes that are associated with high pathogenicity (PB2-E627K, PB1-F2-N66S, HA basic amino acids, NA dele-

tion, or NS-F92D), which in part explains the limited morbidity and mortality during the last pandemic (Forrest and Webster 2010).

Pandemic Preparedness

Planning and Response

Preparedness is vital if the public health impact of an influenza pandemic is to be limited. In 1947, the World Health Organization (WHO) established the now called Global Influenza Surveillance and Response System (GISRS), which monitors circulating influenza viruses, assesses IAVs with pandemic potential, and makes recommendations on seasonal vaccine components, as well as on pandemic planning and response. Before 2004 there was limited national pandemic planning, but as a result of the 1997 Hong Kong and 2003 Southeast Asian HPAI H5N1 outbreaks, international efforts were coordinated in a multi-sectorial way and 2005 WHO published a new pandemic preparedness plan (Nguyen-Van-Tam and Bresee 2013). The plan describes different pandemic phases based on the spread of a novel human pathogenic virus, and gives recommendations on what actions to undertake at each phase (Fineberg 2014). Also in 2005, the WHO member states adopted the International Health Regulations (IHR), stating that member states shall detect, respond and share information on important public health issues. Thereafter, the number of isolates sent to GISRS increased markedly (Nguyen-Van-Tam and Bresee 2013).

The H1N1 pandemic 2009 put the system to a test, and although all preparations had been focused on an HPAI H5N1 virus, evaluations of the WHO efforts were over all positive. It was however concluded that "the world is ill prepared to respond to a severe influenza pandemic" (Fineberg 2014). An important WHO lesson was the need to include a scoring level not only for the spread of a new virus, but also for its pathogenicity in humans, which may modulate which actions to undertake and aid in the communication with the community (Monto and Webster 2013, Fineberg 2014).

Pharmaceutical Preparedness

Neuraminidase inhibitors (NAIs) are the most efficient anti-influenza drugs and therefore constitute a pharmaceutical cornerstone for influenza pandemic preparedness. As opposed to vaccines that take several months to produce NAIs can be used immediately for prophylaxis and treatment (Nguyen-Van-Tam, Openshaw, and Nicholson 2014). Accordingly, many nations have stockpiled the orally available NAI oseltamivir (Tamiflu®) (Patel and Gorman 2009, Wan Po, Farndon, and Palmer 2009). However, during the H1N1/2009 pandemic, large scale oseltamivir usage proved to be complex

with varying success in distribution and administration. For example; in Japan oseltamivir was widely used and the influenza related mortality was very low, while in the UK where prophylaxis and wide indication treatment was also recommended the compliance in taking prescribed drugs was poor (Nguyen-Van-Tam and Bresee 2013, Singer et al. 2013, Monto and Webster 2013). Despite complexity, stockpiling of NAIs and their usage as the first line response in a pandemic situation is still considered beneficial. However, the stockpiling needs to be supplemented by planning for drug access and delivery (Muthuri et al. 2013).

As secondary bacterial infections are responsible for a substantial part of the influenza related mortality, pneumococcal vaccination and antibiotic stockpiling or treatment planning, has also been recommended as part of influenza pandemic preparedness planning (Morens, Taubenberger, and Fauci 2008, Nguyen-Van-Tam and Bresee 2013).

Pandemic vaccines are central for the containment of a pandemic and different vaccine development strategies can be applied. As a vaccine that is highly specific to a novel virus can only be developed after detection and characterization of the virus, and as production and distribution is time consuming, delivery will come late in the pandemic. A less specific vaccine, on the other hand, can be prepared in advance and delivered more rapidly. Pandemic vaccine strategies therefore need to prioritize either antiviral specificity or rapidity in production and delivery (Nguyen-Van-Tam and Bresee 2013).

Non-pharmaceutical interventions are difficult to implement and evaluate. Individual public health measures, like hand hygiene, are probably the most cost effective interventions, though individual measures may be undertaken in combination with community efforts, like closing of schools (Nguyen-Van-Tam and Bresee 2013). During the H1N1/1918 pandemic, the only intervention that could efficiently prevent disease was complete isolation of a defined community (like a college or a military facility) at a remote place during a prolonged period of time (Markel et al. 2006).

Monitoring of Potentially Pandemic Viruses

Potentially pandemic IAVs are continuously monitored, but, as illustrated by the last pandemic, a new pandemic virus may come as a surprise. Prior to 2009, an HPAI H5N1 virus was considered to be the most probable pandemic IAV, with H7N7, H7N2, H9N2 or H2N2 viruses as other possible candidates (Nguyen-Van-Tam and Bresee 2013). The H1N1/pdm09 virus clearly underscored that IAV surveillance needs to be broadened to also include surveillance of viruses in swine and birds, and that studies on cross species adaptations is an important priority (Vijaykrishna et al. 2011, Monto and Webster 2013). The H7N9/2013 virus in China, which evolved in live poultry market environments over a few years, as well as recent human cases

with H10N8, H5N6 and H6N1 viruses, suggest that new human pathogenic viruses are to expect from Chinese and Southeast Asian bird markets and point out surveillance needs in these settings (Figure 6) (To et al. 2014, Jernigan and Cox 2015, Yang et al. 2015).

After the H1N1/pdm09 pandemic, the US governmental Centre for Disease Control developed another influenza risk assessment tool (IRAT), in addition to the WHO GIRS system. IRAT aims to assess the risk of emergence and impact of IAVs with pandemic potential and its evaluation list contains H7N9, H5N1, H9N2, H3N2v and other novel viruses (Jernigan and Cox 2015). Reintroduction of H2N2 strains to humans is also considered a pandemic possibility (Joseph et al. 2015, Jones et al. 2014).

Figure 6. Chicken market in Xining, Qinghai province, China. Live bird markets constitute risk environments for the evolution and spread of new human pathogenic influenza viruses. (Reproduced from Wikimedia Commons by the (CC-BY-SA 2.0) license.)

Prevention and Treatment of Human Influenza

Vaccination

The ideal prevention of influenza virus infections is vaccination, which reduces morbidity and mortality (Fiore et al. 2010). The primary antigenic determinant is HA, and current vaccines are designed to primarily target HA with the aim to produce antibodies towards relevant strains (Webster and Govorkova 2014, Keitel, Neuzil, and Treanor 2013). Currently used vaccines

are produced from viruses that have been cultured in embryonated chicken eggs (ECEs) or in cell cultures. Inactivated whole virus vaccines can be administered by intranasal deposition or intramuscular injection, while purified subunit vaccines are administered intramuscularly (Keitel, Neuzil, and Treanor 2013).

Seasonal Vaccines

Seasonal influenza vaccines are produced according to yearly recommendations by WHO, based on the antigenic specificity of circulating IAV strains (WHO 2014). Since 1977, inactivated vaccines have been trivalent, or since 2013/14 quadrivalent, and target an H1N1 strain, at present (2015/2016) an H1N1/pdm09- like strain, an H3N2 strain and one or two influenza B virus strains (Keitel, Neuzil, and Treanor 2013, WHO 2014). Due to antigenic drift and the use of one or two B strains, specificity and immunogenicity may vary (Webster and Govorkova 2014, Fiore et al. 2010).

Pandemic Vaccines

Pandemic IAV vaccines have been based on licensed seasonal vaccines with inactivated viruses. The most significant challenges for pandemic vaccine production are the need for very fast up-scaled production (Partridge and Kieny 2010) and for a high immunogenicity (Keitel, Neuzil, and Treanor 2013). Approaches to increase the immunogenicity of pandemic vaccines are either prime boost regimens or addition of adjuvants; both approaches can increase the antibody titers but either demands an additional vaccination or increases the risk of adverse events (Keitel, Neuzil, and Treanor 2013). A suggested alternative for fast production and distribution of pandemic vaccines is stockpiling of "candidate" pandemic vaccines, with antigenicity based on assumptions about future pandemic strains (Jennings et al. 2008).

During the last H1N1 pandemic in 2009, vaccines could be distributed after approximately 6 months (Partridge and Kieny 2010, Nguyen-Van-Tam and Bresee 2013). Later, vaccination with Pandemrix®, that contained a new largely non-evaluated squalene based adjuvant, proved to be associated with narcolepsy in children (Barker and Snape 2014, Läkemedelsverket 2011). The unexpected side effect by the pandemic vaccine has increased the skepticism in the community towards any influenza vaccination, mirrored as lower seasonal influenza vaccine coverage (Nylén et al. 2012).

New Vaccines

Several new vaccines based on various principles are at different stages of development. Protein subunit vaccines are attractive because of the safety profile and the possibility of up-scaled production. Several candidates directed against IAVs with pandemic potential (H5N1, H7N9 and H1N1/pdm09), and some that can generate cross-protection to divergent strains or clades, are under development (Zhang et al. 2015). Broad cross-

protective universal subunit vaccines based on conserved regions of the NP, extracellular sites of the M2, or the stem region of group 2 HA (which is more conserved than group 1 HA) are interesting candidates for pandemic vaccines (Zhang et al. 2015). The potential of a new time perspective of days or weeks for vaccine production was recently demonstrated with synthetic self-amplifying mRNA vaccines. Protective immunity was acquired in mice toward the H7N9/2013 virus shortly after the release of the H7 sequence, though studies on human safety and efficacy remain (Ulmer, Mansoura, and Geall 2015).

The ultimate vaccine that would change the entire influenza treatment and prevention scenario is a universal vaccine that protects against all influenza subtypes with long lasting immunity; but although a research priority it still lies in the future (Webster and Govorkova 2014).

Anti-influenza Drugs

Globally approved human anti-influenza agents are the M2 ion channel inhibitors amantadine and rimantadine (1960s), and the neuraminidase inhibitors (NAIs) zanamivir (ZA) (1999) and oseltamivir (1999).

The intravenous formulation NAI peramivir (2010) and the long lasting inhalation powder laninamivir (2010) are approved in a few countries (Japan, China, S Korea) (Komeda 2014, Ikematsu 2014, Ison and Hay 2013, Webster and Govorkova 2014). The broad spectrum HA fusion inhibitor arbidol is approved in Russia (1990) and in China (2006) (Ison and Hay 2013).

Usefulness of Licensed Drugs

Due to extensive resistance of circulating strains, adamantanes are no longer recommended for treatment of seasonal influenza (Webster and Govorkova 2014, WHO 2012).

The usefulness of NAIs was 2014 the subject of a Cochrane review assessing the clinical study reports underlying the registration of oseltamivir and ZA. The authors concluded that both ZA and oseltamivir had small and non-specific effects in reducing time to alleviation of influenza symptoms, but that they reduce the risk to develop symptomatic influenza when used for prophylaxis treatment (Jefferson et al. 2014). However, the registration studies were mainly done on healthy outpatients, and accumulated clinical experience and post registration studies confirm that NAI treatment, especially when started early, reduces morbidity and mortality in patients with severe influenza disease (Nguyen-Van-Tam, Openshaw, and Nicholson 2014, Lee et al. 2010, Chan et al. 2013, McGeer et al. 2007, Muthuri et al. 2014, Muthuri et al. 2013, Ison and Hay 2013). Accordingly, resistance to NAIs is associated with poor clinical outcomes in patients with severe disease (Hu et al. 2013, Eshaghi et al. 2014). It can thus be concluded that NAIs are useful

for prophylaxis of influenza infections and for treatment of severely ill patients, but that they are of little or no use for treatment of seasonal influenza in young and otherwise healthy individuals (Nguyen-Van-Tam, Openshaw, and Nicholson 2014).

Drugs under Development

Several new anti-influenza drugs that target various viral and host cellular structures are under development. The novel influenza drug that has reached furthest in clinical trials is the broad spectrum RNA polymerase inhibitor favipiravir, which also has a mutagenic activity and that is active against all influenza virus types and against several other RNA viruses (Baranovich et al. 2013, Ison and Hay 2013, Webster and Govorkova 2014, Furuta et al. 2013).

A number of monoclonal antibodies that can neutralize a broad range of influenza viruses have been identified, whereof some are in clinical trials (Webster and Govorkova 2014). These are, in contrast to targeting the antigenic sites, primarily directed to conserved sites of the stem region of HA, and are cross reactive for group 1 and/or group 2 HAs (Webster and Govorkova 2014).

DAS181 is a fusion protein with sialidase catalytic activity that is administered by inhalation and that removes the cell surface sialic acid receptors required for HA binding (Ison and Hay 2013, Webster and Govorkova 2014). Nitazoxanide, which is licensed as an anti-parasitic agent, inhibits HA maturation and induces interferon production in the host (Webster and Govorkova 2014, Ison and Hay 2013). Another potential drug for prophylaxis treatment is an engineered sialic acid binding protein (Sp2CBMTD) that masks the receptors of the respiratory epithelium (Govorkova et al. 2015, Connaris et al. 2014). In addition, a number of components that interfere with the replication complex proteins, with vRNA and with the host immune responses are promising drug candidates (Dunning et al. 2014, Webster and Govorkova 2014, Ison and Hay 2013).

Combination therapies aimed to improve the antiviral effect and to overcome or avoid drug resistance have not been extensively evaluated for IAVs. Among the available drugs, an NAI combined with rimantadine has an additive and synergistic anti-influenza effect in vitro (Govorkova et al. 2004), and a triple combination of amantadine, ribavirin and oseltamivir (TCAD) has a synergistic effect against both sensitive and amantadine or oseltamivir resistant strains (Dunning et al. 2014). Combinations of an NAI with several of the components under development seem to have additive antiviral effects. Of note is that combining oseltamivir with ZA has no positive, but possibly an antagonistic effect (Dunning et al. 2014).
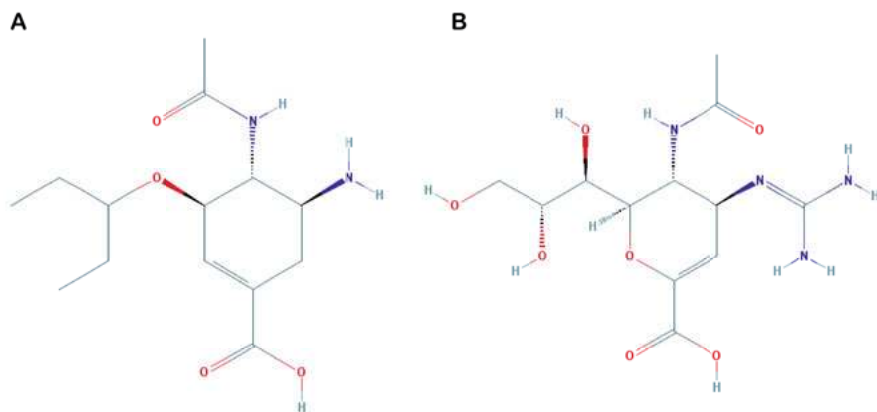
Figure 7. Two dimensional structure of (A) oseltamivir carboxylate (OC) and (B) Zanamivir (ZA). (From National Center for Biotechnology Information. PubChem Compound Data-base; CID=449381, CID=60855, accessed Oct. 21, 2015.)

