Tamiflu® - Use It and Lose It?

JOSEF D. JÄRHULT
Dissertation presented at Uppsala University to be publicly examined in Gustavianum, Auditorium minus, Akademigatan 3, Uppsala. Saturday, December 17, 2011 at 10:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Influenza A viruses cause seasonal and pandemic outbreaks that range from mild infections to the disastrous Spanish Flu. Resistance to neuraminidase inhibitors (NAIs) is a growing problem as these drugs constitute a vital part of treatment strategies and pandemic preparedness plans worldwide. Oseltamivir (Tamiflu®) is the mostly used NAI. Its active metabolite, oseltamivir carboxylate (OC), is excreted from treated patients and degrades poorly in sewage treatment plants and surface water. Thus, OC can enter aquatic environments where the natural influenza reservoir, dabbling ducks, can be exposed to the substance and resistance could develop. If NAI resistance is established in influenza viruses circulating among wild birds, the resistance can form part of a virus re-entering the human population either by reassortment or by direct transmission.

In this thesis, evidence is presented that OC is present in the waterways during a seasonal influenza outbreak in Japan, a country in which oseltamivir is liberally used. Furthermore, when mallards were infected with an influenza A/H1N1 virus and subjected to low, environmental-like concentrations of OC, resistance developed through acquisition of the well-known resistance mutation H274Y. The influenza infection in the mallards was mainly intestinal, had a rapid onset and was progressing in a longitudinal fashion in the intestine. Finally, influenza A viruses isolated from wild mallards in Sweden and containing resistance-related mutations were examined by a neuraminidase inhibition assay. The viruses did not have a decreased sensitivity to NAIs, but had mutations with a resistance-enhancing potential.

Thus, OC is present in the environment and environmental-like concentrations of OC induce resistance in influenza viruses of dabbling ducks. The present resistance situation among wild birds is not well understood but the existence of H274Y among wild birds, though rare, and the spread of the former seasonal A/H1N1 virus containing H274Y among humans indicate that resistance mutations could establish themselves also among wild birds. An oseltamivir-resistant pandemic or a human-adapted highly-pathogenic avian influenza virus are frightening scenarios as oseltamivir is a cornerstone in the defense in those situations. There is a need for further studies, surveillance in wild birds and for a prudent use of antivirals.

Keywords: influenza, oseltamivir, Tamiflu, resistance development, H274Y, environment, pharmaceuticals, mallard, dabbling duck, avian influenza

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To my beloved wife and my three wonderful children
List of Papers

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


IV  Goran Orozovic, Josef D. Järhult, Kanita Orozovic, Conny Tolf, Neus Latorre-Margalef, Johan Lennerstrand, Björn Olsen Osel-tamivir- and Zanamivir-Resistance Related Mutations in Influenza A Viruses Isolated from Wild Mallards in Sweden Studied by a Colorimetric NA Inhibition Assay, manuscript.

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<th>Description</th>
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<tbody>
<tr>
<td>A/H1N1</td>
<td>influenza A virus of subtype H1N1</td>
</tr>
<tr>
<td>CT</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>dpi</td>
<td>day(s) post infection</td>
</tr>
<tr>
<td>ECMO</td>
<td>extracorporeal membrane oxygenation</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>H274Y</td>
<td>mutation at amino acid residue 274 resulting in a substitution from histidine (H) to tyrosine (Y)</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HPAI</td>
<td>highly-pathogenic avian influenza</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% inhibitory concentration</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LPAI</td>
<td>low-pathogenic avian influenza</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby canine kidney</td>
</tr>
<tr>
<td>MUNANA</td>
<td>2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>NA</td>
<td>neuraminidase</td>
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<tr>
<td>NAI</td>
<td>neuraminidase inhibitor</td>
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<td>NAIA</td>
<td>neuraminidase inhibition assay</td>
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<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>OC</td>
<td>oseltamivir carboxylate</td>
</tr>
<tr>
<td>OP</td>
<td>oseltamivir phosphate</td>
</tr>
<tr>
<td>rRT-PCR</td>
<td>real-time reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>SA</td>
<td>sialic acid</td>
</tr>
<tr>
<td>STP</td>
<td>sewage treatment plant</td>
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</table>
Introduction

Influenza Viruses

General Aspects
Influenza viruses are single-stranded RNA viruses that belong to the Orthomyxoviridae family. There are three different types: A, B and C [1]. Influenza B causes seasonal outbreaks while C is virtually non-pathogenic to humans. Neither B nor C cause pandemic outbreaks and in this thesis, only influenza A will be discussed.

The genetic content of influenza A viruses form eight segments that code for a total of 11 proteins. From an immunological point of view, the most important genes are hemagglutinin (HA) and neuraminidase (NA) as they code for surface proteins that are exposed to the immune defense of the host [1]. An important ability of influenza A viruses is reassortment. This means that two or more viruses infecting the same cell can recombine their genetic segments in the manner of “shuffling a deck of cards”, i.e. that segments from different original viruses can combine to form a new virus strain [2]. By reassortment, influenza A viruses achieve genetic shift, rapid changes of viral properties that can give rise to a new strain unrecognized by host immune defense and thus capable of pandemic spread. By point mutations in immunogenic parts of the genome, influenza A viruses also makes much more gradual changes termed genetic drift, these changes allow the viruses to escape some host immunity and to cause seasonal influenza outbreaks. Influenza A viruses are subtyped on the basis of HA and NA. There are currently 16 HA and 9 NA variants described, giving 144 possible distinct subtypes. The NA subtypes can be divided into two distinct phylogenetic groups: the N1-group (subtype N1, N4, N5, N8) and the N2-group (subtype N2, N3, N6, N7, N9) [3] that differ in protein structure and relative sensitivity to antiviral drugs.

Disease in Humans
Influenza A viruses causes seasonal outbreaks, or epidemics, that typically occur during the cold season in temperate countries. These epidemics have virtually zero mortality in the young and healthy. However, in large epidemiological studies of total numbers of deaths influenza epidemics normally
cause excess mortality, mainly in the elderly and those with severe underly-
ing diseases. Clinical symptoms of influenza include fever, malaise, cough and pain in joints and muscles and the onset is often abrupt. The incubation period is 1-3 days and in a healthy person, acute symptoms normally subside in 3-4 days [4].

Pandemics
During the last century, influenza A viruses have caused four pandemic, or worldwide, outbreaks. The rapid, global spread of the pandemics are possible because the pandemic strain has acquired so dramatic changes in surface structures that the human immune defense meets a “new virus” and has very limited use of the memory of previous influenza infections. Thus, the population has no, or very little, immunity and in our present world with frequent travel and tight global contacts, the pandemic can rapidly spread around the globe. The last three pandemics were caused by a reassortment event, but there has been some debate regarding the Spanish Flu, see below.

The 1918 H1N1 “Spanish Flu” Pandemic
In 1918-1920, during the First World War, an influenza pandemic swept four times over the world. The first wave was relatively mild, while the second and third waves were associated with a very high morbidity and mortality, especially in young adults. The fourth wave hit remote areas such as Greenland, Alaska and many of the small island societies in the Southern Pacific Ocean. It is estimated that at least 50 million, perhaps up to 100 million people lost their lives in this pandemic [5]. Probably, some of the mortality was due to secondary, bacterial infections in the airways as demonstrated in a pathological study on people deceased in the pandemic [6]. There has been a controversy regarding the genetic origin of the Spanish Flu, with some authors claiming a direct transmission of an avian virus [7,8]. However, phylogenetic evidence strongly suggests that the 1918 pandemic strain was the result of a reassortment of human, avian and possibly swine viruses [9].

The 1957 H2N2 “Asian Flu” and the 1968 H3N2 “Hong Kong Flu” Pandemics
Both these pandemics were milder than the 1918 pandemic and caused much less mortality. They were the result of a reassortment of human and avian viruses. Little is known about the sequences of swine viruses at that time and thus the involvement of swine viruses in the reassortant cannot be precluded [10].
The 2009 H1N1 “Swine Flu” Pandemic
The recent A/H1N1 pandemic stirred a great concern worldwide. Early reports from Mexico indicated a high morbidity and mortality among young adults. Many countries used oseltamivir (Tamiflu®) for massive treatment campaigns in the early phase and a prompt development of vaccines was initiated. As the pandemic moved on, it became clear that the high mortality rates from the early reports were not seen in other countries. For most people, the symptoms resembled a normal seasonal influenza [11]. There was an overrepresentation of young adults being sick and a few of those developed a rapidly deteriorating viral pneumonia that often required mechanical ventilation and in some cases the use of extracorporeal membrane oxygenation (ECMO). A number of people, some healthy and without predisposing illnesses, died – in Sweden 29 people died from the A/H1N1 virus during the 2009-2010 pandemic [12]. In conclusion, the 2009-2010 pandemic probably caused less excess mortality in the elderly but an increased, though still small, mortality in the young and healthy. The pandemic A/H1N1 virus was a result of a reassortment of three different strains from swine, but all three had genetic elements recently derived from avian viruses [10].