Oseltamivir

Oseltamivir is currently the most used anti-influenza drug. The active component oseltamivir carboxylate (OC) (Figure 7) has a poor oral bio-availability, and the drug is therefore administered as an ethyl ester prodrug, oseltamivir phosphate (OP) (Tamiflu ®, Roche). After transformation by liver-esterases, 75% of an oral dose reaches the plasma as active OC, which is then eliminated unchanged in the urine (LIFproduktresumé 2015b, Smith et al. 2011). OC concentrations in bronchoalveolar lavage of rats (Eisenberg, Bidgood, and Cundy 1997), and in lung tissue, middle ear and nasal mucosa of ferrets (He, Massarella, and Ward 1999), are similar to or exceed the plasma concentrations. The correlation of OC concentrations in plasma to lung-tissue in humans has not been validated. The OC concentration in saliva of healthy volunteers was however less than 5% of that in plasma, an observation that led to the suggestion of increased oseltamivir dosage in severely ill patients (Wattanagoon et al. 2009).

OC and all other NAIs act by competitive binding to the extracellular enzymatic site of the NA protein. Thereby, the NA binding and catalysis of the sialic acid substrate is inhibited and the release of newly formed virions from infected cells and viral spread through respiratory secretions is reduced (Ison and Hay 2013).

OC binding to NA is mainly mediated by the hydrophobic pentyl ether moiety, which binds to the hydrophobic pocket of the enzymatic site. In order to accommodate the large diethyl extension, a conformational change with re-orientation of the E276 residue needs to take place (Figure 8) (Ison and Hay 2013, Russell et al. 2006). As the active sites of group N1 and group N2 NAs differ, the OC binding differs between the groups. Group N1

proteins can accommodate OC binding with the 150-loop in two different conformations; either in an "open" (low energy) conformation or, as a result of higher drug concentrations or longer exposure time, in a "closed" (high energy) conformation (Figure 8). OC binding to group N2 NAs resembles the closed conformation binding of group N1 proteins (Russell et al. 2006).

Zanamivir

ZA is commonly used as a second line NAI in critically ill patients if there is treatment failure or resistance to oseltamivir (Dulek et al. 2010, Ho et al. 2014). ZA (Relenza®, Glaxo-Smith-Kline) is currently approved as an inhalation powder. In average, 78% of a dose is deposited in the oropharynx and further eliminated via the gastrointestinal tract, while 10-20% is absorbed and excreted unchanged in the urine (LIFproduktresumé 2015a) . An intravenous formulation for treatment of severe influenza is under clinical evaluation, and is available for compassionate use (CDC 2015, Watanabe et al. 2014, Marty et al. 2014, Ho et al. 2014).

The ring structure of ZA (Figure 7) and the binding mechanism to its target differ from that of OC. The guanidine side chain of ZA binds to the acidic amino acid residues E119, D151 and E227 of the enzymatic site of NA, without the need for conformational changes (Ison and Hay 2013).

Resistance to Neuraminidase Inhibitors

Prevalence and Definitions of NAI Resistance

Already during the drug development phases resistance to OC and ZA was described *in vitro*, and the results were accompanied by the demonstration of reduced viral fitness of resistant variants (Tai et al. 1998, Gubareva et al. 1997, McKimm-Breschkin et al. 1998). NAI treatment of IAV infected individuals can also generate resistant viruses. Resistance is primarily described following treatment with oseltamivir and occurs both in human (Kiso et al. 2004, Whitley et al. 2013, Gubareva et al. 2001) and avian viruses (de Jong et al. 2005, Hu et al. 2013, Samson et al. 2013). Clinically, resistance to NAIs is associated to prolonged infections in children and in immunosuppressed patients (Eshaghi et al. 2014, Kiso et al. 2004), and in H7N9 and H5N1 cases to high viral loads and severe clinical outcomes (de Jong et al. 2005, Hu et al. 2013).

Following market introduction, the overall OC resistance in clinical settings was reported to be less than 1% in adults and 4% in children under 12 years of age, but higher in hospitalized children, immunocompromised individuals and in HPAI H5N1 infected patients (Samson et al. 2013). Although much

less common than OC resistance (Thorlund et al. 2011), ZA-resistance in ZA treated patients is also described (Nguyen et al. 2012, van der Vries, Stelma, and Boucher 2010, Samson et al. 2013, Gubareva et al. 1998).
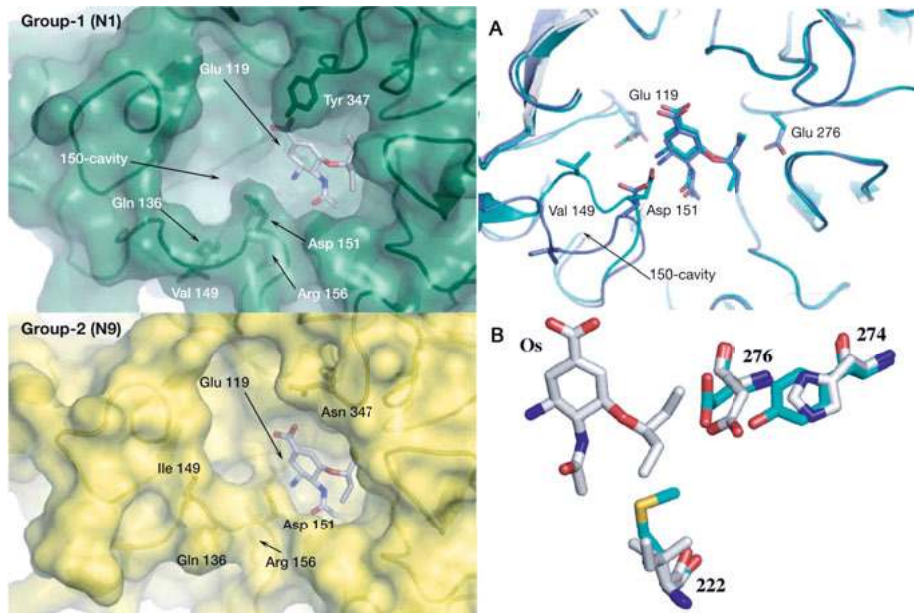
Figure 8. Left panel: Crystal structure surfaces of group N1 and group N2 NAs with bound OC. The group difference of the 150-cavity is determined by the conformation of the 150-loop and the position of Gln136. Right panel **A:** OC binding to the active site of group N1 (N8) with the change from an open (dark blue) to a closed (cyan) conformation of the 150-loop, as a result of long exposure time. **B:** Stick representation of a wild type group N1 protein (N1) (white sticks) superimposed on substituted residues H274Y and I222M (cyan sticks) displaying the interactions with the diethyl moiety of OC. (Reprinted with permission from Macmillan Publishers Ltd: Nature (Russell et al. 2006), copyright 2006, and from Antimicrobial Agents and Chemotherapy (Hurt, Holien, and Barr 2009), copyright © American Society for Microbiology.)

Circulation of OC Resistant Influenza A Viruses

However, raising more concern than selection for resistant variants by clinical treatment is the circulation of NAI resistant human strains in the absence of selective drug pressure. This feature was primarily described in the seasonal H1N1 virus 2007 – 2009 when the circulating strain was OC resistant by an H274Y substitution in NA, without selective drug exposure (Moscona 2009). In 2009, the resistant seasonal H1N1 virus was entirely replaced by the NAI susceptible (though adamantane resistant) pandemic H1N1/pdm09 virus. But since 2010/2011, an increasing number of community cases with OC resistant H1N1/pdm09 viruses has been reported without previous oseltamivir exposure; in all instances the resistance has been conferred by the

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H274Y substitution in NA (Storms et al. 2012, Lackenby et al. 2011, Takashita et al. 2015, Hurt et al. 2011).

Avian IAVs of wild waterfowl have been tested for NAI susceptibility only to a very limited extent compared to human viruses, especially regarding subtypes other than those infecting humans (Stoner et al. 2012). There are however a few large resistance screening studies on avian N1 and N6 subtypes; these confirm that naturally occurring OC resistance is rare among wild bird avian IAVs (Orozovic et al. 2014, Stoner et al. 2010, Stoner et al. 2012).

Determination and Definitions of Drug Susceptibility

Resistance to NAIs is commonly defined by phenotype as a reduced drug susceptibility, which can be confirmed by the presence of a resistance inducing mutation; or sometimes vice versa. An expert working group on antiviral susceptibility (AVWG) supports the WHO GISRS on surveillance methodology and strategies regarding antiviral resistance of human influenza, and have presented recommendations on methods and definitions of NAI resistance (WHO 2012).

As the molecular reasons for NAI resistance are complex and not entirely known, drug susceptibility testing should primarily be phenotypic, followed by genotypic specification/confirmation; the exception being resistance screening for the NA-H274Y substitution in clinical H1N1/pdm09 isolates (WHO 2012). The method of choice for phenotypic susceptibility testing is determination of the NA enzyme activity, reporting the drug concentration that inhibits 50% of the activity (IC_{50}) (WHO 2012). For consistency in the reporting NAI susceptibility the WHO made defining criteria for normal, reduced and highly reduced drug susceptibility. A less than 10-fold increase in IC_{50} compared to a reference strain is defined as "susceptible" or "normal", a 10 to 100- fold increase in IC_{50} as "reduced", and a > 100-fold increase in IC_{50} as "highly reduced" susceptibility (WHO 2012). These criteria however, do not necessarily correspond to the efficiency of NAIs in a clinical context.

Resistance Mechanisms to Oseltamivir and Zanamivir

Resistance to NAIs is primarily caused by amino acid substitutions in the NA protein that reduce the binding of the drugs, either by substitution of active site residues or of framework residues (Samson et al. 2013, Colman, Hoyne, and Lawrence 1993). However, as NA and HA activities are balanced, HA mutations may as well alter the NAI susceptibility of a virus (McKimm-Breschkin et al. 1998). Resistance mutations are NA group specific, and as a result of the different binding moieties, OC and ZA give rise to different resistance profiles (Table 1) (Samson et al. 2013, McKimm-Breschkin 2012).

The most common OC resistance substitutions seen *in vivo*, and which confer high level resistance in the respective groups, are H274Y in group N1, and R292K and E119V in group N2 viruses (Samson et al. 2013, Ferraris and Lina 2008).

Table 1. *Resistance Substitutions and Relative Susceptibility to NAIs*

Type/ subtype	Amino acid substitution**	Oseltamivir	Zanamivir	Peramivir
H1N1	N70S	S	M	?
H1N1	Q136K	S	H	H
H1N1	Y155H	M	H	H
H1N1	I222V	L	S	S
H1N1	I222M	L	S	S
H1N1	H274Y	H	S	H
H1N1pdm(09)	I222V	M	S	?
H1N1pdm(09)	I222R	M	L	L
H1N1pdm(09)	H274Y	H	S	H
H3N2	E119A	H	H	H
H3N2	E119D	H	H	H
H3N2	E119G	S	H	M
H3N2	E119V	H	S	S
H3N2	E119I	H	L	H
H3N2	Q136K	S	M	?
H3N2	I222V	M	S	S
H3N2	R292K	H	M	H
H3N2	N294S	H	S	S
B	E119A	H	H	H
B	E119D	H	H	H
B	E119G	M	M/H	H
B	R152K	H	H	H
B	D198N	H	M	L
B	D198E	M	L	M
B	I222V	L	S	L
B	I222T	H	M	?
B	S250G	M	H	?
B	G402S	H	M/H	?
H5N1	V116A	M	M	L
H5N1	I222L	M	S	?
H5N1	I222V	L/H***	S	S
H5N1	I222M	M/H***	S	S
H5N1	I222T	M/H***	S	S
H5N1	S246N	M	S	?
H5N1	H252Y	M	S	S
H5N1	H274Y	H	S	H
H5N1	N294S	M/H	L	L

Resistance related amino acid substitutions in NA of viruses causing human disease as described in the literature. S = sensitive. L = low, <10-fold increase in IC_{50}. M = medium, 10–50-fold increase in IC_{50}. H = High, $\geq$ 50-fold increase in IC_{50} compared to wild type viruses. ***Clade 2 isolates have higher IC_{50}s than clade 1 isolates. Nb. that the substitutions above are derived both *in vivo* and from cell cultures, why all may not be compatible with selection *in vivo*. (From (McKimm-Breschkin 2012) © 2012 Blackwell Publishing Ltd. Reprinted with permission from Wiley).

NA substitution H274Y

The different consequences of the H274Y substitution in group N1 and group N2 NAs can be structurally explained by a tighter conformation of the group N1 active site and the interaction between H274 and E276. When H (histidine) is replaced by the larger Y (tyrosine) at residue 274 in group N1 NAs, the nearby E276 is displaced and cannot move to expose the hydrophobic pocket essential for binding of the hydrophobic OC side-chain (Figure 8B). At the active site in group N2 NAs there is more space which allows accommodation of the Y at residue 274 without hindering the essential E276 reorientation for OC binding (Russell et al. 2006). Binding of ZA is not influenced by the E276 reorientation (Collins et al. 2009) and therefore the H274Y substitution does not reduce ZA susceptibility.

NA substitution R292K

The R292K substitution on the other hand, interferes with OC binding in group N2 but not in group N1 NAs. When K (lysine) replaces R (arginine) at residue 292 in group N2 proteins, the K interacts with E276 and hinders the movement needed to accommodate the OC binding; and in addition, a hydrogen bond between R292 and OC is lost (Russell et al. 2006). In group N1 proteins, the weak interaction between OC and K292 (as compared to R292) is supported by an additional hydrogen bond with the conserved Y374 residue, which is believed to be sufficient for OC binding and the explanation why R292K does not confer resistance in viruses with group N1 NAs (Russell et al. 2006).

NA substitutions of I222

The NA-I222 residue is highly conserved across all influenza A and B viruses and, together with E276, R224 and S246, forms the hydrophobic pocket of the active site (Hurt, Holien, and Barr 2009). Unlike H274Y and R292K, I222 substitutions are not NA group specific and can reduce drug susceptibility in both group N1 and group N2 viruses (McKimm-Breschkin 2012). Substitution of the isoleucine leads to loss of a hydrophobic interaction between the I222 side chain and the pentyl ether group of OC (Hurt, Holien, and Barr 2009, McKimm-Breschkin 2012). In addition, as in the case of I222M, the substituting residue may project into the hydrophobic pocket and obstruct OC binding (Figure 8B). And further, if combined with an H274Y substitution it results in an extensive occlusion of the active site so that the large pentyl ether moiety of OC can no longer be accommodated (Hurt, Holien, and Barr 2009).