Disease in Birds
Ecology
Natural Hosts
Influenza A virus is a zoonosis, the natural hosts are wetland birds, mostly Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, waders) [1,13]. Dabbling ducks such as the mallard (Anas platyrhyncos), are particularly well suited for the perpetuation of influenza viruses in several aspects. They feed in shallow water which facilitates spread via the fecal – oral route [1,14]. Furthermore, they congregate in flocks, have a large population size, migrate to interact with new individuals and every year new, immunologically naïve juveniles are added to the population. Influenza A viruses are well adapted to aquatic environments; a pioneer study showed that the virus can be infective in non-chlorinated river water for at least 32 days at 4°C [15]. A later study has confirmed the importance of water temperature showing that some viral strains remain infective for well over a year at 4°C but only for days at 37°C [16]. Dabbling ducks are considered the major natural reservoir of influenza A viruses [13].

Low-Pathogenic Avian Influenza (LPAI)
Most viruses circulating among wild birds are low-pathogenic (low-pathogenic avian influenza, LPAI). The prevalence of LPAI viruses among dabbling ducks vary with season with a higher percentage of birds infected during fall migration. One contributing factor to this variation is the high
proportion of non-immune juveniles in the fall. There are also geographic
differences; a study from Sweden found 15% infected mallards during fall
and 4% in the springtime [17], whereas data from North America show a
higher fall prevalence but a lower spring prevalence [1]. All 16 HA and 9
NA variants of influenza A viruses discovered so far have been found in
birds and most of them in dabbling ducks. Exceptions are H13 and H16 that
are predominantly isolated from gulls and terns [18]. Most subtypes of LPAI
viruses have a large geographical spread, possibly through migration. H14
and H15 are exceptions, being mostly isolated in Russia and Australia
[19,20]. Phylogenetically, LPAI viruses studied so far belong to either the
Eurasian or the North American lineage [21]. However, several findings of
viruses with a lineage-mixed genome have been reported [22,23,24] indicat-
ing that the separation is not complete. Hence, the naturally circulating gene
pool of LPAI viruses in wild birds can be considered large and variable in
many aspects.

Highly-Pathogenic Avian Influenza (HPAI)
The definition of highly-pathogenic avian influenza (HPAI) is based on in
vivo pathogenicity tests in chickens and not on HA type. However, the abso-
lute majority of HPAI viruses belong to H5 or H7. There is evidence of
HPAI development in wild birds, e.g. H5N3 virus in terns in South Africa in
1961 [25] but in most cases HPAI development occurs in poultry. A poultry
farm is a gigantic monoculture with up to several hundred thousand birds
and furthermore chickens (Gallus gallus domesticus) have no natural immu-
nity to influenza A viruses. If a suitable LPAI virus (mostly H5 or H7) enters
such an environment, a massive replication and transmission can take place.
In this process, genetic changes that include the introduction of multiple
basic amino acids at the HA0 cleavage site can transform the virus into an
HPAI virus. As it no longer requires trypsin-like enzymes to cleave HA0, the
HPAI virus is able to infect other tissues than the airways and the gastroin-
testinal canal [26]. The virus can then spread among poultry e.g. through
bird transportation or live bird markets.

Currently, we are experiencing an outbreak of HPAI H5N1 that originates
from a virus initially isolated in China [27] and that has spread to Asia and
Europe. To stop the spread of HPAI H5N1 a “stamping out” strategy has
been used – in this process, several hundred millions of poultry have proba-
bly been culled worldwide [28]. There is evidence of ongoing HPAI H5N1
activity in many countries today, including Indonesia, Egypt and China [29].
Wild birds have been infected with HPAI H5N1 but the role of wild birds in
the spreading of the virus is controversial and difficult to assess [30].

Clinical Course
LPAI in dabbling ducks has a mild clinical course but smaller “costs” for the
host are difficult to study. In an infection experiment registering physiologi-
cal parameters, only a slight, short-lasting increase in body temperature was seen [31] and in another experiment mallard hens had a transient decrease in egg production [32]. Furthermore, migrating dabbling ducks positive for influenza A had a 20 g lower mean weight than uninfected birds [33] and the migration of LPAI infected swans was delayed [34]. Interestingly, a recent study found mallards in normal body condition more sensitive to LPAI infection when compared to mallards -10% and -20% in body weight [35]. This is contradictory to the hypothesis that birds in worse body condition would be more sensitive to infection.

Pathology
LPAI infection in ducks is thought to be located mainly in the gastrointestinal tract and more specifically in the distal parts [15,36,37,38,39], although a recent study has shown involvement also of the ileum [40]. Most previous studies have not been able to detect inflammatory changes in proximity of demonstrated viral antigen. However, in one study viral antigen was detected near lymphocytes in the caecal mucosa [36] and other authors found a mild inflammation in the ileum [40].

HPAI infection causes high mortality and morbidity in gallinaceous birds. In wild birds, sensitivity to HPAI H5N1 varies among species. Dabbling ducks are normally more resistant to HPAI infection than other wild birds. Probably, the disease is milder if the bird has previously encountered a similar LPAI virus. When causing lethal infection HPAI viruses are widely spread in the body of infected birds [30,41].

Transfer from Birds to Humans
Influenza A viruses can spread from birds to humans in two distinctly different ways. As discussed above, two or more strains from birds, humans and/or other mammals like swine can form a human-adapted virus through reassortment. Another way of transfer is through a direct transmission of an avian virus to humans – also termed de novo introduction. HPAI can be transmitted directly from birds to humans but a very high infectious dose is required and it has mostly been observed in people dealing with dead birds, e.g. after a HPAI outbreak. The mortality in human HPAI H5N1 infection is 59% according to the cumulative number of cases and deaths reported to WHO since 2003 [42]. There have been no unequivocal reports of human to human transmission of HPAI.

Treatment and Prophylaxis of Influenza
There are two different strategies regularly used in the treatment and prophylaxis of influenza A: antiviral drugs and vaccines.
Antiviral drugs

Admantanes

The adamantanes, amantadine and rimantadine, block the M2 protein and thus inhibit viral replication at an early stage [43,44]. However, due to a massive development of resistance both in human and avian strains [45,46] the clinical use of adamantanes has virtually stopped. Another disadvantage of adamantanes is their high rate of adverse events such as nausea, insomnia and hallucinations [47].

Neuraminidase Inhibitors (NAIs)

As the name implies, the neuraminidase inhibitors (NAIs) inhibit the viral enzyme NA. The action of NA can be thought of as a “pair of scissors” – it cuts sialic acid (SA) as opposed to HA which works as a “glue” by adhering to SA. NA is needed for the release of newly formed virions from the infected host cell [48,49] but also for the process of viral entry through the airways of the host by cutting mucoproteins [50]. The fact that NAIs hinder viral release and not viral entry in the cell or replication means that treatment effectiveness is heavily dependent on starting early in the course of the infection. The possibility to decrease viral entry in the airway epithelium through hindering of NA – mucoprotein interaction may contribute to the successfulness of NAI prophylaxis [51]. There are two commercially available NAIs: oseltamivir (Tamiflu®) and zanamivir (Relenza®). Zanamivir cannot be administered orally but is inhaled or in some cases used in an intravenous formulation. More NAIs are in the pipeline of drug production; perimivir is the one closest to introduction on the market.

Vaccines

Vaccines are an effective and safe way to prevent many infections including influenza. An obvious advantage of vaccines is that there is no resistance development as the vaccines trigger the human immune response and do not have a direct antiviral activity. On the other hand, influenza viruses tend to escape vaccine induced immunity – just like immunity from the disease itself – by genetic drift or shift. Therefore influenza vaccines must be tailor-made to match the strains they are to protect against. A trivalent vaccine containing antigens from H1N1, H3N2 and B strains is the standard preventive measure for seasonal influenza [52].

In a pandemic scenario, the rapid mass-production of vaccines is problematic as most production techniques still depend on embryonated hen eggs. According to the Global Action Plan developed by WHO, the goal in a pandemic situation is to have produced 2 billion doses 6 months after a vaccine candidate is available. However, in a WHO study evaluating the 2009 pandemic it was demonstrated that in 6 months, only 534 million doses had
been produced and that it took 5 months from the identification of the pandemic A/H1N1 virus strain until the first vaccines were available. Furthermore, the supply of vaccines to developing countries is especially hard to accomplish; this is problematic as a severe pandemic is expected to hit particularly hard in those countries [53].