ZA is structurally close to the transitional state analog of the natural sialic acid substrate and no conformational changes are needed for binding to the NA active site. Therefore, it has been reasoned, the probability of substitu-

tions that lead to ZA resistance without compromising enzyme activity - and viral fitness - would be low, and the main reason aside the lower use why ZA resistance has remained quite infrequent in clinical settings as compared to OC resistance (McKimm-Breschkin 2012, Ison and Hay 2013, Samson et al. 2013).

A substitution conferring high level resistance to ZA in clinical settings is the Q136K in NA. It has been observed both in human H3N2 isolates after NAI treatment (Eshaghi et al. 2014), and in human seasonal H1N1 viruses (Hurt et al. 2009). The NA-136 residue is not part of the active enzymatic site, but the Q136K substitution generates a deviation of the 150-loop of the active site, hindering the ZA binding (Han, Liu, and Mu 2012).

Combinations of NA amino acid substitutions can potentiate or modulate resistance. Especially, as described above, several substitutions of the framework residue I222 can generate reduced susceptibility to NAIs in human H1N1, H3N2, and influenza B viruses (McKimm-Breschkin 2012, van der Vries et al. 2012), as well as in HPAI H5N1 viruses (McKimm-Breschkin et al. 2013). But additionally, substitutions at the 222 site can increase the phenotypic impact of resistance both due to H274Y in group N1 (Huang et al. 2014, Pizzorno et al. 2012, LeGoff et al. 2012, Hurt, Holien, and Barr 2009) and E119V in group N2 viruses (Baz et al. 2006). Interestingly, the dual substitutions I222R+H274Y in H1N1/pdm09 confer reduced susceptibility to both OC and ZA (LeGoff et al. 2012, Pizzorno et al. 2012).

Compensatory Mutations

Until 2008, IAV mutations that generate resistance to NAIs were believed to interfere with the NA enzyme to such a degree that viral fitness would be reduced, and that removal of selective drug pressure would restore the wild type genotype, i.e. that NAI resistant IAVs could not circulate in competition with wild type viruses. The resistant human seasonal H1N1/H274Y virus that circulated 2007-2009 tilted the concept (Moscona 2009). Subsequent studies on the resistant H1N1/H274Y/2007-2009 virus have increased the understanding of the evolution of resistance in IAVs. Sequential emergence of mutations in NA, HA and possibly in other gene segments that facilitate the acquisition of subsequent resistance mutations and compensate for decreased fitness, can accumulate over time and allow the evolution of resistant viruses with retained fitness (Duan et al. 2014, Bloom, Gong, and Baltimore 2010, Samson et al. 2013, Butler et al. 2014).

In the H1N1/H274Y/2007-2009 virus, the NA substitutions R221Q, D343N and V233M were demonstrated to restore replication, infectivity and transmissibility by increased NA affinity, activity and surface expression (Bloom, Gong, and Baltimore 2010, Abed et al. 2011). In addition, the subsequent acquisition of a D353G substitution in NA rendered the resistant

variant highly transmissible, which may have contributed to its predominance (Bouvier, Rahmat, and Pica 2012).

In a resistant H1N1/pdm09/H274Y variant circulating in the community in Australia in 2011, the NA substitutions V240I, N368K and N385S were demonstrated to upregulate NA surface expression and enhance enzyme activity, and were suggested to be permissive for the H274Y resistance substitution and thus for the maintenance of the cluster without a selective drug pressure (Hurt et al. 2012). Over the subsequent years, H1N1/pdm09 variants with the V240I and N368K substitutions have continued to increase in prevalence and since 2014 constitute > 99% of the globally circulating H1N1/pdm09 viruses. If the circulating H1N1/pdm09 viruses are now more permissive to an H274Y substitution the risk is increased for the emergence and spread of an OC resistant H1N1/pdm09 virus (Butler et al. 2014).

In addition to increasing resistance, several substitutions of the I222 residue in NA can restore reduced fitness; I222T/V/R restore fitness when it is combined with H274Y in N1 viruses (Huang et al. 2014, Pizzorno et al. 2012), as does I222V when combined with E119V in N2 viruses (Simon et al. 2011). Substitutions in HA may as well have a permissive or compensatory role when combined with NA resistance substitutions, as observed both in H1N1 (Yang et al. 2011, Zaraket et al. 2010) and in H3N2 clinical isolates (Cohen-Daniel et al. 2009). Deep sequencing and phylogenetic analysis of H3N2 isolates repeatedly sampled from a chronically infected immunosuppressed child who was intermittently treated with ZA and oseltamivir, revealed a complex interplay with multiple evolutionary processes in parallel in several viral subpopulations, demonstrating multiple driving forces for both selection and diversity (Rogers et al. 2015).

Clearly, permissive or compensatory mutations in an IAV are of importance whether a virus can harbor a resistance mutation with retained fitness (Butler et al. 2014, Bloom, Gong, and Baltimore 2010, Duan et al. 2014).

Environmental Pollution with Neuraminidase Inhibitors

Occurrence of Neuraminidase Inhibitors in the Environment

After administration of both oseltamivir and ZA, 75-80% of the active OC and ZA is excreted by urine or feces (LIFproduktresumé 2015b, a). Both components are poorly removed by conventional sewage treatment and therefore end up in aquatic environments (Fick et al. 2007, Jain et al. 2013). OC is the most studied substance, both regarding drug measurements in aquatic environments and regarding experimental studies on degradation and removal of the metabolite from water.

There is a correlation between the amount of oseltamivir that is prescribed to patients and the OC concentrations detected in effluents of sewage treatment plants (STPs) and in river water, with multiples higher drug concentrations at STP effluents (Azuma et al. 2012, Leknes, Sturtzel, and Dye 2012, Singer et al. 2014, Ghosh et al. 2010b, Prasse et al. 2010, Azuma et al. 2015). It is however the presence of active drugs in aquatic environments that can be expected to have an ecologic effect.

In Japan, which accounts for over 70% of the global oseltamivir prescription (Tashiro et al. 2009, Hoffman-La-RocheINC 2007), numerous environmental measurements over the last five to ten years have detected OC in river water in the range of a few hundred ng/L up to 865 ng/L (Söderström et al. 2009, Takanami et al. 2010, Takanami et al. 2012, Ghosh et al. 2010b, a, Azuma et al. 2012, 2013). Studies in a number of European countries have detected OC in river water at average concentrations of approximately 50 ng/L, with a range up to 200 ng/L (Prasse et al. 2010, Leknes, Sturtzel, and Dye 2012, Singer et al. 2013, Singer et al. 2014, Goncalves et al. 2011). Samples from the Rhine river at the border between Germany-France-Switzerland, contained high concentrations of non-metabolized OP relative to OC (OP/OC ratio 13.1 as compared to < 1 in STP effluents) indicating release from drug manufacturing (in Switzerland) aside from sewage discharge (Prasse et al. 2010).

The lower use of ZA, peramivir and laninamivir as compared to OC is reflected in lower levels of active drugs released to aquatic environments. In Japan, drug concentrations of up to 59 ng/L of ZA, 11 ng/L of peramivir and 9 ng/L of laninamivir have been measured in river water, with dynamics that are synchronized with the number of influenza cases during the influenza season (Takanami et al. 2012, Azuma et al. 2013, Azuma et al. 2015).

Removal of Oseltamivir by Sewage Water Treatment

Conventional STPs use different techniques to remove waste products, usually a combination of mechanical treatment followed by chemical and biological (active sludge) treatment (Fick et al. 2007, Ghosh et al. 2010a, Prasse et al. 2010). Measurements of pharmaceuticals from influents and effluents of conventional STPs have demonstrated between 0% (Leknes, Sturtzel, and Dye 2012, Ghosh et al. 2010a) and 59% OC removal by the treatment processes (Prasse et al. 2010). Experimental studies have demonstrated that OC is not removed by conventional sewage treatment (Fick et al. 2007), nor degraded by UV light exposure (Fick et al. 2007, Bartels and von Tumpling 2008), which often is an important degradation mechanism of drug metabolites in the environment (Boreen, Arnold, and McNeill 2003).

In studies investigating other potential degradation methods, bacterial strains that use OC as their sole carbon and energy source (*Nocardioides* sp and *Flavobacterium* sp) were isolated from environmental water sediments,

suggesting biological degradation pathways of OC (Accinelli, Sacca, et al. 2010). Accordingly, in several degradation studies in water, microbial processes were demonstrated to be important for the dissipation of OC from waste water. OC removal can be increased by addition of active microbial sludge (Accinelli, Caracciolo, and Grenni 2007, Slater et al. 2011), active sediments from natural waters (Sacca et al. 2009, Accinelli, Sacca, et al. 2010) and by fungal (*Phanerochaete chrysosporium)* exposure (Accinelli, Saccà, et al. 2010). The results of biodegradation studies have led to suggestions for bioremediation approaches in sewage treatment (Sacca et al. 2009, Accinelli, Sacca, et al. 2010), though their efficiency may be questioned if the OC load would be very high, i.e. in a pandemic situation (Slater et al. 2011).

Adding ozone treatment to the conventional methods of waste water treatment has proven very efficient in removing OC and other NAIs experimentally (Mestankova et al. 2012, Azuma et al. 2015). Confirming the experimental data, drug measurements from STP influents and effluents at units that use ozone treatment in addition to conventional techniques have repeatedly demonstrated significantly lower concentrations of released drugs as compared to conventional STPs. Over 85% of all NAIs are removed by adding a tertiary sewage treatment with ozone (Figure 9) (Ghosh et al. 2010a, Azuma et al. 2013, Azuma et al. 2015, Ghosh et al. 2010b).
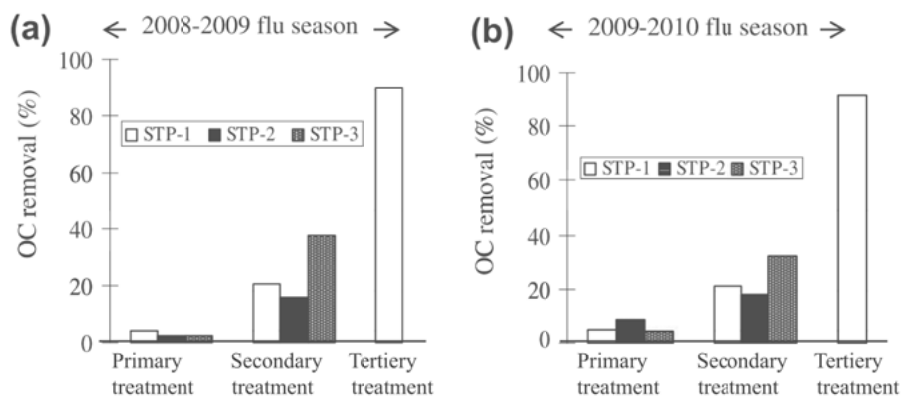
Figure 9. OC measurements from sewage influents and effluents of three sewage treatment plants (STP-1,2,3) during two influenza seasons in Japan. STP-1 had an additional tertiary ozone treatment. (Reprinted from Chemosphere (Ghosh et al. 2010a) Copyright (2010), with permission from Elsevier)

Effects of Oseltamivir in Aquatic Environments

Environmental risk assessment of NAIs in aquatic systems includes evaluation of eco-toxicological effects and of direct antiviral effects on naturally circulating IAVs, including the potential for resistance development.

It is unknown whether there are any eco-toxicological effects by OC and prediction studies by mathematical modelling come to varying conclusions regarding toxic effects on algae and fish (Chen, Lin, and Liao 2014, Straub 2009, Singer et al. 2008, Singer et al. 2007).

Resistance screening of avian IAVs carried by wild waterfowl has been limited, as discussed above. *In vivo* experimental studies by our group have demonstrated resistance development in an avian H1N1 virus, conferred by the H274Y substitution in NA, when infected Mallards were exposed to 0.95µg/L of OC in their water (Järhult et al. 2011). Achenbach et al demonstrated *in vivo* selection for the E119V substitution in NA in an avian H5N2 virus when infected Mallards were exposed to 1µg/L of OC in water. The resistant H5N2/E119V variant dominated the viral population and was transmissible between Mallards, but it was outcompeted by wild type virus when drug exposure was removed (Achenbach and Bowen 2013).

Hypothesis

Wild waterfowl is the natural reservoir of IAVs and may be exposed to OC in aquatic environments as a result of environmental pollution. If birds that host IAVs in their intestinal tract are exposed to OC, the viruses may become resistant to OC. If an avian IAV can maintain fitness despite a resistance mutation, the resistant virus may circulate among wild birds without drug pressure. If a resistance trait in a wild bird avian IAV can persist through an evolutionary process to a novel human pathogenic IAV, the novel virus may be inherently resistant to OC already when it is introduced to humans.

Aims

General Aim

The aim of the studies was to test the hypothesis on the development of OC resistance in avian IAVs of different subtypes when natural host birds were exposed to OC in their water, and if acquired resistance may persist without drug pressure. With the intention to approximate an environmental situation, the hypothesis was tested experimentally in an *in vivo* Mallard model.

Specific Aims

I. In a previous *in vivo* Mallard experiment OC resistance was induced in an avian H1N1 virus when infected birds were exposed to OC in their water (Järhult et al. 2011). As group N1 and group N2 NAs are structurally different and susceptibility to NAIs and OC resistance mutations differ between the groups (Russell et al. 2006), we wanted to test the hypothesis on development of OC resistance also on a virus with a group N2 NA protein. In the same Mallard model, ducks were therefore infected with an avian H6N2 virus and exposed to OC in their water, followed by genotypic and phenotypic resistance analysis of virus from fecal samples.

II. N9 is part of the phylogenetic group N2 and differs slightly from the N2 protein (Baker et al. 1987). In March 2013, there was an outbreak in China with severe human infections caused by a multiple reassortant avian H7N9 virus (Jernigan and Cox 2015). To test if an avian IAV with the H7N9 genotype may acquire OC resistance in the natural wild bird host, we exposed Mallards infected with an LPAI H7N9 virus (phylogenetically distant to the Chinese strain) to OC in their water and analyzed excreted virus for resistance related genotypic and phenotypic changes.

III. If a resistant wild bird avian IAV has emerged during OC exposure in the environment, the resistance can pose a problem to humans only if the resistance trait can be retained and circulate among wild birds without drug pressure; it may then potentially evolve to a resistant human-pathogenic virus. In this study we tested if the OC-resistant avian H1N1/H274Y virus of

a previous exposure study (Järhult et al. 2011) could retain the resistance when it was allowed to replicate in Mallards while OC exposure was gradually removed.

IV. In study (I), the avian H6N2 virus acquired the NA resistance substitution R292K when infected Mallards were exposed to OC. In addition to the R292K substitution, variants that also contained either a D113N or D141N substitution in NA and E216K in HA evolved during the experiments. For the same reasons as in study (III), we tested if resistance in this N2 virus could persist in infected Mallards without drug pressure, and if the amino acid substitutions additional to R292K influenced viral fitness and persistence of resistance.

Materials and Methods

Overview

The general methodology was similar in all four studies included in this thesis. Experiments were designed to allow continuous replication of LPAI viruses in Mallard hosts while they were exposed to varying levels of OC in their water. Viral evolution was investigated by sampling and analysis of virus from feces of the ducks. Viral properties were primarily examined regarding genotypic and phenotypic resistance to OC and ZA, and regarding replication and transmission in and between Mallards. Additionally, changes of genotype at culture in chicken eggs, detection of virus in intestinal tissue and evaluation of the NA activity contributed to the estimations of viral fitness. The investigated viruses and the detected amino acid substitutions were put in context by comparing the sequences to those of influenza sequence databases.

Mallards

Mallards *(Anas platyrhynchos)* are normally hatched during springtime; therefore experiments were performed during the autumn when the ducks were between ten weeks and six months old. Mallard ducks were purchased from commercial breeders at two to three months of age for study (I) and (III), and - in order to minimize the risk for previous IAV infection - newly hatched, one day old, for study (II) and (IV). All Mallards were male and were housed in isolation indoors at the animal house facilities of the National Veterinary Institute in Uppsala (Figures 10A, 10B), after ethical approvals (Ethics Committee on Animal Experiments in Uppsala, permit C201/11 and C63/13) and according to the legislation and the recommendations by the Swedish Agricultural Board. Animal welfare and procedures during housing and experiments were optimized together with the responsible veterinarian to minimize the suffering of the birds. Mallards were tested for previous IAV infection by blood serology (FlockCheck Avian influenza virus antibody test, IDEXX) and prior to inclusion in the experiments for present infection by PCR detection of virus in feces (described below).

Drugs

Oseltamivir carboxylate (OC) used in the experimental water of the Mallards and for susceptibility testing of viral samples, and deuterium labelled OC used as an internal standard in the concentration analysis method were obtained from the manufacturer F Hoffman la Roche Ltd, Basel, Switzerland. Zanamivir (ZA) used for susceptibility testing of viral samples was purchased locally as Relenza®. Both compounds were dissolved in double distilled water and were stored as stock solutions at minus 20°C.

Viral Isolation, Propagation, Typing and Quantification

All viral isolates used in the experiments were originally collected from wild Mallards during autumn migration at the Ottenby bird observatory in Southeast Sweden (Latorre-Margalef et al. 2009).

There are several methods for isolation, propagation and titration of IAVs. Avian IAVs are preferentially isolated in specific pathogen free (SPF) embryonated chicken eggs (ECEs). Cell cultures is an alternative, and although mammalian cell lines (i.e. Madin Darby canine kidney (MDCK) cells) have often been used, chicken embryo fibroblast lines are usually better for culture of avian viruses (Spackman and Killian 2014, Lombardo et al. 2012).

The primary method for detection of IAV in cell culture supernatants or in allantoic fluid of ECEs is by a HA hemagglutination assay with chicken erythrocytes, which is a sensitive method if IAV is present at a concentration above 10^5 50% egg infectious dose (EID_{50}) (Reed and Muench 1938, Killian 2014). To determine the HA subtype, and to rule out other reasons for hemagglutination, the hemagglutination assay can be followed by a hemagglutination inhibition (HI) assay, in which a panel of HA subtype specific antisera is added that can inhibit the hemagglutination reaction (Pedersen 2014).

The samples collected from wild Mallard ducks and used in our studies were isolated by inoculation of the chorioallantoic sac of 10 or 11 days old SPF ECEs according to protocols from WHO (Cox 2005). Confirmation of IAV growth was done by a hemagglutination assay, and HA typing with an HI assay. NA typing was done by PCR amplification and sequencing, using universal primers for conserved non-coding NA regions (Hoffmann et al. 2001). Experimental samples subjected to NAI susceptibility testing (Study I, II, III, IV), were also propagated in SPF ECEs, as high viral titers are required for the functional NA inhibition assay.

Determination of Evolutionary Relationships

In study (II) with an LPAI H7N9 virus, the phylogenetic relationships of NA and HA were determined in context of Eurasian H7, N9 and North American sequences, downloaded from the Influenza Research Database (http://www.fludb.org/), by building Bayesian trees (Huelsenbeck and Rannala 2004, Larget 2008). Trees were built using resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX).

Mallard Model

Experiments were designed to allow continuous viral replication in the gastrointestinal tract of the Mallards by successive introduction of influenza-naïve ducks to those already infected. Viral transmission between dabbling ducks is primarily fecal-oral (Webster et al. 1992). The replication and fecal shedding peak after two days and the infection begins to be cleared after approximately five to seven days (Bröjer et al. 2013). Each experiment started with inoculation of two Mallards by injection of the relevant LPAI viral stock solution in the esophagus, where after they were put in an isolation room with a single water pool (170 L) for swimming and drinking and with feed *ad libitum* (Figures 10C, 10D). OC aliquots were each day added to the water pool to obtain the intended drug concentration. In order for viral replication to continue with the latest evolved variant, generations (two Mallards) of uninfected ducks were introduced after three days and housed together with the infected ones during two days to allow transmission, where after the previous generation was euthanized (with intravenous injection of 100 mg/kg sodium pentobarbital [Pentobarbital vet. 100 mg/mL]). The following day a new generation of two uninfected birds was introduced in the experimental room, and so on, with successive generations of birds each staying 5 days in the experiment. The number of generations varied between the studies depending on the study design (Table 2).

   Daily fecal samples were collected from each bird by putting them in clean cardboard boxes for five to twenty minutes where after swabs were taken from feces left in the boxes. On a few occasions when no feces were left, cloacal swabbing was done to obtain a sample. Samples were immediately frozen in influenza transportation medium (Fouchier et al. 2005) in minus 80°C, until RNA extraction or viral isolation. Water samples were as well taken daily for viral analysis. A number of birds were post mortem necropsied, including tissue collection for subsequent histopathology and immunohistochemistry staining.

Figure 10. **A, B**: Male Mallards were raised in isolation indoors at the Swedish Veterinary Institute, according to legislation and ethical approval. **C**: Experiments started by inoculation with an avian IAV by esophagus injection of two Mallards, thereafter placed in the experimental room. **D**: Virus was allowed to replicate and transmit by successive introduction of uninfected birds. The available water was each day spiked with OC to desired concentrations.

Table 2. *Experimental designs in the Mallard Model.*

Day	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
0	■									
1	■									
2	■									
3	■	■								
4	■	■								
5	■	■								
6		■	■							
7		■	■							
8		■	■							
9			■	■						
10			■	■						
11			■	■						
12				■	■					
13				■	■					
14				■	■					
15					■	■				
16					■	■				
17					■	■				
18						■	■			
19						■	■			
20						■	■			
21							■	■		
22							■	■		
23							■	■		
24								■	■	
25								■	■	
26								■	■	
27									■	■
28									■	■
29									■	■
30										■
31										■
32										■

Day = experimental day. G1= generation one consisting of two Mallards, etc.
Study (I), indicated in brown, included three experiments, each with four generations of H6N2 infected Mallards exposed to 0.12µg/L, 1.2µg/L or 12µg/L of OC.
Study (II), indicated in pink, included seven generations of H7N9 infected Mallards exposed to 2.5µg/L of OC during nine days, 7.4µg/L during nine days and 24µg/L during six days.
Study (III), indicated in grey, included 10 generations of H1N1/H274Y infected Mallards exposed to stepwise decreasing OC concentrations every new generation from 79µg/L to 0.008µg/L at day 26, and without OC day 27 to 32 (G10).
Study (IV), indicated by bold borders, consisted of three identical experiments, each with five generations of Mallards infected with three different H6N2/R292K variants exposed to stepwise decreasing OC concentrations from 10µg/L to 1µg/L at day 8, and without OC during the last 8 days (G4 and G5).

Nucleic Acid Amplification Methods

The polymerase chain reaction (PCR) method, by which a specific part of a DNA chain can be amplified biochemically, was developed during the 1980s, and is the major method for amplification of any nucleic acid (and rendered Mullis and Smith a Nobel Prize) (Bartlett and Stirling 2003). In the present studies, we used a real-time reverse transcriptase PCR (RRT-PCR) method for detection of virus in fecal samples, and a reverse transcriptase PCR (RT-PCR) method to obtain amplicons for sequencing of the NA and HA genes.

The first important principle step of any PCR method is the extraction of the nucleic acid template from its biologic context; suitable extraction methods vary depending on several factors including the material, the concentration of template and the nucleic acid. (Bartlett 2003).

The contents needed for PCR reactions are: the nucleic acid template, primers, a DNA polymerase enzyme, deoxynucleoside triphosphates (dNTPs), and magnesium containing buffer solution.

Primer oligonucleotides of approximately 20-30 bases are designed to be complementary to the templates' both strands; ideally with a melting temperature of 50 to 65 °C and with a low tendency to bind as hairpins or primer dimers. A thermostable DNA polymerase, usually the highly efficient *Taq* enzyme (originally from the thermophilic *Thermus aquaticus* bacterium of hot springs), extends the primers from their 3' end along the template strand at high temperatures, usually at 72°C. High fidelity polymerase enzymes, with 10-fold less errors than the *Taq* enzyme (which have 1 to 2×10^{-6} errors per nucleotide per duplication), may be used to obtain a lower error frequency of the amplicons (Grunenwald 2003).

Thermal cycling of the contents allows hybridization of primers to the template (annealing), elongation by the polymerase synthesis (extension), and denaturation of double stranded DNA (melting, usually at > 90°C) to be repeated as long as the enzyme is active, usually during 30 – 40 cycles. The result of the reaction is an exponential amplification of the template sequence. Optimization of the primers, the cycling-temperatures, and the concentration of each component, can improve the PCR results.

As only DNA is stable enough to be amplified by PCR, original RNA extracts need to be reverse transcribed to complementary DNA (cDNA) by a reverse transcriptase enzyme before the amplification process. The reverse transcription can be done in a separate reaction or included in the PCR reaction (one-step RT-PCR) (Spackman 2014).

Extraction of RNA

Efficient extraction and purification of RNA prior to PCR amplification and sequencing is crucial for sensitivity and specificity. RNA extraction from

allantoic fluid following ECE culture is usually easier than RNA extraction from clinical samples as the viral concentration is high and the content of the sample is well defined. Processing of fecal samples are typically difficult as the samples also contain RNA from numerous other microorganisms aside from the host, as well as RNAses that degrade RNA once it is extracted from cells, and PCR inhibiting factors. Therefore, RNA extraction from fecal samples require specific methods with reagents that inactivate RNAses and remove or dilute PCR inhibitors (Spackman and Lee 2014).

In our studies, RNA from all samples (fecal, allantoic fluid from ECEs and water) was primarily extracted with automated magnetic particle methods, either with a Magnatrix 8000 extraction robot (Magnetic Biosolutions) using the Vet viral RNA kit (NorDiag ASA), or with a Maxwell® 16 instrument using the Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega Biotech AB). In study (III), when deep targeted sequencing was done of the NA, RNA was extracted from feces with a manual organic method using a protocol with TRIzol® (Life technologies).

Real-time Reverse Transcriptase PCR for Viral Detection in Feces

RRT-PCR methods that detect IAVs independent of subtype are cost-effective and up-scalable and are therefore standard screening methods for IAV surveillance in domestic and wild birds, as well as for IAV detection in human clinical samples.

In sequence specific real-time PCR methods a probe is added to the reaction; the method we used included a hydrolysis dual–labelled TaqMan probe (Holland et al. 1991). The probe oligonucleotide is labelled with a fluorescent reporter dye at the 5' end and a quencher, which in proximity absorbs the reporter's fluorescence, at the 3´end. The probe binds to the DNA template during annealing, and during the primer extension the *Taq*-polymerase degrades the probe by its 5´exonuclease activity whereby the reporter dye is released and its fluorescence signal can be detected by the PCR instrument (Spackman and Suarez 2008). During the log phase of the amplification, the fluorescence signal corresponds to the amplicon concentration, visualized in real-time. Aside the rapidity, real-time PCR is therefore also quantitative, represented by the number of reaction cycles required for detection of the fluorescence signal. The use of one-step real-time RT-PCR assays decrease the labor and the contamination risk as compared to a separate reverse transcriptase reaction (Spackman 2014).

In our studies, we used a previously published and well validated one-step RRT-PCR protocol that targets the highly conserved IAV matrix gene and that detects most avian viruses (Spackman and Suarez 2008). Primers and a probe labelled with FAM (6-carboxyfluorescin) and BlackHoleQuencher-1

dyes were used together with the iScript one-step RT-PCR kit for probes (Bio-Rad) and reactions were run in a Corbett Research Rotor-Gene 2000 Real-time Thermo Cycler (Corbett Research).

Reverse Transcriptase PCR for NA and HA Amplification

In order to sequence the NA gene, RNA from all fecal and water samples that were found IAV positive by RRT-PCR and all ECE isolates were subjected to RT-PCR of the NA gene. In study (I) also the HA gene (H6) was amplified and sequenced to investigate if additional mutations had evolved in HA in parallel with the resistance mutation in NA. Primers were designed to be used both for RT-PCR amplification (one forward and one reverse) and for sequencing (two forward and two reverse) (Table 3). Reverse transcription and cDNA amplification was done with a one-step RT-PCR protocol using the SuperScript^{TM} III One-Step RT-PCR System with the Platinum® *Taq* High Fidelity polymerase (Life Technologies). The protocols were optimized for each study. PCR products were confirmed by gel electrophoresis and purified from primers and unused nucleotides by exonuclease treatment with ExoSAP-IT (Affymetrix Inc.).