Oseltamivir

Pharmacokinetic Aspects
Oseltamivir is administered orally as a prodrug, oseltamivir phosphate (OP) due to the poor bioavailability of the active substance oseltamivir carboxylate (OC). OP is readily absorbed and rapidly converted to OC by esterases, mainly in the liver. More than 75% of an oral dose reaches the circulation as OC. The active metabolite is then excreted from the body in unchanged form predominantly via the urine [54]. OC is likely as poorly absorbed in the intestine of ducks as in the intestine of humans. However, as the LPAI infection in ducks takes place in the intestine, replicating virus and OC co-exist setting the stage for resistance development.

Stockpiling and Pandemic Planning
Oseltamivir has been extensively stockpiled; e.g. the US had 40 million treatment regimens in stock as of April 2009 [55]. Worldwide, more than 220 million treatment courses have been stockpiled, and the shelf life has been extended to 7 years [56]. The development of vaccines is an attractive measure to fight a pandemic. However as discussed above, the mass-production of vaccines is a process of several months, leaving antiviral drugs as the only option in the early phase. Thus, oseltamivir is a cornerstone of pandemic preparedness plans all over the world.

Degradation
OC is stable in the aqueous phase and is not removed or degraded in normal sewage treatment plants (STPs) [57]. Persistence of oseltamivir in surface water ranged from non-detectable degradation to a half-life of 53 days in another study [58]. Thus, there is reason to believe that OC is present in the aquatic environment near STPs when oseltamivir is used extensively. Japan has had the highest per-capita consumption of oseltamivir during several seasonal influenza outbreaks. In one study, it was estimated that more than 10 million treatment courses – corresponding to almost 10% of the population – were used during the 2004-2005 season [59]. The manufacturer Roche
estimated that 6 million people out of 16 million infected with influenza used the drug during the same season [60]. A promising means to reduce OC in the environment is to increase the degradation through ozonization. A study from Japan demonstrated that the addition of ozonization as a tertiary treatment in an STP increased the removal of OC to >90% [61].

Bioremediation

One strategy to lower environmental levels of OC is to improve sewage treatment through bioremeditative measures. When granules with a bioplastic formulation of the fungus *Phanerochaete chrysosporium* was added to wastewater samples, the removal of OC was approximately doubled compared to controls [62]. Furthermore, two bacterial strains growing on OC as the sole carbon source has been isolated from the sediment of Japanese rivers [58].

Resistance to Neuraminidase Inhibitors

General Aspects

Terming NA mutations poses some problems as the position for the respective amino acid varies between the different NAs. In this thesis, the N2-numbering system – i.e. the numbering of the NA sequence compared to the NA of N2 – is consequently used. Thus, in some literature the mutation names do not match; e.g. H274Y can be termed H275Y.

When the NAIs were introduced, the general belief was that resistance development would not be a practical problem for these substances. Resistant viruses were observed after drug pressure assays e.g. in cell lines and in 4% of volunteers in an early study of oseltamivir [63], but the mutants had severely reduced viral fitness. Therefore, it was deduced that resistance development to NAIs interfered too much with the key function of NA to be a problem *in vivo*. However, resistance was observed also in clinical isolates and in some settings it reached considerable levels as in the study by Kiso et al where 14% of Japanese children carried resistant viruses after oseltamivir treatment [64].

There are 19 amino-acid residues that are well conserved among NAs of all subtypes. They are divided into *catalytic* residues involved in the interaction of the substrate and the active site of NA (R118, D151, R152, R224, E276, R292, R371 and Y406) and *framework* residues important for the structure and stabilization of the active site (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294 and E425) [65]. Resistance could potentially arise from a mutation at or near any of those residues and many are previously described [66].
H274Y

The mutation H274Y, conferring resistance to oseltamivir, was the principal reason for the resistance development mentioned above. However, it was rarely seen in clinical practice until the season 2007-2008. That season, H274Y was observed in seasonal H1N1 viruses, first in Norway and then in the rest of Europe [67]. Low percentages of H274Y was reported from the rest of the world 2007-2008, but in the next season 2008-2009, resistant viruses constituted the absolute majority world-wide [68]. There was no correlation to the use of oseltamivir [68,69].

In *in vitro* studies of H274Y, sensitivity to oseltamivir was decreased by 200-750-fold in an H1N1 virus [70,71] but not affected in an H3N2 virus [71]. In clinical isolates, a 100-700-fold decrease of sensitivity to oseltamivir has been reported in H1N1 viruses [72]. As H274Y causes resistance in the studied N1 viruses but not in N2 viruses it is tempting to assume that the resistance is specific to the phylogenetic N1-group of NAs. However, resistance has been shown in an N9 virus (belonging to the N2-group) [73]. N9 viruses are resistant also when carrying the R292K mutation that normally confers resistance in the N2-group, suggesting that the N9 NA shares some properties of both the N1- and N2-group of NAs. H274Y only marginally affects the sensitivity to zanamivir [70,73].

D151N

The role of D151N (as well as D151E/G/V) is unclear. A study on influenza A viruses from before the introduction of NAIs found D151N/G/E/V mutants that in some NA inhibition assays (NAIAs) had a 50% inhibitory concentration (IC_{50}) above the 95% confidence interval for that subtype. However, the increase in IC_{50} was not consistent among different assays (chemiluminescent or fluorescent, see Materials and Methods) or different isolates [74]. In a more recent study, D151N/G mutants enhanced the resistance of H274Y but did not increase IC_{50} by themselves [75]. In this study it was argued that the development or at least accumulation of the D151N/G mutants was due to culturing in Madin Darby canine kidney (MDCK) cells as the mutations mostly were present in mixed populations and were not detectable in the original clinical specimens. The same phenomenon was observed with Q136K and zanamivir resistance; here the Q136K mutant displayed greater viral fitness than the wild-type in MDCK cells but equivalent infectivity and transmissibility in a ferret model [76]. Interestingly, the viruses in the study from before NAI introduction [74] were cultured in MDCK cells before analysis. Thus, if accumulation of D151N occurs in MDCK cells, the pre-analysis culturing might have contributed to the increased IC_{50}s found in this study.
I222V

When examining serial samples from an immunocompromised child with a chronic influenza A/H3N2 infection initially treated with oseltamivir, Baz et al detected several mutations. The first one to appear was the established resistance mutation E119V, followed by I222V. When studied in recombinant NA, I222V alone increased the IC$_{50}$ for OC 2-fold, but 290-fold in combination with E119V (130-fold with only E199V) [77]. After serial passaging in increasing concentrations of OC, Hurt et al found that two H5N1 viruses both acquired the H274Y mutation. In addition, one H5N1 strain acquired I222M and that mutation increased resistance to OC (3-9-fold compared to H274Y alone). I222V was introduced by reverse genetics and also had a synergistic effect with H274Y but to a somewhat lesser extent than I222M. I222V alone increased the IC$_{50}$ for OC 3-fold in an H1N1 virus and 7-fold in an H5N1 virus; when I222V was added to H274 the IC$_{50}$ increased from 1200 to 1500 and from 700 to 1500 respectively [78]. Another mutation at amino acid 222, I222R, was recently found in a pandemic A/H1N1 virus isolated from an immunocompromised patient. The mutation I222R caused resistance to all available NAIs by decreasing the sensitivity to OC 45-fold, zanamivir 10-fold and perimivir 7-fold [79].
Aim

Overall, this thesis aims to assess the following hypothesis (Figure 1):

As OC degrades poorly in STPs and surface water, it can enter aquatic environments where dabbling ducks can be exposed to the substance. Dabbling ducks are the natural influenza reservoir and have a perpetual circulation of influenza A viruses in their population. Thus, there is a risk of resistance development in the intestine of the ducks where replicating virus and OC co-exist. Through reassortment or direct transmission, an oseltamivir-resistant influenza virus could spread to humans.

*Figure 1. Schematic display of the general hypothesis. Illustration by S.J. Järhult.*
Specific Aims

Paper I: To determine if OC is present in the aquatic environment when oseltamivir is used extensively during a seasonal influenza outbreak.

Paper II: To study if an LPAI virus in mallards exposed to low levels of OC develops oseltamivir resistance.

Paper III: To examine the pathobiology and pattern of viral shedding in mallards infected with an LPAI virus.

Paper IV: To functionally investigate LPAI viruses isolated from wild mallards in Sweden.

Figure 2. Illustration of the aim of the papers in this thesis in relation to the general hypothesis.
Materials and Methods

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) is a general method extensively used to detect pharmaceutical substances in environmental samples. The method is previously well described [80]. In this thesis, a LC-MS/MS system was used to measure OC in water in paper I, II and III. The access to deuterium-labeled OC in this work has allowed for the use of an internal standard. The internal standard gives the method high quality control and assurance which is important when analyzing potentially dirty samples with high matrix effects.