Table 3. *Primers for RT-PCR Amplification and Sequencing of NA and HA*

Primer	Sequence (5' to 3')	Application
	NA primers	
N2_FWy	TGAACCCAAATCAGAAGATAATAACA	RT-PCR, sequencing
N2_FWi	GTGTGCATAGCATGGTCCAG	sequencing
N2_REVy (ic)	GCGAAAGCTTATATAGGCATGAA	RT-PCR, sequencing
N2_REVi (ic)	AACCTGAGCGTGAATCCTTG	sequencing
N9_FWy	TCTATGCACTTCAGCCACTGCTA	RT-PCR, sequencing
N9_FWi2	TCACCGCCCACAGTATACAA	sequencing
N9_REVy (ic)	ATCAGGCCAGTTCCATTGTCCTA	RT-PCR, sequencing
N9_REVi (ic)	TACCTGGATAAGGGTCATTACACT	Sequencing
N1_FWy	GCAGGAGTTCAAAATGAATCCAAATC	RT-PCR, sequencing
N1_FWi	CTCATGCTCCCACTTGGAAT	Sequencing
N1_REVy (ic)	TGTTCAAAAAACTCCTTGTTTCTACT	RT-PCR, sequencing
N1_REVi (ic)	GTGTCCTCTAACGGGGCATA	Sequencing
	HA primers	
H6_FWy	ATTGCAATCATTGTAATAGCGATACTGGC	RT-PCR, sequencing
H6_FWi	TGAATGTGGAATCTAATGGAAATCTAATCG	RT-PCR, sequencing
H6_REVi (ic)	AGTCTGGCATGTGGCATCGCAGTTCTCG	RT-PCR, sequencing
H6_REVy (ic)	TGCATTGCATTGAACCATTTGAACACATC	RT-PCR, sequencing

Sequencing Methods

The DNA molecule was described in 1953 by Watson and Crick (Watson and Crick 1953) (later awarded with a Nobel Prize), and during the 1970s DNA sequencing techniques were developed. In 1977, Sanger published the chain terminating sequencing method (also rendering a Nobel Prize), in which labelled dideoxynucleoside triphosphates (ddNTP) terminate DNA chains while they are being synthesized by a DNA polymerase (Sanger, Nicklen, and Coulson 1977). The different size DNA fragments can thereafter be sorted by gel or other capillary electrophoresis methods and each added nucleotide identified by its label. Still being the standard sequencing method, major improvements include thermostable DNA polymerases that enable higher temperatures and cycling of the process, labeling of the ddNTPs with different wavelength fluorescent dyes instead of radiolabeling, and automation of the process (Stirling 2003, Hagemann 2015). The first automated sequencer was released in 1987 by Biosystems and represented the first generation high throughput DNA sequencing system, sequencing 84 kilobases per run (Illumina 2015). In 2005, the first of several "next generation sequencing" (NGS) systems was launched, still sequencing amplified single molecules, followed in 2009 by the first single molecule "third generation" sequencing system (Wierstraete 2012).

Next Generation Sequencing

The main principal difference between first and next generation sequencing is the fragmentation of the template and massive parallel sequencing of short reads instead of sequencing a single DNA fragment; this multiplies the data output to a gigabase per run.

The first step in all NGS methods is the library preparation of the template, in which it is fragmented and adaptor and barcode sequences are added to the ends of the fragments. Second, the library/libraries are amplified by a PCR that includes attachment of the amplicons to a material (emulsion PCR on spheres or bridge amplification on a slide) (Illumina 2015, Wierstraete 2012); this allows identification of the clonally amplified sequences during the parallel sequencing. Identification of the incorporated dNTPs during the sequencing are based on different principles in different platforms; specific fluorescent dyes connected to reversibly terminating nucleotides or probes, pyrophosphate release with a subsequent chemistry emitting light, or, which was the method used in study (III), release of H^+ ions registered by a pH sensor in semiconductor sequencing of Ion Torrent, (Hagemann 2015, Wierstraete 2012).

Sequencing Quality

Sequencing results are quality scored, whereby Q20 indicates the probability of one incorrect base incorporation in 100 and a base call accuracy of 99%;

Q30 indicates one incorrect base in 1000 and an accuracy of 99.9%, etc. (Wierstraete 2012). Sequencing sensitivity, defined as the probability that the assay will detect a sequence variation depends on several factors including read quality, depth and uniformity of the sequencing and the alignment of sequences. Sequencing that includes amplification of a template includes the risk of PCR problems with duplicate fragments or poor quality of the sequence material, which can influence sensitivity and specificity (Hagemann 2015, Gargis, Kalman, and Lubin 2015).

The Ion Torrent Platform sequencing yield is maximized by size selection of template fragments and by enrichment of the emulsion PCR product, so that only beads that contain DNA are selected for sequencing (Wierstraete 2012). In contrast to other platforms, the Ion Torrent chemistry uses natural dNTPs, which reduces sequencing bias compared to unnatural nucleotides; reads are relatively long and reaction times short. A disadvantage compared to Illumina platforms is a higher error rate (Hagemann 2015).

Standard Sequencing of NA and HA

NA (and HA in study I) PCR products from water, fecal samples and ECE isolates were sequenced with automated Sanger sequencing, by Macrogen inc. in South Korea or in the Netherlands. Two forward and two reverse primers were used with the intent to cover most of the gene with two or more high quality sequences. Sequencing results were analyzed with the SeqScape v2.7 (Applied Biosystems) software, comparing NA and HA sequences from experimental samples with the NA and HA sequences of the original viruses (GenBank accession numbers of NA: JX912288 (H6N2), AHZ41932 (H7N9), and JF710317 (H1N1)). To consider a sequence result reliable at least two quality sequences (by looking at the electropherograms) were required at any given nucleotide.

Next Generation Sequencing of NA

In study (III), in which the H1N1/H274Y virus was studied regarding maintained resistance without drug pressure, re-emergence of a small subpopulation of wild type virus could go undetected by standard Sanger sequencing. Therefore, deep targeted sequencing, with the Ion Torrent system, was performed in addition to Sanger sequencing on selected samples from the beginning and from the end of the experiment.

Reverse transcription of RNA, PCR and sequencing was done using the PathAmp^{TM} Flu A Reagents with high fidelity polymerase on the Ion PGM^{TM} System (Life Technologies) (Rusk 2011). Thereby reliable detection of variant sequences composing $\geq 1\%$ was obtained. Sequencing and bioinformatics analysis was done by the National Genomics Infrastructure (NGI)/Uppsala Genome Center.

Phenotypic Resistance Testing

Viral susceptibility to antiviral agents can be determined by measuring viral infection or by biochemical assays that measure the inhibiting effect on the target enzyme (Tisdale 2000).

Among cell culture based methods, plaque reduction assays have previously been the standard for drug susceptibility testing of viral isolates and it is still an important method in the development of new drugs. Advantages of the plaque reduction method are the independence of inoculum size for determination of IC_{50}, and the ability to detect antiviral activity and resistance mutations at sites that are not related to the primary drug target (Tisdale 2000).

Other cell based methods are dye-uptake assays that determine the cytopathic effect by detection of metabolizing cells, and yield reduction assays that quantitate a viral antigen or a reporter gene harvested from the cell culture. These methods can be automated and are specifically suitable to determine susceptibility of viral isolates treated with a combination of drugs (Tisdale 2000).

However, measuring IAV infection and susceptibility to antiviral drugs with cell based assays have substantial limitations. Plaque reduction assays are not useful for routine monitoring or high throughput routine screening as IAV isolates differ in plaque structure and size and the process is slow and cumbersome (Tisdale 2000). Regarding NAI susceptibility testing, cell based assays may be difficult to interpret as a result of the balanced HA-NA activity in the IAV lifecycle, as changes in the HA activity can obscure the drug effect on the NA activity (Tisdale 2000, Zambon and Hayden 2001).

As the NA activity is extracellular, biochemical assays that specifically target the enzyme activity are suitable for testing NAI susceptibility. There are several methods that determine the NA activity with the common principle to quantify the sialic acid that has been released by the enzymatic process. Quantification can be done by labelling sialic acids with radioactive, colorimetric or fluorometric side chains which can be measured, preferentially without prior chromatographic separation (Potier et al. 1979). In 1979, a sodium (4-methylumbelliferyl-α-D-N-neuraminate) (MUNANA) substrate for a fluorometric NA-assay was developed (Potier et al. 1979). The method has been modified (Gubareva, Webster, and Hayden 2002) and is currently the WHO recommended standard method for surveillance of NAI susceptibility of influenza virus isolates, along with genotyping (WHO 2012, Lackenby 2008 , Zambon and Hayden 2001).

Functional NA Inhibition Assay

In all the studies of this thesis, viral susceptibility to OC and ZA was tested in a functional NA inhibition assay with the MUNANA substrate (MU-

NANA; Sigma). In the reaction NA hydrolyses the substrate to N-acetylneuraminic acid and free 4-methylumbelliferone, which can be excited by light at 365nM and emits fluorescence (maximum at 460nM) at high pH. The fluorescence signal is proportional to the NA enzyme activity, and if an NAI that inhibits the enzyme is added to the virus and substrate mix, hydrolysis is decreased and the fluorescence signal is proportionally lowered (Gubareva, Webster, and Hayden 2002). We used a MUNANA protocol by the European network for management of drug resistant viruses Virgil Clinvir (Lackenby 2008). In study (I) and (III) the fluorescence signal was determined in a GloMax® Multi Microplate Multimode Reader (Promega), and in study (II) and (IV) in an Infinite® M1000 PRO (Tecan) microplate reader. IC_{50}s of the drugs were obtained from best fit dose response curves using the Prism5 or Prism6 GraphPad (GraphPad) software.

Histopathology and Immunohistochemistry

To evaluate tissue damage, inflammation and intracellular presence of IAV in infected Mallards, necropsies with macroscopic examination and staining of tissue sections were done on selected birds in study (I) and (II). Hematoxylin and eosin staining for histopathology, and immunohistochemistry (IHC) staining with an anti-influenza A nucleoprotein monoclonal antibody (HB65, EVL, The Netherlands) for intracellular viral detection, were carried out on sections from parenchymal organs and from multiple levels of the gastrointestinal tract.

Quantification of Oseltamivir Carboxylate in Water

Measuring drugs and their metabolites in environmental surface water includes challenges of very low concentrations and polarity of the substances. There are several methods for detection and quantification of pharmaceuticals in biological and environmental materials, but for trace concentrations of polar compounds liquid chromatography for separation coupled to mass spectrometry for detection has been most successful (LC-MS or LC-MS/MS) (Khan et al. 2012). A preceding solid phase extraction (SPE) step for enrichment can overcome some of the difficulties of quantification, and automated coupling of the SPE to an LC-MS system can make the method efficient (Khan et al. 2012, Trenholm, Vanderford, and Snyder 2009). Matrix effects due to co-elution, which result in enhanced or suppressed signals, must be minimized when substances are analyzed in a LC-MS/MS system, and the difficulty of large water sampling volumes must be overcome for a reliable and efficient analytic method (Azuma et al. 2014, Khan et al. 2012).

To verify the OC concentrations in the water of our experiments, and to evaluate the variability within and between experimental days, water samples were taken daily, before and after changing the OC spiked water of the Mallards. The samples were analyzed with an on line SPE/LC-MS/MS method with deuterium labelled OC as an internal standard for quantification (Khan et al. 2012, Järhult et al. 2011).

Screening of the NCBI Influenza Virus Sequence Database

In order to obtain an estimation of their prevalence among wild birds, the Influenza Virus Sequence Database of the National Center for Biotechnology Information (NCBI) (Bao et al. 2008) was screened for the sequence variants that evolved in study (I) (R292K in N2) and study (II) (I222T in N9). As many thousand sequences are collected in the database, the prevalence of specific sequence variants can give indications on the underlying evolutionary driving forces and on the potential of a variant to circulate in the wild. A high prevalence among wild bird avian viruses increases the probability that the substitution can arise spontaneously and that it does not reduce viral fitness. In study (I) the database was accessed on April 9th 2012 and in study (II) on February 15th 2015, and alignments were done with the BioEdit v7.0.5.3 and v7.2.5 software, respectively.

Statistic Testing

In study (II) and (III), hypothesis testing on equal IC_{50} means was done with a two-sample (unpaired) *t*-test, with the generation of p-values and 95% confidence intervals (CI) for difference in means. In study (I) and (IV) the assumption of equal variances was violated, therefore the non-parametric Mann-Whitney U-test was used for hypothesis testing on equal IC_{50} medians and calculations of p-values. 95% CI were calculated for medians of each group. Hypothesis testing was computed with the Statistica® v.12 (StatSoft) software.

Results

OC Resistance Induced in Avian IAVs by Drug Exposure of Infected Mallards

H6N2/R292K (Study I)

Study (I) consisted of three experiments, each including 4 generations of Mallards over 14 days. The Mallards were infected with an H6N2 virus and exposed to 0.12µg/L (0.42nM), 1.2µg/L (4.2nM) or 12µg/L (42nM) of OC in the respective experiments. Exposure to 0.12 or 1.2µg/L of OC did not result in any genotypic or phenotypic changes, i.e. the virus remained wild type.

At day four in the first generation of birds exposed to 12µg/L of OC, a viral subpopulation was detected with the R292K (arginine to lysine) substitution in NA, and from day five throughout the experiment only R292K variants were detected. The R292K substitution generated 13,000 fold and 7.8 fold increases in the IC_{50}s of OC and ZA respectively, as compared to the wild type virus (Table 4).

Necropsies with histopathology and IHC staining were done on two birds per experiment (n=6), whereof one was infected with an R292K variant and five with wild type virus. No macroscopic changes were observed. Epithelial intracellular IAV was detected in one intestinal IHC section of a duck with wild type virus (exposed to 1.2µg/L of OC), and in one section of the duck with R292K variant virus (exposed to 12µg/L of OC). Minor infiltration with inflammatory cells was observed in several intestinal sections of several birds but this did not correlate to the presence of intracellular IAV.

In the 0.12 and 1.2µg/L experiments (with persisting wild type virus), virus was infective, was transmitted between Mallards and was excreted in feces with similar dynamics as seen in previous LPAI experiments in the Mallard model (Figure 11.I) (Bröjer et al. 2013).

At exposure with 12µg/L of OC, all birds were likewise infected and transmitted virus, though viral excretion appeared to be lower in generation one and two, to be restored to "wild type levels" in generation three and four (Figure 11.I).

The functional MUNANA NA assay indicated an up to 70% reduced NA activity of R292K variant virus compared to wild type virus, and when the samples of generation one that contained the R292K variant (one with mixed

wild-type/R292K genotype and two with only R292K genotype) were propagated in ECEs, all reverted to wild type.

However, in later samples of the 12 μg/L experiment (generation three and four), amino acid substitutions additional to R292K were detected in the NA (D113N or D141N) and HA (E216K). Aside restored viral excretion levels, their presence coincided with maintained R292K genotype in the ECE propagation process.

Table 4. *H6N2 (I) and H7N9 (II) Susceptibility to Oseltamivir and Zanamivir*

Virus	IC_{50} OC	IC_{50} ZA
H6N2	nM (SEM)	nM (SEM)
50908 wt	0.26*	0.55*
R292 (n=14)	0.21 (0.043)	0.68 (0.069)
R292K (n=9)	2,900 (53)	5.3 (0.40)
H7N9		
1621 wt	0.45[#]	0.58[#]
I222 (n=3)	0.51 (0.050)	0.50 (0.12)
I222T (n=10)	4.1 (0.25)	1.2 (0.13)

IC_{50}= the drug concentration that inhibits 50% of the NA activity, determined with the functional MUNANA assay on duplicate samples. R292= arginine at NA residue 292. R292K= lysine at NA residue 292. I222= isoleucine at NA residue 222. I222T= threonine at NA residue 222. * Means of 8 repeated assays (SD 0.18 and 0.095 for OC and ZA respectively). [#] Means of two repeated assays (SD 0.048 and 0.010 respectively). (Modified and reprinted from PLoS ONE 8(8): e71230 by the (CC-BY) license and from Antimicrob Agents Chemother 59:5196–5202 by the (CC-BY-NC 3.0) license).
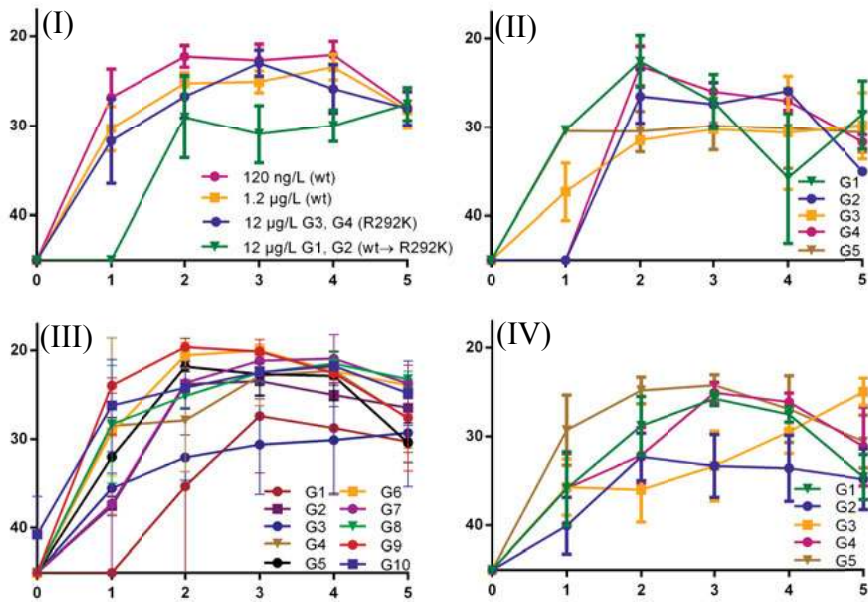
Figure 11. Viral shedding in feces, quantified by RRT-PCR of the matrix gene. X-axis: day in the experiment. Y-axis: Cycle threshold (CT) values. A sample with a CT value ≥45 was determined negative. Error bars display SEM. G1 = generation one consisting of two birds etc. **(I).** H6N2, n=8 at 120 ng/L and 1.2 μg/L OC exposure, n=4 in the two groups of the 12 μg/L experiment. **(II).** H7N9 exposed to increasing OC concentrations. For clarity, only the positive sample was included in the graph if one of the two was negative for a specific day, i.e. G1 day 1, G2 day 5, G3 day 2, G4 day 3, G5 day 1. **(III).** H1N1/H274Y exposed to decreasing concentrations of OC, G10 was unexposed. **(VI).** H6N2/R292K exposed to decreasing concentrations of OC. G1= two birds in each of three experiments (n=6), etc. G1 and G2 were OC exposed, G3 was OC exposed day 0-2, and G4 and G5 were entirely unexposed. (Modified and reprinted from PLoS ONE 8(8): e71230, PLoS One 10(9):e0139415 by the (CC-BY) license; Antimicrob Agents Chemother 59:5196–5202 by the (CC-BY-NC 3.0) license; and with permission from Applied and environmental microbiology 2015. 81:2378-2383, Copyright © American Society for Microbiology).

H7N9/I222T (Study II)

In study (II), Mallards were infected with an H7N9 virus that was allowed to replicate in seven generations of ducks over 23 days while the OC exposure was stepwise increased. The LPAI H7N9 virus was found to have H7 and N9 genes that were phylogenetically related to European wild waterfowl viruses and only distantly to any poultry virus (Figure 12).

Viral infection and transmission was successful in five generations of Mallards over 17 days, while OC exposure was 2.5μg/L (8.8nM) and

7.4μg/L (26nM, from day 9), but was not transmitted further to generation six and seven when the OC exposure was increased to 24μg/L (84nM, from day 18) (Table 5).

At day two in generation one the NA substitution I222T (isoleucine to threonine) was detected in a viral subpopulation, and from day five the I222T variant dominated all subsequently sequenced samples as long as virus was excreted (Table 5). The IC_{50}s of the I222T variant were 8.0 fold increased for OC and 2.4 fold increased for ZA (Table 4).

The I222T variant was excreted in feces to similar levels as wild type virus of other subtypes in previous experiments in the Mallard model (Figure 11.II) (Bröjer et al. 2013) and the I222T substitution was retained in all samples that were propagated in ECEs.
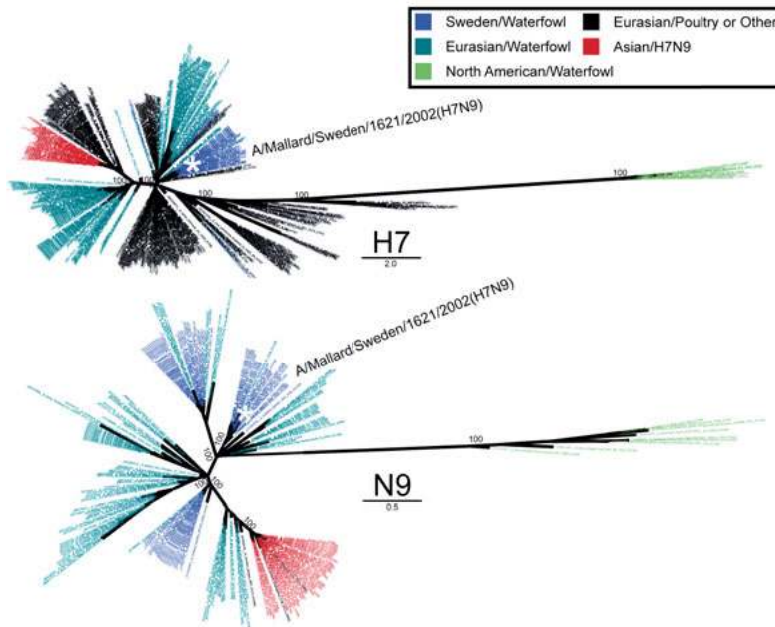

Figure 12. HA and NA phylogenetic trees of Eurasian H7 and N9 virus segments by Bayesian inference. * indicates phylogenetic placement of the H7N9 virus of study (II). Scale bar represents number of substitutions per site. Posterior probabilities of major clades are included at corresponding nodes. (Reprinted from Antimicrob Agents Chemother 59:5196–5202, by the (CC-BY-NC 3.0) license).

Table 5. *Mallard Model study (II) with H7N9 Detection and NA 222 residues*

Day	OC conc. in water µg/L (RSD)	Presence of Mallard generations and detection of IAV							
		G1	G2	G3	G4	G5	G6	G7	W
0	2.5 (11%)								
1									
2		I T*							
3		I							
4		T*							
5		T	T						
6									
7			T T						
8									
9	7.2 (11%)								
10				T					
11					T T				
12					T				
13					T T	T			
14									
15						T			
16						T			
17						T			
18	24 (6%)								
19									
20									
21									
22									
23									

G1= generation one consisting of two Mallards etc. W= water. RSD= relative standard deviation. Dashed lines indicate change of the OC concentration. Rectangles indicate presence of Mallards in the experimental room. Shading indicates detected IAV with RRT-PCR of fecal samples. Amino acid residues of NA at site 222: I=isoleucine, T=threonine, T*=threonine as mixed genotype with isoleucine. (Modified and reprinted from Antimicrob Agents Chemother 59:5196–5202, by the (CC-BY-NC 3.0) license).

Oseltamivir Resistant IAVs in Mallards Without Drug Exposure

Maintained Resistance in H1N1/H274Y (Paper III)

In paper (III), Mallards were infected with a resistant H1N1/H274Y virus that was allowed to replicate in 10 generations of birds over 32 days, while OC exposure was reduced by a factor $10^{0,5}$ each generation, beginning at 79µg/L to be entirely removed at day 27.

All birds were readily infected and transmitted virus. With the exception of generation one, in which viral excretion was detected one day later, excretion patterns in feces were similar throughout the experiment, independent of decreasing drug exposure (Figure 11.III). The OC resistant genotype and

phenotype persisted through the entire experiment with no detectable reemergence of wild type variants. The deep targeted sequencing that was done on selected samples in addition to the Sanger sequencing had a reliable detection sensitivity of 1% for variant sequences at a single base, and thus the sequencing results over the NA codon for amino acid 274 had at least 99% sensitivity (Table 6).

The OC-susceptibility of the H274Y variant, with a 340 fold increase in IC_{50} compared to wild type virus, did not change over the experiment and was similar to that observed in previous experiments with the virus (Järhult et al. 2011). As expected, there was no reduced susceptibility to ZA (Figure 13).

Table 6. *Study (III): Targeted deep sequencing of the codon for amino acid residue 274 of NA*

Viral sample	No. of reads at base 820 of the NA-gene				Consensus codon for NA amino acid 274
	A	C	G	T	
A/51833(H1N1)/wt	0	550	0	3	CAC
A/51833(H1N1)/H274Y	3	4	1	742	TAC
g 1:1, 4 dpi	2	2	0	283	TAC
g 2:1, 6 dpi	2	1	0	202	TAC
g 2:2, 6 dpi	5	3	3	423	TAC
g 10:1, 30 dpi	4	1	1	654	TAC
g 10:2, 30 dpi	3	0	3	863	TAC
g 10:1, 31 dpi	1	1	0	606	TAC

A/51833(H1N1)/wt = original wild type H1N1 isolate. A/51833(H1N1)/H274Y= OC resistant isolate used for inoculation in study (III). Labeling: "g 2:1, 6 dpi" = fecal sample from generation 2, Mallard number 1, taken 6 days post inoculation. A= adenosine. C= cytosine. G=guanine. T= thymidine. (Copyright © American Society for Microbiology, reprinted with permission from Applied and environmental microbiology 2015. 81:2378-2383).
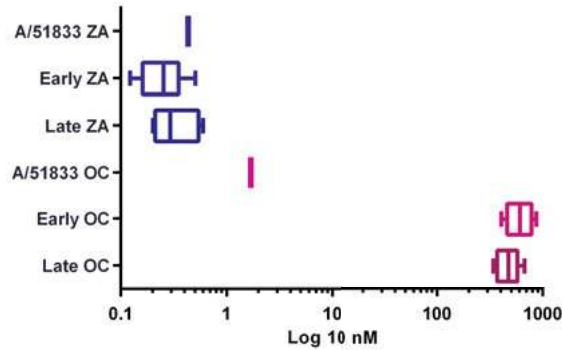
Figure 13. Study (III): H1N1/H274Y susceptibility to OC and ZA tested in a functional NA inhibition assay**.** X-axis: Log IC_{50}. Y-axis: the tested viruses: A/51833/wt= original H1N1 wild type isolate. Early ZA and Early OC= experimental samples with H1N1/H274Y virus from Mallard generation 1 and 2 (OC exposed) tested for ZA and OC susceptibility. Late ZA and Late OC = experimental samples with H1N1/H274Y virus from Mallard generation 10 (unexposed to drugs) tested for ZA and OC susceptibility. Experimental values are means of 8 duplicate samples. Boxes indicate 95% CI of the mean, whiskers indicate minimum and maximum values. IC_{50}s of the original isolate are means of 6 repeated assays (with SD 0.56 and 0.24 for OC and ZA values resp.). (Copyright © American Society for Microbiology, reprinted with permission from Applied and environmental microbiology 2015. 81:2378-2383).

Loss of Resistance in H6N2/R292K (Study IV)

Study (IV) consisted of three identical experiments with three OC resistant H6N2/R292K variants (R292K, R292K+D113N and R292K+D141N) that were allowed to replicate in five generations of birds over 17 days while the OC exposure was stepwise reduced and removed.

All birds in all three experiments were infected and transmitted virus throughout the experiments, with similar levels of viral excretion independent of route of infection, drug exposure and genotype (Figure 11.IV). The resistant R292K genotype reverted to wild type in all birds within three days after removal of drug exposure (Table 7). Variants with the additional NA substitutions D113N or D141N were equally outcompeted by wild type virus, indicating that these did not have a significant compensatory effect for reduced fitness. The drug susceptibility of the R292K variants were similar as in study (I), with 15,000 fold and 10 fold higher IC_{50}s of OC and ZA respectively, as compared to the wild type H6N2 virus.

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Table 7. *Study (IV): Genotype at the NA residue 292*

Day	OC conc. in water µg/L (RSD)	G1			G2			G3			G4			G5		
		E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3
0	10 (4-7%)															
1																
2	3 (9-11%)			K												
3				K												
4																
5	1 (3-10%)															
6																
7					K	K	K									
8							K	K	K							
9	0									K						
10								R		R						
11								R	R	K		k				
12												R	R			
13											R	R			R	
14											R	R	R	R		R
15														R		R
16														R	R	
17																R

G1= generation one consisting of two Mallards in each experiment, etc. E1 = experiment with the NA substitutions R292K + D113N. E2 = experiment with the NA substitutions R292K + D141N. E3 = experiment with the single NA substitution R292K. N.b. that all generations of each experiment consisted of two birds, here displayed in combination for graphic simplicity. Red displays lysine (K), blue displays arginine (R), and orange displays a mixed proportion of R and K at NA residue 292, as determined by Sanger sequencing of fecal samples. (Modified and reprinted from PLoS One. 10(9):e0139415 by the (CC-BY) license).

Oseltamivir Carboxylate in Experimental Water

The SPE/LC-MS/MS analytical method for quantification of OC in the experimental water was sensitive and stable with limits of quantification between 1 and 2.5 ng/L and a linearity of calibration curves (R^2) of 0.9999 to 1.0000.

In all studies, OC concentrations in the water of the Mallards were stable within and between experimental days. The variations in the results were within the general variation of chemical analysis of pharmaceuticals. The relative standard deviations at each concentration level varied from 0 to 25% in study I and from 0 to 15% in study (II), (III) and (IV). In study (I) the differences in concentration between water samples collected immediately and 24 hours after OC was added (and had exposed the Mallards) were up to +/- 30% , while in studies (II), (III) and (IV) the concentration differences were between 4 and 8 % higher at 24 hours.

Table 8. *Summary of Results Study (I), (II), (III), (IV)*

	Avian influenza A virus		
	H6N2 (I, IV)	**H7N9** (II)	**H1N1/H274Y** (III)
OC exposure of infected Mallards (μg/L)	12	2.5	0.95 *
NA substitution	R292K	I222T	H274Y
IC_{50} OC (fold change)	13,000	8.0	340
IC_{50} ZA (fold change)	7.8	2.4	none
OC resistant variants replicate in Mallards without drug pressure	Reversion to wild type	n.d.	H274Y persistence

* The resistant variant had been selected from a wild type H1N1 virus in a previous study (Jarhult et al. 2011)

Discussion

OC Exposure Generates Resistant Avian IAVs in Mallards

Acquisition of NA Substitutions by OC Exposure

In the present *in vivo* Mallard studies, the propensity for OC resistance development in different avian IAV subtypes have been tested in the most common natural IAV host species (Munster et al. 2007, Olsen et al. 2006). Previous results from the same Mallard model concluded that an avian H1N1 virus (with group N1 NA) acquired OC resistance by the amino acid substitution H274Y in NA, when infected Mallards were exposed to 0.95µg/L of OC in their water (Järhult et al. 2011).