Water Sampling for OC

As previously discussed, Japan uses oseltamivir extensively for seasonal influenza. Therefore the Japanese river system Yodo River was chosen for water sampling (paper I). This river system supplies the cities of Kyoto and Osaka with water and receives the outlets of the STPs in the area. Thus, in its course from the mountains to the sea, the river should get successively more OC added from STPs. Surface water was collected in between influenza seasons (June 2007), in the early phase of the influenza season 2007-2008 (December 2007) and at the peak of that season (February 2008).

In paper II, daily samples were taken to measure the OC concentration in the water source in the experiment room. Generally, samples of “old” water (i.e. water that had been in the experiment room for 24 hours) were used for analysis. However, three days in each experiment samples were also taken just after adding OC to the water. The concentrations were similar before and after the 24 hours had passed (data not shown), consistent with the fact that OC is stable in surface water.

Real-time Reverse Transcriptase PCR (rRT-PCR)

RRT-PCR targeting the matrix gene is routinely used for clinical diagnostics and has been extensively evaluated for human clinical samples and found to
have a high specificity and sensitivity [81,82,83]. Furthermore, rRT-PCR has been evaluated more specifically for avian viruses and proved to be a reliable tool [84,85,86,87]. The rRT-PCR assay of Spackman et al based on the matrix gene [88] was used for detection of influenza A viruses throughout the experiments in this thesis.

NA Sequencing
DNA sequencing is a standard technique widely used in modern molecular biology. In this work all the sequencing was performed using traditional Sanger sequencing by the company Macrogen Inc. (Seoul, Korea). In order to generate sequencing templates, six primers were designed covering the NA gene of the influenza A/H1N1 virus used in paper II and III. The complete gene was covered by the six primers with two pairs at the 5’ and 3’ end of the NA gene, and two gap primers (Table 1).

Table 1. NA gene amplification and sequencing primers.

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Sequence (5´- 3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GCAGGAGTTCAAAATGAATCCAAATC</td>
</tr>
<tr>
<td>2</td>
<td>TGTTCAAATAACTCCTTGTTCAGGTCATGC</td>
</tr>
<tr>
<td>3</td>
<td>CCATTGGGTCAATCTGTATGGTGA</td>
</tr>
<tr>
<td>4</td>
<td>GTTGCCCATTTCACCATTGACAGATCT</td>
</tr>
<tr>
<td>5</td>
<td>CTCACTGCTCCACCTTGGAGAT</td>
</tr>
<tr>
<td>6</td>
<td>GTGTCCTCTTAACGCGGCGATA</td>
</tr>
</tbody>
</table>

Isolation
In this work, isolation of influenza viruses was performed in embryonated hen eggs. This method is established as the golden standard and is recommended by WHO as the preferred method to isolate influenza viruses from animal samples [89]. Briefly, sample medium was injected in the allantoic cavity of 10-day-old specific pathogen free embryonated hen eggs. The allantoic fluid was harvested three days later and if the isolate was to be used for inoculation, the viral titer was determined by 50% Embryo Infectious Dose (EID₅₀) [90].

Surveillance at Ottenby Bird Observatory
Ottenby Bird Observatory (56°12′N, 16°24′E) is located on Öland, an island in the Baltic Sea off the south-east coast of Sweden. Here, a surveillance
program for avian influenza A viruses has been conducted since 1999. Birds, mainly dabbling ducks and particularly mallards, were caught in a funnel live-trap by the seaside. Captured birds were identified for species, sex and – when possible – age. Sampling of feces, rRT-PCR and isolation of positive samples in embryonated hen eggs was performed in the same fashion as described in this thesis. Isolates were then subtyped by an HA inhibition assay and by sequencing of the NA gene [17].

Neuraminidase Inhibition Assay (NAIA)

There are three established neuraminidase enzyme activity based inhibition assays available; chemiluminescent, fluorescent and colorimetric. These assays are used to test the sensitivity of influenza viruses to antiviral drugs. It has been shown that the three different assays give comparable results for determination of strain sensitivity or resistance to antiviral drugs. However, the IC_{50}s are not directly comparable and each method has its own advantages [91]. In this thesis, the fluorescent method based on the substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) [92] was used in paper II and a colorimetric method [93] (slightly modified protocol, e.g. reduced sample volume) was used in paper IV.

It is crucial to critically revise a positive result in the NAIA. An important lesson can be learned from the work in paper IV. Here, an H12N3 virus did not show any inhibition with OC or zanamivir even at the highest concentrations tested (10,000 nM). This could be perceived as a highly resistant influenza virus. However, further testing showed that the same NAIA results were present also after a second passage. In the material after the second passage, no influenza A virus could be demonstrated neither by rRT-PCR nor by virus culturing in eggs or MDCK cells. The NA activity drastically decreased when the solution was filtered through a 0.22 µm filter suggesting a bacterial origin of the activity. The sample had a small amount of hemagglutinating activity, but this and the rest of the findings were attributable to streptococci that was cultured from the sample. Thus, bacterial or other contaminations must always be suspected when unexpected results are achieved in the NAIA.

Mallard Model

A key question in the hypothesis behind this work was if influenza A viruses in mallards exposed to low levels of OC can develop resistance to the drug. To test this, a new mallard model was developed with the aim to mimic the serial passing of virus which occurs between ducks in the wild. First, two ducks were artificially infected with virus solution through the esophagus
and then two new ducks were introduced every third day. In this way, most ducks in the experiment were infected by the natural route, i.e. mainly through fecal-oral transmission. Figure 3 summarizes the set-up of the model used in paper II and III. OC was added to the sole water source in the experiment room.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure3.png}
\caption{Set-up of the mallard model. Blue blocks represent set-up in experiments with 80 ng/L and 1 µg/L of OC, red dotted blocks 80 µg/L of OC.}
\end{figure}

Several aspects had to be considered when constructing the mallard model. Firstly, the timing of when to introduce every new duck “generation” had to match the virus shedding pattern of the preceding generation. From an earlier study [31], the shedding maximum of mallard ducks infected with LPAI virus was estimated to occur at 2-3 days post infection (dpi) and thus 3 dpi was chosen for the introduction of a new generation. As shown in Figure 4, this corresponds well with the shedding pattern seen in the actual experiment.
Secondly, it was unknown whether the shedding pattern of the intra-esophageally inoculated ducks (hereafter termed “artificially” infected) would differ from the shedding pattern of the ducks infected via the natural route from a preceding generation (termed “naturally” infected). As earlier data only concerned artificially infected ducks [31], a distinctly different shedding pattern of ducks with a natural infection could mean that the intended chain of infection would be broken. However as seen in Figure 5, the shedding pattern of ducks with artificial and natural infections appeared to be similar.
Figure 5. Viral shedding in the mallard model – comparison of artificial and natural infection. Light grey = artificial infection, dark grey = natural infection. CT-values represent cycle threshold values in rRT-PCR where negative samples are set to CT = 35. Error bars represent ± standard deviation. The number above each error bar denotes the number of ducks in that group (n).

Thirdly, it could be argued that the different levels of OC used in the experiments would affect viral shedding and thus risk the transmission between duck generations in the model. Figure 6 displays shedding patterns in relation to OC concentrations; no difference in shedding patterns can be observed when comparing concentrations of OC from 80 ng/L to 80 µg/L.
Finally, a potential problem with the serial transmission of virus would be infection of newly introduced ducks from remaining virus in the experiment room. This would mean that one or more generations of viral evolution could be by-passed – limiting the value of the many serial transmissions under drug pressure to allow the viral population to acquire relevant mutations. To minimize this problem, the experiment room was thoroughly cleaned before each introduction of new ducks. After the cleaning, most random samples of the experiment room and the water pool showed CT-values around the negative detection limit of CT = 35 with a maximum rRT-PCR result of CT = 28. Considering that ducks in the experiment room at that time shed virus with an average CT-value of around 20 (see Figure 4), the freshly excreted virus from those ducks should quickly outnumber any remaining virus. When comparing the same spot in the experiment room before and after cleaning in two different pairs of samples, a drop in CT-value of 11 was observed, corresponding to a difference in viral amount of more than 1,000-fold. Furthermore, considering that the rRT-PCR targets only a fraction of the matrix gene, small fragments of nucleic acid remaining in the experiment room after cleaning could yield a weakly positive result even if no or very few viable virus particles were present.