In study (I) and (II), OC exposed Mallards were infected with viral subtypes containing NAs belonging to the structurally and functionally different NA group N2 (Fields, Knipe, and Howley 2007, Russel, Gamblin, and Skehel 2013). In study (I), arginine (R) at the functional NA residue 292 was substituted by lysine (K) in an H6N2 virus after four days of exposure to 12µg/L of OC. In study (II), European wild waterfowl H7N9 virus acquired the isoleucine (I) to threonine (T) substitution of the framework NA residue 222 after two days of 2.5µg/L OC exposure.

In both studies, the variant viruses dominated the viral populations entirely once they had emerged, and were transmitted to subsequent Mallards as long as virus was transmitted; i.e. throughout the experiment with the H6N2/R292K virus and until the highest drug exposure level (24µg/L) with the H7N9/I222T virus. We could thus conclude that avian IAVs of different subtypes, with phylogenetically different NAs can acquire resistance substitutions if their natural hosts are exposed to OC in their water.

OC Concentrations Selecting for Resistance

The drug concentrations at which resistant variants outcompeted wild type viruses varied between the studies, reasonably related to the inhibitory effect of the drug and to the fitness cost for the mutation in the subtype and in the specific virus. An additional reason for the concentration differences relates to limitations of the Mallard model; as specific drug exposure levels were

chosen (for resource and ethical reasons) exact titrations of the resistance inducing drug concentrations were not possible.

In study (I), neither 0.12 nor 1.2μg/L of OC exposure induced any NA substitutions, though 12μg/L did. In study (II), there was no un-exposed control group or lower exposure level than 2.5μg/L and therefore the lowest selective OC concentration for I222T in H7N9 is unknown.

In lack of an unexposed control group, it can be argued that the I222T substitution may have occurred by chance. The probability that the I222T substitution was not a result of drug-induced selection is however low as there is no known circulation of avian N9 viruses with the T222 genotype (the variant is absent in the NCBI Influenza Virus Sequence Database). In addition, drug selection of I222T is supported by previous observations of I222R and I222K variant viruses selected by oseltamivir treatment of an H7N9 infected patient (Marjuki et al. 2015), and by *in vivo* selection of I222T in H3N2 virus by OC exposure of infected mice (Pizzorno et al. 2014). Furthermore, OC selection of I222T is supported by the fact that in the experiment, the I222T variant gave rise to a phenotype with decreased sensitivity to the drug that generated the selective pressure.

Reduced Drug Susceptibility by R292K and I222T

NAI resistance conferred by the R292K substitution is well described in human H3N2 viruses (Samson et al. 2013, McKimm-Breschkin 2012) and in avian H7N9 and H10N8 subtypes causing human disease (Qi et al. 2014, Hu et al. 2013). The I222T substitution is described in H3N2, influenza B and in H5N1 isolates (McKimm-Breschkin 2012, Pizzorno et al. 2014), but it is the first time that it is described in an H7N9 virus. The levels of reduced NAI susceptibility observed in the functional NA inhibition assay were in parity with levels described in other IAV subtypes carrying the same substitutions; R292K conferred highly reduced sensitivity to OC and < 10 fold increase in the IC_{50} of ZA, while I222T resulted in < 10 fold increased IC_{50}s of both inhibitors (McKimm-Breschkin et al. 2013, Dapat et al. 2013, Sheu et al. 2008, Hatakeyama et al. 2007, Burnham et al. 2015, Burnham et al. 2014, Kiso et al. 2004, Zurcher et al. 2006, Pizzorno et al. 2014).

The I222T substitution is of concern not only for reducing drug susceptibility by itself, but when combined with other more potent NAI resistance substitutions it can potentiate the phenotypic resistance, as demonstrated in an H1N1/pdm09 virus with dual H274Y+ I222T substitutions (Huang et al. 2014). Resistance potentiating effects have also been demonstrated for I222M/V+H274Y and I222M/V/T+H252Y in H5N1 (Hurt, Holien, and Barr 2009, McKimm-Breschkin 2012), for I222R/V+H274Y in H1N1/pdm09 (LeGoff et al. 2012, Pizzorno et al. 2012), and for I222V+E119V in H3N2 virus (Baz et al. 2006).

Viral Fitness of OC Resistant Variants

Both the mutated variants H6N2/R292K in study (I) and H7N9/I222T in study (II) performed equally well as their wild type counterparts regarding infectivity and transmissibility in Mallards (Figures 11.I, 11.II), and both dominated the viral populations after introduction, indicating that viral fitness was not substantially reduced.

During propagation of mutated variants in ECEs, the I222T NA-genotype persisted unchanged in the H7N9 virus, contributing to the indications of maintained fitness. In other subtypes it has been observed that several substitutions at the I222 site can compensate for reduced fitness caused by other NAI resistance substitutions in the same virus (Hurt, Holien, and Barr 2009, Huang et al. 2014, LeGoff et al. 2012, Pizzorno et al. 2012, Simon et al. 2011). Substitutions of residue I222 of NA thus stand out as having permissive and modulating effects on NAI resistance and on viral fitness.

The genotypic effects by ECE culture of resistant H6N2/R292K variants in study (I) were more complex. Virus from the first Mallard generation, in which R292K was the only substitution, reverted to wild type during the egg propagation, while isolates from generation three and four, containing additional NA (D113N or D141N) and HA (E216K) substitutions, maintained the genotype.

The reversion to wild type genotype during ECE propagation, together with a reduced NA activity of R292K variants in the NA inhibition assay, indicates reduced viral fitness of the mutant. But as the R292K genotype was maintained in the isolates with the additional NA/HA substitutions, it was hypothesized that these may have a compensatory effect for reduced fitness. On the other hand, a high prevalence of N113 and N141 variants in N2 subtypes in the Influenza Sequence Database suggests that their presence may be unrelated to drug pressure or viral fitness. Possibly late experimental samples simply contained smaller wild type populations that could not compete during the ECE propagation.

As the R292 residue is one of the conserved amino acids in the catalytic triad of the NA enzymatic site (Russel, Gamblin, and Skehel 2013), it is not surprising that its substitution leads to reduced enzyme activity. Rather, it is remarkable that no significant reduction in infectivity or transmissibility was noted for the resistant H6N2/R292K variant in Mallards.

Potential for Maintained OC Resistance Without Drug Pressure

H1N1/H274Y

In study (III), it was demonstrated by genotypic and phenotypic methods that OC resistant avian H1N1/H274Y virus maintained resistance without reemergence of wild type subpopulations when OC exposure was withdrawn from infected Mallards.

In order to increase the sensitivity in the detection of a possible reemerging wild type subpopulation that may have gone undetected by Sanger sequencing, the NA sequencing was complemented by massive parallel sequencing. The NGS results could exclude a wild type population larger than 1% and thus strengthen the results of retained H274Y genotype. Maintenance of resistance without drug pressure suggests that the H1N1/H274Y variant has a viral fitness in parity with that of wild type virus; the prerequisite for potential circulation among wild birds.

The study design is thought to have given sufficient evolutionary space for competition with emerging wild type virus. Definite exclusion of a minor difference in viral fitness in favor of wild type virus in this experiment (study III) is however difficult. The within host IAV evolutionary dynamics model of (Russell et al. 2012), accounts the errors (10^{-5} per site per replication) made by the RNA polymerase as the source of mutations. An exponential expansion of the IAV population during the first two days of infection would mean 10^{10} infected cells that each gives rise to 10^{4} new virions until the population reaches a plateau at 10^{14}. In such a model, reasonably as applicable to gastrointestinal duck infection as to airway infection in poultry, a single mutation variant would constitute over 10^{10} virions after two days, corresponding to 1/10,000 to 1/100,000 of the entire viral population. That is without selective pressure or fitness differences included in the model. Applying the calculations on this study (III); an increase in the wild type population size a 100 to a 1000 times over the random may thus have gone undetected.

However, aside replication without OC exposure during the last 5 days of the experiment, the H274Y variant replicated and transmitted at OC concentrations below the selecting concentration of 0.95µg/L (Järhult et al. 2011) during 17 days in six generations of Mallards, which is reasonably a sufficient time and replication span for a wild type population with superior fitness to be detected.

Of note is that the H1N1 virus used in these experiments inherently had asparagine (N) at NA residue 222, instead of the more common isoleucine (I). The influence of N222 on the fitness of this virus has not been investigated, but it can be speculated that it may have a permissive role for the acquisition and maintenance of the H274Y substitution, given the compensato-

ry nature of several I222 substitutions as seen by others (Hurt, Holien, and Barr 2009, Huang et al. 2014, LeGoff et al. 2012, Pizzorno et al. 2012, Simon et al. 2011).

H6N2/R292K

In study (IV), with the resistant H6N2/R292K virus that was selected in study (I), the resistant variants were promptly outcompeted by wild type virus when OC exposure was removed from the Mallards. Variants with additional substitutions in NA (D113N or D141N) and HA (E216K) aside R292K were equally outcompeted. Although a minor fitness compensatory effect by the additional substitutions cannot be excluded, the probability that they would have a significant biologic effect is very low. It can be concluded that the OC resistant H6N2/R292K variants have a reduced viral fitness and cannot compete with wild type virus in the absence of a selective drug pressure, and could thus not circulate among wild birds.

Selection for the resistance substitution R292K in NA was observed *in vitro* already during the development phase of oseltamivir, both in human and avian group N2 viruses (Tai et al. 1998, Gubareva et al. 1997), and it is a well described substitution in human H3N2 isolates following oseltamivir treatment (Cohen-Daniel et al. 2009, Kiso et al. 2004, Samson et al. 2013). Oseltamivir treatment of human H7N9/2013 infections in China is also associated with the selection of resistant R292K variants (Hu et al. 2013). *In vivo* fitness studies in mice and ferrets with R292K resistant H3N2 and H4N2 variants have demonstrated reduced infectivity and transmissibility (Gubareva et al. 1997, Carr et al. 2002, Herlocher et al. 2002). These observations are in accordance with the absence of circulating NAI resistant human H3N2 variants (Samson et al. 2013). They are however contradicted by the maintained fitness of an OC resistant human isolate with an H7N9/2013/R292K variant that was comparable to wild type virus regarding replication in human respiratory cells, virulence in mice and transmission between guinea pigs (Hai et al. 2013). Over all, the R292K substitution in NA appears to generate viruses with reduced viral fitness compared to wild type virus, but the picture is not completely concordant and is probably influenced by subtype, exact genetic context and possibly by host species specificity.

Influence by Resistance Permissive Mutations

In contrast to what has thus far been observed in human H3N2 viruses, resistant human H1N1 variants can circulate without drug pressure. The seasonal H1N1/2007-2009 virus was OC resistant as a result of an H274Y substitution (Moscona 2009), and since 2011 OC resistant clusters of H1N1/2009pdm/H274Y continue to circulate (Hurt et al. 2011, Butler et al.

2014). Permissive mutations that compensate for reduced viral fitness have been identified for these and other H274Y resistant strains, which demonstrates the dependence on the genetic context in which a resistance mutation is acquired (Butler et al. 2014, Bloom, Gong, and Baltimore 2010, Duan et al. 2014, Takashita et al. 2015, Abed et al. 2011).

The results of study (III) and (IV) with two avian viruses with NAs of different phylogenetic groups, combined with the observations of currently circulating resistant human IAVs, makes it tempting to suggest that OC resistant IAVs with group N2 NAs may have less potential to circulate in the wild than OC resistant viruses with group N1 NAs.

On the other hand, the retained fitness of the OC resistant H7N9/2013/R292K virus in China (Hai et al. 2013), supports that also group N2 viruses may harbor resistance mutations with retained fitness.

Clearly, aside different propensity for various avian IAV subtypes to acquire OC resistance mutations, the genetic context in which a resistance mutation is induced is paramount for the potential of persistence without drug pressure and for the potential to circulate in wild birds.

Consequences of Environmental Pollution with OC

Can OC in the Environment Select for Resistant Avian IAVs?

The OC concentrations that experimentally have selected for resistance related NA substitutions in avian IAVs in Mallards are 0.95µg/L for H274Y in H1N1 (Järhult et al. 2011), 1µg/L for E119V in H5N2 (Achenbach and Bowen 2013), 2.5µg/L for I222T in H7N9 (study II) and 12µg/L for R292K in H6N2 virus (study I). In the H1N1 virus, 0.08µg/L did not select for resistant variants, nor did 0.12 or 1.2µg/L in H6N2. The H7N9 virus was not exposed to lower than 2.5µg/L of OC and thus the lowest selective concentration is unknown.

In the environment, numerous measurements of OC in the main river systems in Japan have confirmed concentrations up to 0.86µg/L (Takanami et al. 2010), while European studies at several river sites have detected OC in the range of 0.02 to 0.2µg/L (Singer et al. 2014, Prasse et al. 2010, Goncalves et al. 2011). The drug concentrations at which avian IAVs experimentally developed resistance in Mallards were thus above the environmental ones detected to date. However, they are in the same magnitude, and as the OC levels in river water vary with oseltamivir consumption (Azuma et al. 2012, Singer et al. 2013) and with the quality of sewage treatment (Ghosh et al. 2010b), higher environmental concentrations may occasionally occur. Although the exposure of aquatic birds may be lower at STP effluents, it should be noted that OC concentrations there are multiples higher than in

river water (Azuma et al. 2012, Ghosh et al. 2010b, Leknes, Sturtzel, and Dye 2012).

As IAVs acquire resistance mutations at various drug concentrations, predictions on the amount of pharmaceuticals in the environment that constitute a risk for resistance induction are uncertain. The high genetic variability of avian IAVs (Dugan et al. 2008) provides chance for genetic contexts to be permissive if a selective pressure for resistance occurs. From our exposure studies we conclude that the risk for evolution of resistant avian IAVs in the environment better not be neglected.

The Probability of an Avian Origin Resistant New Human Influenza A Virus

Thus far, all known human pathogenic IAVs have aroused as the result of reassortment of avian IAVs; either with human or swine viruses, as the last century's pandemic strains; or with multiple avian viruses, as the currently transmitting H7N9/2013 and HPAI H5N1 viruses. Therefore, also future novel human pathogenic IAVs are expected to have avian origins (Jernigan and Cox 2015, Cox and Subbarao 2000).