*Figure 6. Viral shedding in the mallard model in relation to OC concentration. White = 80 ng/L of OC, light grey = 1 µg/L, dark grey = 80 µg/L. CT-values represent cycle threshold values in rRT-PCR where negative samples are set to CT = 35. Error bars represent ± standard deviation. The number above each error bar denotes the number of ducks in that group (n).*
Necropsies, Histopathology and Immunohistochemistry (IHC)

Necropsies and histopathological examinations (paper III) were performed according to standard routines [94,95]. Tissue samples from brain, lungs, trachea, air sacs, heart, liver, spleen, kidneys, pancreas, adrenal glands, ventriculus, proventriculus, duodenum (section across midpoint of duodenal loop), jejunum (midpoint between end of duodenal loop and Meckel’s diverticulum), ileum (one piece by Meckels diverticulum and one piece about one centimeter prior to the caecal junction), colon (midway between caecal junction and cloaca), caecal junction, and cloacal bursa, were fixed in 10% neutral buffered formalin for histopathology and immunohistochemistry (IHC). IHC was performed using standard techniques [96,97] and an antibody targeting the nucleoprotein (NP). As NP is expressed primarily in replicating virus, this IHC will mostly stain cells where replication takes place.

Sampling of Intestinal Contents for rRT-PCR

To further assess the localization of LPAI infection in the intestine of the duck, several sectional samples of intestinal contents were analyzed by rRT-PCR in paper III. During necropsies, the contents of the ventriculus, duodenum (duodenal loop), jejunum (to Meckels diverticulum), ileum (to the ileocaecal junction), ceacum and colon were carefully extracted. Each portion was mixed and then sampled using a sterile cotton swab in the same fashion as normal fecal/cloacal samples for rRT-PCR. A four-point serial 10-fold dilution of complementary DNA (cDNA, synthesized from RNA using random hexamers) was included in each experiment to allow relative quantification.

A potential drawback of the intestinal contents approach is the risk of contamination in the process of sampling. Given the mixing of relatively large sample volumes a contamination would not largely affect the results, but it is important to keep in mind that the viral load in negative or weakly positive samples could be overestimated. Another aspect that has to be considered when interpreting these results is that virus originating from a proximal part of the intestine also can be present in the more distal parts. Thus, in the situation of high viral load in both proximal and distal parts it is difficult to judge the contribution of distal replication. Furthermore, a possible source of variation is that different amounts of force could be applied at the extractions of intestinal contents. Thus, in some samples more epithelial cells could be present, potentially containing more influenza virus. All efforts were made to standardize the sampling procedure in order to minimize the variation and contamination.
Results

Paper I

OC was detected in river water in Yodo River in Japan during the influenza season 2007-2008 (Figure 7). During the peak of the influenza season (February 2008), 2 ng/L of OC was found in river water collected in the mountains before the river system enters populated areas (R1). In Kyoto, the first city downstream, the OC concentration was measured to 4-19 ng/L (R2-R4). In Osaka, where the river system meets the sea, the river water contained 12 and 58 ng/L of OC (R5 and R6). In the beginning of the influenza season (December 2007), the OC concentration was measured to 2 and 7 ng/L in Kyoto (R2 and R4). No OC was found before the influenza season had started (R1, R2 and R4).

Figure 7. Summary of the results of paper I. The map shows the Yodo River in the Osaka–Kyoto region of Japan. Arrows from the left side of the figure show sampling points during summertime when no OC was detected and arrows on the right show sampling points and results during the peak of the influenza season 2007-2008.
An influenza A/H1N1 virus isolated from a wild mallard in Sweden was employed to infect mallards according to the model described in Materials and Methods. Three experiments were performed using 80 ng/L, 1 µg/L and 80 µg/L of OC. The concentrations of OC were measured to 83 ng/L ± 23%, 0.95 µg/L ± 24% and 81 µg/L ± 11% (mean value and relative standard deviation) in the respective experiment. The NA gene was sequenced from daily fecal samples. In the 80 ng/L experiment, no resistance mutations were detected. In the 1 µg/L experiment, the mutation H274Y co-existed with the wild-type virus in at least two samples (8 and 23 days from the start of the experiment). However, H274Y did not dominate the viral population and the final result of the experiment was a wild-type virus. When the ducks were exposed to 80 µg/L of OC, H274Y quickly outcompeted the wild-type virus. The sequencing results revealed that the mutation occurred 2 dpi, dominated 3 dpi and from 4 dpi and onwards, only the H274Y genotype was found. Figure 8 summarizes the genotypic results.

Figure 8. Genotypic results of experiments with 80 ng/L, 1 µg/L and 80 µg/L of OC. Sequencing results of the NA gene at amino acid residue 274. Blue = cytosine (C), green = adenine (A) and red = thymine (T), corresponding to uracil (U) in the RNA sequence. Wild-type genotype = CAC = histidine, H274Y = TAC = tyrosine.
13 wild-type, 15 H274Y-containing and 2 mixed genotype samples were isolated in embryonated hen eggs and examined by fluorometric NAIA. The IC$_{50}$s for OC of wild-type viruses were measured to 2-4 nM; this includes repeated testing also of the original isolate used to infect the mallards. The IC$_{50}$s of isolates containing H274Y were 400-700 nM. A sample from the 1 µg/L experiment that contained both wild-type and H274Y according to the sequencing results (Figure 8) was isolated two separate times in embryonated hen eggs. One of the isolations resulted in a wild-type virus with an IC$_{50}$ of 3 nM and the other one yielded an H274Y mutant with an IC$_{50}$ of 500 nM, see Figure 9.

The database at the National Center of Biotechnology Information (NCBI) was searched for H274Y mutations in N1 influenza A viruses from birds, see Table 2.

![Figure 9](image.png)

*Figure 9. Results of two different isolations of a sample with a mixed genotype of wild-type and H274Y.*

<table>
<thead>
<tr>
<th></th>
<th>H274Y</th>
<th>Wild-type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1N1</td>
<td>1</td>
<td>208</td>
<td>209</td>
</tr>
<tr>
<td>H5N1</td>
<td>4 (3)*</td>
<td>2132</td>
<td>2136 (2135)</td>
</tr>
<tr>
<td>(other H)N1</td>
<td>0</td>
<td>459</td>
<td>459</td>
</tr>
<tr>
<td>Total</td>
<td>5 (4)</td>
<td>2799</td>
<td>2804 (2803)</td>
</tr>
</tbody>
</table>

*Table 2. Wild-type and H274Y-containing avian N1 influenza A viruses in the NCBI database. * - indicates that the four H274Y-positive H5N1 viruses probably represent three distinctly different viruses.*
Paper III

Mallards infected with an A/H1N1 LPAI virus (see paper II) were examined with histopathology and IHC. RRT-PCR was performed on fecal samples and samples from portions of intestinal content. One artificially infected duck examined 1 dpi had positive IHC and inflammatory changes in the lung, but apart from that no histopathologic changes or IHC positivity was found in any other organ than the gastro-intestinal tract. In the intestine, degenerating cells in the lamina propria, infiltrating heterophils and lymphocytes and positivity of IHC with an NP-antibody was observed. Figure 10 displays a section of jejunum stained by H&E and IHC.

![Figure 10](image.png)

**Figure 10.** A section of the jejunum of a mallard euthanized 2 dpi. A. H&E stain showing apoptosis of mononuclear cells in the lamina propria accompanied by infiltration of heterophils. Inset shows magnification of area with apoptosis that corresponds to viral antigen seen in B. B. IHC stain showing viral antigen in cytoplasm and nuclei of surface epithelium (arrow) and in mononuclear cells (arrow heads) in the lamina propria of the jejunum as well as diffuse granular staining probably corresponding to apoptotic cells. Inset shows magnification of area with viral antigen that corresponds to apoptotic cells seen in A.

Viral antigen was found in 7/8 artificially infected ducks euthanized 1-4 dpi (the remaining duck was the one positive only in the lung), 2/4 artificially infected ducks at 5 dpi, 4/14 naturally infected ducks at 5 dpi and 0/4 ducks at 7 dpi. See Table 2 for a summary of the distribution of positive sections among ducks with viral antigen detectable by IHC.
Table 3. Distribution of positive sections in ducks positive in IHC. Graded in 0, +, ++, +++ by observing IHC-stained sections. dpi = day(s) post infection, M. div. = Meckel’s diverticulum, Prox. = Proximal.

<table>
<thead>
<tr>
<th>dpi</th>
<th>Type of infection</th>
<th>Lung</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>M. div. ileum</th>
<th>Distal ileum</th>
<th>Caecal tonsil</th>
<th>Colon</th>
<th>Cloacal bursa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>artificial</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>artificial</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>artificial</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>artificial</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>artificial</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>++</td>
<td>na</td>
</tr>
<tr>
<td>5</td>
<td>natural</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>natural</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>++</td>
<td>na</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>natural</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>na</td>
</tr>
</tbody>
</table>

Results of the rRT-PCR analysis on fecal samples have been shown in Materials and Methods. RRT-PCR analysis were also performed on portions of intestinal contents of 11 ducks (n = 1 at 1-4 dpi, n = 7 at 5 dpi). The assay was performed with a standard curve consisting of 4 dilution steps of cDNA, allowing a relative quantification. Portions from the ventriculus, duodenum, jejunum, ileum, caecum, colon and feces were examined. To achieve a better overview, the results were congregated into one proximal and one distal part of the intestine (Figure 11). The ducks from 1-2 dpi and 3-4 dpi were grouped together; the results were very similar within the two groups.

Figure 11. Comparison of proximal (ventriculus, duodenum, jejunum, ileum) and distal (caecum, colon, feces) parts of the intestinal contents of ducks 1-5 dpi assessed by rRT-PCR with relative quantification. Proximal parts shown in light grey, distal parts in dark grey. The number below each pie chart denotes the mean total number of copies in the entire gastro-intestinal tract in that group compared to the mean total number in the 5 dpi group. This is not an exact quantification but allows for a comparison of the relative viral load. n = 2 (1+2 dpi, 3+4 dpi), n = 7 (5 dpi).
Paper IV

21 LPAI viruses collected from wild mallards at Ottenby Bird Observatory were examined with colorimetric NAIA. The isolates had the following NA subtypes: N1, N3, N6 and N9. 13 of the isolates had mutations in conserved regions of the NA gene – of these, 11 isolates had mutations previously discussed in the sense of NAI resistance. The remaining 8 isolates did not have any of those mutations. No NAI resistance was found in the NAIA. Table 3 displays the IC$_{50}$ values from this study.

Table 4. IC$_{50}$s from colorimetric NAIA (paper IV). 1) C = catalytic, F = framework, 2) Susceptibility to zanamivir/oseltamivir, S = sensitive, R = resistant, 3) mutation not previously related to NAI resistance.

<table>
<thead>
<tr>
<th>Virus subtype</th>
<th>Mutation</th>
<th>Type of residue</th>
<th>IC$_{50}$ ± SE (nM)</th>
<th>Susceptibility z/o</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ZA</td>
<td>OC</td>
<td></td>
</tr>
<tr>
<td>H1N1 no</td>
<td>-</td>
<td>1.2 ± 0.15</td>
<td>5.4 ± 0.01</td>
<td>S/S</td>
</tr>
<tr>
<td>H1N1 no</td>
<td>-</td>
<td>5.5 ± 0.12</td>
<td>6.4 ± 0.09</td>
<td>S/S</td>
</tr>
<tr>
<td>H1N1 I222V</td>
<td>F</td>
<td>8.5 ± 0.30</td>
<td>6.9 ± 0.15</td>
<td>S/S</td>
</tr>
<tr>
<td>H8N1 D151K 3)</td>
<td>C</td>
<td>8.2 ± 0.12</td>
<td>3.2 ± 0.09</td>
<td>S/S</td>
</tr>
<tr>
<td>H1N1 R118K</td>
<td>C</td>
<td>4.9 ± 0.23</td>
<td>8.4 ± 0.12</td>
<td>S/S</td>
</tr>
<tr>
<td>H1N1 R118K</td>
<td>C</td>
<td>5.1 ± 0.17</td>
<td>11.0 ± 0.30</td>
<td>S/S</td>
</tr>
<tr>
<td>D151N C</td>
<td></td>
<td>4.3 ± 0.06</td>
<td>9.7 ± 0.28</td>
<td>S/S</td>
</tr>
<tr>
<td>H2N3 no</td>
<td>-</td>
<td>9.7 ± 0.13</td>
<td>2.8 ± 0.03</td>
<td>S/S</td>
</tr>
<tr>
<td>H5N3 no</td>
<td>-</td>
<td>7.9 ± 0.18</td>
<td>0.4 ± 0.03</td>
<td>S/S</td>
</tr>
<tr>
<td>H2N3 R156K 3)</td>
<td>F</td>
<td>11.3 ± 0.53</td>
<td>1.1 ± 0.18</td>
<td>S/S</td>
</tr>
<tr>
<td>H4N6 no</td>
<td>-</td>
<td>25.4 ± 0.58</td>
<td>2.4 ± 0.15</td>
<td>S/S</td>
</tr>
<tr>
<td>H4N6 no</td>
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<td>1.3 ± 0.09</td>
<td>S/S</td>
</tr>
<tr>
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<td>11.1 ± 0.14</td>
<td>1.2 ± 0.09</td>
<td>S/S</td>
</tr>
<tr>
<td>H4N6 R152K</td>
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<td>0.6 ± 0.07</td>
<td>S/S</td>
</tr>
<tr>
<td>H4N6 R152K</td>
<td>C</td>
<td>11.7 ± 0.30</td>
<td>1.1 ± 0.03</td>
<td>S/S</td>
</tr>
<tr>
<td>H11N6 R152K</td>
<td>C</td>
<td>17.0 ± 0.18</td>
<td>0.9 ± 0.09</td>
<td>S/S</td>
</tr>
<tr>
<td>H11N9 no</td>
<td>-</td>
<td>10.1 ± 0.15</td>
<td>2.4 ± 0.12</td>
<td>S/S</td>
</tr>
<tr>
<td>H11N9 no</td>
<td>-</td>
<td>38.6 ± 0.28</td>
<td>1.8 ± 0.12</td>
<td>S/S</td>
</tr>
<tr>
<td>H11N9 R118K</td>
<td>C</td>
<td>17.8 ± 0.11</td>
<td>1.5 ± 0.15</td>
<td>S/S</td>
</tr>
<tr>
<td>H11N9 R118K</td>
<td>C</td>
<td>13.3 ± 0.32</td>
<td>1.4 ± 0.06</td>
<td>S/S</td>
</tr>
<tr>
<td>H11N9 R118K</td>
<td>C</td>
<td>26.1 ± 0.43</td>
<td>1.6 ± 0.09</td>
<td>S/S</td>
</tr>
<tr>
<td>D151N C</td>
<td></td>
<td>4.3 ± 0.06</td>
<td>9.7 ± 0.28</td>
<td>S/S</td>
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</table>
Discussion

LPAI in Mallards
In this thesis, LPAI infection (A/H1N1) in mallards was studied using a variety of techniques. These include rRT-PCR (paper II and III), virus isolation (paper II), histopathology (paper III) and IHC (paper III). In general, the results confirm the current picture of LPAI in dabbling ducks as a primarily intestinal infection with some potential to replicate also in the airways. When going more into detail, the studies add new knowledge of the infection dynamics.

Time Course
Results from viral shedding by rRT-PCR and demonstration of viral antigen by IHC strongly suggest a rapid onset and early peak of LPAI infection in mallards. Data on viral shedding (Figure 4) demonstrate that most, but not all, birds were rRT-PCR positive 1 dpi. All birds were positive as the shedding peaked at 2 dpi and the shedding then successively declined. This was consistent with IHC data on viral antigen (Table 2) which seemed to peak around 1-2 dpi although these data were limited by the small number of birds examined each dpi. The IHC positivity decreased earlier than the viral shedding; this seems logical as the NP antibody used for IHC primarily detects replicating virus. RRT-PCR is also a much more sensitive assay than IHC and as mentioned above, it only needs a small fragment of the matrix gene and not necessarily virus particles capable of replication to yield a positive result. It was possible to isolate virus in the majority of rRT-PCR positive fecal samples; exceptions were some samples with a very high CT-value (data not shown). The exceptions may be due to very low number of virus in the samples or the previously mentioned high sensitivity of the rRT-PCR assay.

Localization
In this study, the LPAI infection was localized to the gastro-intestinal canal of the mallards with one exception. One artificially infected bird euthanized at 1 dpi had signs of influenza pneumonia with positivity in IHC and consistent histopathological changes. There were also older lesions in the form of
two small granulomas. In the intestine, no histopathological lesions were seen and no viral antigen was detected. Contents of the entire gastrointestinal canal were rRT-PCR negative apart from the sample from the ventriculus that was moderately positive. The most probable cause of the influenza pneumonia was believed to be intratracheal deposition of virus, either by a mistake in the inoculation process or by regurgitation. This is because: a) no other birds in the study had histopathological changes or viral antigen in the lung and b) the bird was negative by IHC and rRT-PCR in the entire intestinal canal apart from the ventriculus. The older lesions in the lung might have contributed as they could constitute a *locus minoris*. The possibility of secondary spread from e.g. the intestine seems improbable as only one day had passed since the inoculation and as the intestine was negative by IHC and rRT-PCR.

The intestinal infection appeared to progress in a proximal-to-distal manner. This was supported by the facts that more proximal sections of the intestine was positive by IHC earlier in the infection (Table 2) and that relatively more virus was detected by rRT-PCR in proximal portions of the intestinal contents (Figure 11). The IHC data was hampered by a small number of birds euthanized early in the infection but taken together the results still strongly suggest a longitudinal progression of LPAI infection in mallards.