Although the selective pressure for antiviral resistance is much higher on human viruses, and there are no indications that resistance mutations per se make wild waterfowl IAVs better adapted to poultry or to mammals, the concern of an inherently resistant novel human IAV to arise in the wild is reasonable. That is because of the much greater number of wild bird avian viruses with a much higher variability (Dugan et al. 2008) as compared to the few and more homogenous human adapted strains. Therefore the probability of a permissive resistance-accommodating genetic context is higher in wild bird avian IAVs than in human viruses, if resistance would be selected for.

The environments surrounding live bird markets are considered to constitute risk settings for the evolution of new human pathogenic IAVs (Jernigan and Cox 2015). It may be speculated that the close contacts between different wild and domestic bird species and humans in a condensed urban setting, given NAI treatment of human influenza cases, may also facilitate the selection of resistant avian IAVs.

Risk Reducing Interventions

Prudent use of NAIs

Preventive measures to reduce the environmental pollution with active NAIs are mainly less usage and less release from STPs.

NAIs used in clinical settings for severely ill patients can save lives and are indeed indicated for severe cases of seasonal and pandemic influenza and for the few cases of severe avian (primarily H5 and H7 subtypes) influenza (Nguyen-Van-Tam, Openshaw, and Nicholson 2014, Muthuri et al. 2014). In the case of a new pandemic, widespread prophylaxis may also become indicated to maintain vital functions in the society (Monto and Webster 2013, Patel and Gorman 2009). On the other hand, there is little to gain from NAI treatment of the large majority of all influenza cases: otherwise healthy individuals with seasonal influenza, in which only a minor reduction in time to alleviation of symptoms can be obtained (Jefferson et al. 2014). As the usage of NAIs is reflected in the amounts of active drugs released to river water (Azuma et al. 2012), there are very good reasons to use the drugs only when a clinical effect is truly to expect.

Upgraded Waste Water Treatment

Improved waste water treatment for removal of active pharmaceuticals is a priority (not only for NAIs), and several technologies based on separation or oxidation can, in addition to conventional sewage treatment, increase removal of pharmaceuticals from water. Ozone treatment methods, which can reduce the amount of NAIs that leave STPs with over 85% (Azuma et al. 2015, Azuma et al. 2013, Ghosh et al. 2010b, a), has reached furthest in implementation and can in principle be applied for waste water treatment either at the source, like hospital or industry sewage outlets, or as an additional method in STPs. However, thus far ozone treatment of sewage water has only been done to a limited extent (Gottschalk, Libra, and Saupe 2010, Mestankova et al. 2012, Dahl 2015, Karlsson-Ottosson 2015, Ghosh et al. 2010a). Challenges for broad implementation of ozone treatment of waste water include the technically complex process, very high energy requirements and a possible accumulation of toxic non-organic by-products like bromate (Gottschalk, Libra, and Saupe 2010).

Resistance Surveillance in Avian IAVs

Although oseltamivir is stockpiled for pandemic preparedness and a new pandemic virus is expected to have avian origins, surveillance of avian IAVs has included drug susceptibility testing only to a very limited extent. Especially if compared to the extensive resistance screening of human isolates. Despite absence of highly resistant IAVs in wild waterfowl according to a few screening studies from North America and Europe (Stoner et al. 2010, Stoner et al. 2012, Orozovic et al. 2014), extended resistance surveillance of IAVs in wild and domestic birds is warranted as that is the only way to detect emerging resistance in the avian IAV pool.

Revising Pandemic Preparedness Plans

Stockpiling of anti-influenza drugs for a future pandemic rely much on oseltamivir, which is reasonable given an OC susceptible virus is expected (Patel and Gorman 2009). Awareness of the possibility of a new human virus being OC resistant and knowledge on the circulation of OC resistant human H1N1 variants may however be reasons to consider stockpiling also other drugs, primarily ZA but possibly also other new antivirals.

Conclusions

Avian IAVs of different subtypes, with NAs of both the N1 and N2 phylogenetic groups, can become resistant to oseltamivir when infected Mallards are exposed to low levels of OC in their water (Study I, II). What OC concentration that selects for resistance and which resistance substitution that evolves depend on the viral subtype and on the exact genetic make-up of a specific virus.

Some avian IAVs that have become OC resistant by drug exposure of the host bird can retain the resistance also without drug pressure, while others cannot (Study III and IV). Though only one N1 and one N2 virus have been tested for maintained OC resistance without drug pressure in the present *in vivo* model, the results, in combination with human IAV resistance epidemiology (Samson et al. 2013, Hurt et al. 2012), suggest that group N1 viruses may harbor resistance substitutions more easily than group N2 viruses. It is however clear that the main determining factor for the maintenance of NAI resistance without drug pressure is a permissive genetic context (Duan et al. 2014, Butler et al. 2014, Bloom, Gong, and Baltimore 2010).

As a result of drug exposure in clinical settings, the selective pressure for OC resistance is greater on human IAVs than on avian IAVs, but the probability of a genetic context that will be permissive for a resistance mutation is greater in a wild waterfowl virus (Dugan et al. 2008). As OC is released to river water (Takanami et al. 2010), IAVs of wild waterfowl may be exposed to resistance selective OC in the environment. Study III and IV confirm that the propensity for maintained OC resistance vary, but if a resistant variant can circulate among wild birds, we hypothesize that the resistance substitution may be carried through an evolutionary process with reassortment events and species crossing adaptations, to a new human pathogenic virus. An OC resistant novel human IAV would pose a substantial public health problem, and if the virus would have pandemic potential preparedness plans that rely on oseltamivir would not be useful.

In conclusion, environmental pollution with active OC may generate resistant avian IAVs of wild birds. This may become a problem to humans, should the resistance trait become part of a new human pathogenic virus.

Preventive measures include prudent use of NAIs and high quality sewage treatment.

Future Perspectives

In a broad human perspective, resistance to IAV needs to be addressed at multiple levels. The *in vivo* Mallard model used in the present studies has been developed to imitate an environmental situation of a natural IAV host. The hypothesis testing on resistance induction by OC exposure and persistence of resistance without drug pressure can be extended to the suggested subsequent evolutionary steps towards a human pathogenic resistant IAV. The model can as well be used to evaluate resistance dynamics of other drugs potentially polluting the environment.

Studies in the Mallard model are under way that address:

- Resistance development to second line or alternative NAIs. ZA and peramivir are not used to the same extent as oseltamivir and therefore environmental concentrations are lower than those of OC (Azuma et al. 2013, Azuma et al. 2015). Increased usage and release to the environment may however lead to drug exposure of wild waterfowl. Therefore, it is of interest to test the propensity for ZA and peramivir resistance development in wild bird avian IAVs in the natural host already before the use is widespread, to be able to adjust usage and pandemic preparedness accordingly.
- The capacity of the H1N1/H274Y variant (study III) to compete with wild type virus. Simultaneous infection of Mallards with the resistant virus and its original wild type counterpart may better conclude whether the resistant H1N1/H274Y variant may truly have the potential to circulate in the wild.
- The propensity for double resistance to both OC and ZA. Allowing the H1N1/H274Y variant (study III) to replicate in Mallards under ZA exposure can reveal if the H274Y substitution remains and if additional resistance substitutions can emerge with maintained viral fitness.
- The potential of the resistant H1N1/H274Y variant (study III) to be part in reassortment events. Co-infection of Mallards with the resistant H1N1/H274Y variant and another avian IAV aims to answer the question whether the NA gene can maintain the resistance mutation when it is transferred to another genetic context by reassortment with another virus.

- The potential of the resistant H1N1/H274Y variant (study III) to transmit to chickens. Allowing transmission from infected Mallards to chickens will give an indication on its potential for direct species crossing. Further potential can be evaluated by allowing reassortment between chicken adapted virus and the H1N1/H274Y variant by co-infection in Mallards and chickens.

If some of the new drug candidates directed against new viral targets will prove safe and efficient, treatment options for severe influenza will be improved (Webster and Govorkova 2014, Ison and Hay 2013). What role the new drugs will take in the treatment and prevention of influenza and in pandemic preparedness planning aside NAIs, remains to be seen. The development of new influenza drugs needs to be complemented by studies on their ecologic and environmental impact, including their potential to generate resistance in IAVs of wild birds. For this purpose the present Mallard model may be useful.

Only limited screening for drug resistance in IAVs of wild birds has been done to date, especially in areas where there is a high usage of NAIs. Adding resistance screening to IAV surveillance programs is a priority. As the mechanisms for resistance are complex and not entirely known, both genotypic and phenotypic resistance testing is required.

There are methods that substantially reduce the release of NAIs and other active drug metabolites to the environment, especially ozonation is highly efficient (Ghosh et al. 2010a, Azuma et al. 2015). Development of the methods and their implementation in sewage treatment need to be prioritized.

The ultimate disarmament of the threat of a severe influenza pandemic is a universal influenza vaccine that generates long-lasting immunity to all IAV subtypes in humans and in other species; its development remains an overall top research priority.

Sammanfattning på svenska

Tamiflu® i vattnet: Resistensdynamik hos influensa A virus i gräsänder exponerade för oseltamivir

Influensa A virus (IAV) förekommer hos många djurarter men den naturliga värden är vattenlevande fåglar, i första hand simänder och måsfåglar. En stor och mycket variabel pool av aviära IAV upprätthålls genom ständig cirkulation av virus mellan fåglar i form av asymptomatiska tarminfektioner. Den virala spridningen och variabiliteten följer mönster som influeras av värdfåglarnas ekologi, immunsystem och migration.

Alla kända humana IAV har sitt genetiska ursprung i aviära virus och har uppkommit genom genetisk blandning (reassortment) mellan virus från fågel och människa eller gris. När ett helt nytt humant influensavirus introduceras bland människor ger det i regel upphov till en snabb och omfattande spridning, ofta med svår luftvägssjukdom och hög dödlighet, så kallad pandemisk influensa. Det senaste århundradets influensapandemier är "Spanska sjukan" 1918-1919 (H1N1), "Asiaten" 1957 (H2N2), "Hongkong" 1968 (H3N2) och "Svininfluensan" 2009 (H1N1). När ett pandemiskt virus fortsätter att cirkulera i befolkningen ger det efterhand upphov till en mildare sjukdomsbild i form av säsongsinfluensa, vilket främst är ett resultat av ökande immunitet i befolkningen.

I sällsynta fall kan aviära IAV med högpatogena egenskaper smitta direkt till människor och ge upphov till svår influensasjukdom ("fågelinfluensa") med hög dödlighet. För närvarande ger aviära H5N1 och H7N9 virus upphov till sporadiska humana fall med upp till 30-60% dödlighet, men hittills har inga aviära virus kunnat spridas mellan människor.

Eftersom nya humanpatogena IAV uppkommer ur aviära virus är de evolutionära processerna hos aviära IAV av intresse också ur humanmedicinskt perspektiv.

De bästa tillgängliga läkemedlen för att behandla svår influensa är neuraminidashämmare och bland dessa är oseltamivir (Tamiflu®) och zanamivir (Relenza®) registrerade globalt. Resistens mot neuraminidashämmare är väl dokumenterat både laborativt och vid klinisk användning av läkemedlen och är förenat med sämre klinisk prognos. I de flesta länder där man har pandemiberedskapsplaner förlitar man sig till stor del på det oralt tillgängliga preparatet oseltamivir, vilket finns lagrat i stora beredskapslager. Om ett nytt

pandemiskt IAV vore resistent mot oseltamivir skulle dagens beredskap vara otillräcklig och man skulle stå inför ett besvärande folkhälsoproblem.

Resistenstestning av IAV från vilda fåglar har endast gjorts i mycket begränsad omfattning, men de studier som finns har hittills inte upptäckt någon höggradig antiviral resistens. Det finns dock förutsättningar för att simänder, och virus i deras tarm, skulle kunna utsättas för oseltamivir i miljön eftersom den aktiva metaboliten oseltamivirkarboxylat (OC) är mycket stabil och inte elimineras i vattenreningsverk. OC läcker därför ut i vattendrag där simänder har sin naturliga hemvist. Upprepade studier i Japan och i Europa har detekterat OC i flodvatten nedströms reningsverk; i Japan i koncentrationer upp till 0,86μg/L, i Europa i genomsnitt i en tiondel så höga halter.

Hypotesen bakom studierna i denna avhandling är att IAV i tarmen på simänder kan bli resistenta om deras värdfåglar exponeras för OC i vattnet. Om ett resistent virus sedan kan behålla resistensen trots att det selektiva läkemedelstrycket upphör skulle resistenta virus kunna cirkulera bland vilda fåglar.

Hypotesen har testats *in vivo* i en gräsandsmodell (*Anas platyrhynchos)* där änder infekterats med olika aviära IAV och utsatts för OC i sitt vatten. Virus som utsöndras med avföringen har analyserats avseende resistens mot OC, både genotypiskt genom sekvensering och fenotypiskt genom en funktionell enzym-inhibitionsmetod.

Resultaten av studierna visade att H6N2 virus (studie I) och H7N9 virus (studie II) förvärvade resistens genom aminosyrasubstitutionerna R292K respektive I222T i neuraminidas-enzymet när infekterade änder exponerades för 12 respektive 2,5μg/L OC i sitt vatten.

Vidare att det resistenta H6N2/R292K viruset snabbt blev utkonkurrerat av vildtypsvirus när läkemedelstrycket upphörde (studie IV). Däremot kunde ett OC resistent aviärt H1N1 virus, som i en tidigare studie förvärvat resistens genom en H274Y substitution, förbli resistent i gräsänder också utan OC exponering (studie III).

De OC koncentrationer som experimentellt selekterat för resistens i gräsänder ligger något över hittills uppmätta miljönivåer. Men om man beaktar att olika virus blir resistenta vid olika OC koncentrationer och att miljöhalter varierar över tid och plats kan man inte bortse från att OC i miljön är en riskfaktor för uppkomst av resistenta aviära IAV.

Sammantaget har vi alltså kunnat konstatera att IAV med neuraminidasenzym tillhörande båda de fylogenetiska grupperna N1 och N2, kan bli resistenta om deras värdfåglar exponeras för OC. Vidare att olika virala subtyper har olika förutsättningar att förbli resistenta när läkemedelstrycket försvinner. Detta hänger samman med vilken övrig genetisk uppsättning som finns i viruset, om det finns andra samtidiga mutationer som kan kompensera för

sänkt viral fitness orsakad av resistensmutationen. Om en resistensmutation skulle selekteras fram i ett vildfågel-IAV som har kompensatoriska egenskaper skulle resistensen potentiellt kunna kvarstå genom en evolutionär process till ett nytt resistent humanpatogent virus.

Förebyggande åtgärder mot resistensutveckling hos IAV i miljön består främst i att minska miljöbelastningen av OC, dels genom rationellt begränsad läkemedelsanvändning och dels genom förbättrad vattenrening. Därtill kan man vid framtida pandemiberedskapsplanering behöva överväga alternativa antivirala läkemedel.

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