**Mallard Model**

The *in vivo* mallard model described in paper II and III provides a promising means to study viral evolution under dynamic conditions, e.g. drug pressure. It is possible to control the experimental conditions, yet the virus is subjected to both replication and transmission as in the natural situation. As demonstrated in this thesis, the model is reliable in terms of inter-generation transmission. Furthermore, artificial intra-esophageal inoculation and natural infection between birds produce similar patterns of viral shedding. Possible future uses of the mallard model, apart from drug exposure studies, include persistence analysis of resistant viruses and *in vivo* testing of viruses isolated from wild birds.

**OC in the Environment**

The findings in paper I demonstrate – for the first time – that OC is present in the aquatic environment at detectable levels during a seasonal influenza outbreak. As the number of sampling sites and occasions were limited it is difficult to draw detailed conclusions but the levels seemed to increase further downstream in the Yodo River system (Figure 7). Furthermore, the levels were in the magnitude of biological activity and correlated well with
levels estimated from data on oseltamivir consumption. During the influenza season 2008-2009, another study in Japan measured levels of OC in river water up to 190 ng/L and in outgoing water from STPs up to 293 ng/L [98]. Very limited sampling for OC has been performed in aquatic environments, thus higher levels certainly exist under circumstances yet to be examined. Furthermore, both studies were performed during seasonal influenza outbreaks; during a pandemic, usage and thus environmental levels of OC are expected to be considerably higher, reaching µg/L-levels.

Interestingly, in a study from Germany a high OP/OC ratio was found in river water at the German – Swiss border. The high ratio suggested outlet from a pharmaceutical factory in the vicinity as the origin of OP. Sectional sampling of the river showed higher ratios on the side of the river where the factory is situated and no OP was detected upstream of the factory [99]. As OP can be converted to OC by naturally occurring esterases, the findings highlight that manufacturing of oseltamivir is a potential source of environmental OC that needs to be addressed. This is in analogy with the outlet of antibiotics from pharmaceutical factories, e.g. in India, that can cause high environmental levels and contaminate sources of drinking water [100].

Resistance Development in Mallards

Paper II demonstrates that an influenza A/H1N1 virus in mallards exposed to low levels of OC develops resistance through acquisition of the well-known mutation H274Y (Figure 8). A resistant phenotype was confirmed by NAIA. The IC50 difference between wild-type isolates (2-4 nM) and H274Y isolates (400-700 nM) is consistent with findings in human clinical isolates [72]. This is the first time that a drug exposure experiment in influenza-infected mallards was performed. Thus, the levels of OC needed to induce resistance under the experimental conditions were unknown. Therefore, a wide range of OC concentrations were used in order to increase the chances of detecting resistance development. Hence, the gap was wide between the level where H274Y was detected but did not outcompete the wild-type virus (1 µg/L) and the level where H274Y quickly outnumbered the wild-type (80 µg/L). However, the first priority in this experiment was to demonstrate the mechanism of resistance development as such and to find the magnitude of OC concentration that causes it. As µg/L-levels of OC are expected in the environment, the experimental conditions correspond to a realistic scenario. This means that oseltamivir resistance could be induced in influenza A viruses of wild ducks when the drug is widely used, but this is only true for limited periods of time during pandemic or seasonal influenza outbreaks. As earlier in vitro studies have indicated a decreased viral fitness in strains with NAI resistance mutations the question arises: Will the resistance prevail when OC disappears from the environment?
In this sense, it is interesting to study the results from isolations of a sample with mixed genotype (i.e. a virus population consisting of both wild-type and H274Y, Figure 9) in paper II. During the replication process in the embryonated hen eggs, no OC is present and hence there is no drug pressure. The fact that two different isolations gave rise to one wild-type and one H274Y-positive isolate demonstrates that either genotype can dominate the replication and outcompete the other. Although not being a fitness test in a true sense, this is still a good indication that the fitness of the wild-type and the mutant are not dramatically different when H274Y is induced in a randomly chosen virus from a wild mallard in Sweden. Sweden uses oseltamivir conservatively and the sampling was performed in a rural area on an island by the seaside. Therefore, this strain cannot possibly have been exposed to any drug pressure around the time of sampling. Another interesting fact is the accumulation of H274Y in seasonal influenza A/H1N1 in the seasons 2007-2008 and 2008-2009. As there was no correlation between the spread of resistance and the use of oseltamivir [68,69], i.e. the drug pressure, the H274Y mutant must have been fit enough to outcompete the wild-type strain(s). It has been demonstrated that this is probably due to compensatory, “permissive” mutations (V234M and R222Q) which restore the decreased surface expression of NA caused by H274Y [101]. Another study has also demonstrated a compensatory effect on NA activity in H274Y mutants by D344N [102]. Thus, it seems that the genetic makeup of the virus strain where resistance mutations such as H274Y develop will determine whether the mutation results in a decreased viral fitness or not.

Resistance in Wild Birds

The probability of a virus strain with a permissive genetic makeup appears to be higher in LPAI viruses of wild birds, and especially dabbling ducks, as they are the natural reservoir of influenza A viruses [10]. This means that many more strains co-circulate in the wild bird population at a given time and that there is a larger genetic variation. An example of this is that the sensitivity to oseltamivir in avian A/H1N1 viruses showed a much larger variation when compared to mammalian viruses [103]. The analysis of sequences from the NCBI database [104] in paper II revealed that H274Y has been reported in wild birds, though rarely. H274Y has been found both in H5N1 and H1N1 – interestingly, the H1N1 isolate originated from a duck in Minto Flats in Interior Alaska, a habitat with high densities of nesting ducks. The interior of Alaska is scarcely populated and certainly oseltamivir use is negligible, thus there is no drug pressure. The occurrence of H274Y under these circumstances further supports the idea that H274Y does not require drug pressure to prevail when present in a virus with a suitable genetic makeup.
In paper IV, 21 influenza A viruses isolated from wild birds were examined by a colorimetric NAIA (Table 3). The viruses were isolated from wild mallards at the same location as the virus used in paper II – Ottenby Bird Observatory. 13 of the viruses had mutations in conserved regions of the NA gene and 11 viruses carried mutations previously discussed in the sense of NAI resistance [105]. However, when examined by NAIA, the mutants did not differ from mutation-free controls of the same subtypes. The mutations could be divided into four groups regarding previous studies:

a) Tested in the same group of NAs as where they were found in wild birds and had a synergistic effect on H274Y but no or little effect on their own (D151N [75], I222V [78]).

b) Showed resistance in influenza B, a small or no decrease in sensitivity in other phylogenetic NAs, not tested in the same group of NAs as were they were found in wild birds (R152K [73,106,107], D198N [108,109]).

c) Instable when introduced by reverse genetics, therefore not possible to investigate sensitivity to NAIs (R118K [107]).

d) Not associated with decreased sensitivity to NAIs (D151K, R156K).

Thus, no mutation had previously been described as causing a substantial decrease in NAI resistance in the phylogenetic group of NAs where it appeared in the avian viruses. In that sense, the NAIA results are not controversial. However, even if not causing resistance in the genetic background where they were found in this study, the results still highlight the LPAI gene pool among wild birds as large, diverse and containing genetic material that can cause resistance in other backgrounds. The finding of the mutations D151N and I222V is particularly interesting as these mutations could boost the effect of H274Y and as they were present in the subtype H1N1 which is highly relevant to humans. If H274Y is induced in a virus with such a synergistic mutation, the effects would be even worse if the resistance spread to the human population. The recent report of a multidrug-resistant pandemic A/H1N1 virus with the I222R mutation [79] further highlights the resistance potential of the 222 residue.

Given the importance of LPAI viruses in wild birds, there is a need for further surveillance campaigns to achieve a better picture of the resistance situation in nature and to monitor it over time.

Spread of Resistance to Humans

As discussed above, resistance can spread from birds to humans via reassortment or direct transmission. An alarming scenario is an oseltamivir-resistant pandemic where an NA gene containing resistance mutation(s) has
been recruited from an avian virus during the reassortment process. This would render pandemic preparedness plans and stockpiles of oseltamivir useless and make treatment and prophylaxis extremely difficult. Another possibility is that oseltamivir resistance is established in the pool of circulating HPAI viruses with the risk that such a virus acquires human-to-human transmissibility while retaining high mortality and morbidity.

A crucial question to address regarding the importance of this work is the following: Even if resistance can occur and spread as described here, why is it so important? Is it not more important to study resistance development in humans themselves; in the treated patient replicating virus and NAIs obviously co-exist – why take the detour involving wild birds? There are two major arguments to answer this question:

1) The size and diversity of the influenza gene pool among wild birds is overwhelming compared to the pool of circulating human influenza viruses. At any given time, more or less only three different strains circulate among humans. In wild birds, all subtypes described to date have been found. Furthermore, there is a constant circulation of virus year-round and a large proportion of the population is exchanged each year and juveniles without immunity to influenza are added. Thus, it is perhaps more common that resistance develops in treated humans, but it is more probable in the bird population that a resistance mutation occurs in a virus with a suitable genetic makeup.

2) If resistance spreads to humans via a pandemic or a human-adapted HPAI virus, the consequences are far worse than if it arises in a strain already circulating in the human population. In the latter case there is already some immunity in the population and the resistant virus is probably one of the more harmless circulating seasonal strains (like the development of H274Y in the former seasonal A/H1N1 virus). In the former case, preparedness plans rely on oseltamivir both as an attempt to blanket the outbreak and as treatment and prophylaxis especially during the first wave.

**Strategies to Lower Environmental Levels of OC**

Measures to lower the environmental levels of OC and thus the drug pressure include bioremeditative and degradation-enhancing efforts as described in *Introduction*. Of these efforts, the addition of ozonization in STPs appears to be the most attractive measure. Apart from increasing the degradation of OC, the ozonization process can also reduce the outlet of antibiotics and antibiotic-resistant bacteria.

However, the most important measure remains a prudent use of antiviral drugs. The effect of NAIs in healthy people suffering a seasonal influenza infection is limited; oseltamivir 75 mg twice daily or a corresponding dose in children only shortens duration of symptoms with approximately one day if
administered within 48 hours [110,111,112]. On the contrary, in immunosuppressed patients there is growing evidence that a combination therapy is favorable. *In vitro* and *in vivo* experimental data suggest an additive or synergistic effect and a decrease in resistance development and therefore the use of combination therapy is increasingly advocated [113,114,115]. To strictly limit the use in the young and healthy in non-pandemic periods and to consider combination therapy for those with a suppressed immune defense appears to be a reasonable strategy.

**Clinical Consequences of Resistance**

Generally, resistance in influenza is a problem to physicians especially when treating immunosuppressed patients. Numerous reports exist on resistant viruses recovered from such individuals. An interesting example is the finding of I222R in a pandemic A/H1N1 virus isolated from an immunocompromised Dutch patient. This mutation caused resistance to all available NAIs [79]. As the pandemic A/H1N1 virus is naturally resistant to adamantanes, there are no treatment options left in this case.

In the event of an oseltamivir-resistant pandemic with morbidity in the same magnitude as the Spanish Flu, the consequences are almost unimaginable. A fraction of the morbidity is enough to rapidly outnumber ventilator capacity in intensive care units worldwide. The same goes for the scenario of a human-adapted HPAI virus capable of human-to-human spread still retaining (some of) its pathogenicity. The latter possibility does not seem too far-fetched when considering a recent interview with Ron Fouchier – scientist at the Erasmus Center in the Netherlands – where he claims to have found that five mutations might be enough to make a HPAI H5N1 virus transmissible in ferrets and that it retains its pathogenicity [116].

Apart from limiting the use of NAIs, the development of new anti-influenza drugs is essential. However, it is extremely important to consider the risk of resistance development – including what happens in the environment – and to limit the use of new drugs from the beginning. Each new antiviral, or antibiotic, has a limited life span which is heavily dependent on its use.
Summary

This thesis presents evidence that OC is present in the aquatic environment during a seasonal influenza outbreak (Figure 12). Thus, the natural influenza reservoir, dabbling ducks, can be exposed to the substance. Furthermore, it is demonstrated that influenza A/H1N1 virus in mallards subjected to low, environmental-like, concentrations of OC develop oseltamivir resistance through acquisition of the resistance mutation H274Y. Therefore, there is reason to believe that resistance development occurs in influenza A viruses of wild ducks when oseltamivir is used widely. It is shown that the H1N1 infection in mallards has a rapid onset, is mainly intestinal and has a longitudinal progression in the bowel. Mutations in influenza viruses from wild mallards are presented that – although they did not result in a resistant phenotype – have the potential to enhance other resistance mutations. The occurrence of H274Y in NCBI sequences from wild birds and the fact that H274Y became established in the pre-pandemic seasonal human H1N1 virus support the thought that once induced, oseltamivir resistance can prevail if present in a virus with a suitable, permissive genetic makeup. Through direct transmission or reassortment, the NA gene conferring oseltamivir resistance can spread to a human-adapted influenza virus with pandemic potential, threatening to disable oseltamivir, a cornerstone in pandemic preparedness planning. In a bigger perspective, the results in this thesis exemplify that antiviral drugs, like antibiotics, have a limited life span due to resistance development. The substances can have complex environmental effects that include humans, necessitating a broad, multi-disciplinary research approach.
Figure 12. Schematic display of the summary in relation to the general hypothesis.
Future Perspectives

Science often poses more new questions than what it answers. This work is no exception and there are numerous paths from here that deserve to be explored. The following areas appear particularly important:

- To learn more about OC in the environment. Studies performed so far are few and small. A global approach including pandemic periods is desirable. The environmental perspective established in this thesis needs to be applied on other NAIs and antivirals including those in the pipeline of drug development.

- To extend the knowledge of the influenza situation among wild birds. This concerns influenza ecology in general, but the resistance situation in particular. An extensive screening for influenza viruses and resistance mutations among wild birds in different parts of the world is important, also to follow the development over time. Genotypic analysis might not always be sufficient; to some extent the screening should be complemented with functional methods such as NAIA to achieve a broader picture.

- To understand the dynamics of influenza resistance development in dabbling ducks exposed to OC. It is crucial to study if, and how, resistance mutations are affected by decreasing levels OC and thus how the resistance prevails in between influenza seasons and pandemic periods. The mallard model described in this thesis appears to be a very promising tool to further examine this.

- To broaden the understanding of influenza resistance development in dabbling ducks. To date, only one subtype of influenza is examined in the sense of resistance development in the environment. It is still unknown how environmental levels of OC affect the large and important phylogenetic N2-group of NAs that have distinct resistance mutations. The mallard model employed here is an attractive set-up to answer also this question.
Infektioner med influensa A virus drabbar mänskligheten som årlig säsongsinfluensa (epidemiska utbrott) och mer sällan som världsomfattande (pandemiska) utbrott. Allvarlighetsgraden varierar från lindrig sjukdom med lått ökad dödlighet för gamla och sjuka till svåra pandemier; exempelvis tog spanska sjukan sannolikt 50-100 miljoner människoliv. Resistens mot influensaläkemedel är ett växande problem – sedan många år används t.ex. inte de s.k. admantanerna p.g.a. resistensutveckling. Mot den andra tillgängliga läkemedelsklassen, neuraminidashämmare, har en oroväckande utveckling ägt rum med uppkomst av resistensmutationen H274Y i säsongsinfluensan A/H1N1 som cirkulerade fram till den senaste pandemin. Den mest använda neuraminidashämmaren är oseltamivir, med handelsnamnet Tamiflu®. Detta preparat används både som behandling och profylax vid säsongsinfluensa och finns dessutom i stora lager som del av beredskapsplaner världen över inför en allvarlig pandemi. Den aktiva metaboliten av oseltamivir, oseltamivirkarboxylat (OC), är mycket stabil och utsöndras till största delen oförändrad från behandlade patienter via urinen. OC är också stabilt i ytvatten och bryts heller inte ner i reningsverk. Därför hamnar OC till slut i vattenmiljön vid reningsverkens utlopp. I dessa miljöer uppehåller sig gärna simänder såsom gräsand. Änder är den naturliga reservoaren för influensa; de har levtt med infektionen under miljontals år och därmed successivt utvecklat ett samspel med viruset så att de blir betydligt mindre sjuka än vad människor blir. Det finns alltid en viss andel av änderna i varje population som är infekterad med influensa. Influensa hos andar är i huvudsak en tarminfektion och om det finns OC i vattnet där änderna befinner sig finns det risk för resistensutveckling i tarmen där läkemedel och replikerande virus möts.

Om oseltamivir-resistenta virus etablerar sig bland influensavirus som cirkulerar bland vilda fåglar kan det bli ett problem även för människa. De pandemiska influensavirus vi känner till har nämligen bildats som en sammanslagning, s.k. reassortment, mellan influensavirus från olika vårddjur, däribland fåglar. Om resistens mot oseltamivir på detta sätt finns med från början i ett virus som orsakar en pandemi är det ett enormt problem eftersom beredskapslagren då blir värdeflösa. Ett annat skrämmande scenario är om ett högpotentt fågelinfluenzavirus (t.ex. H5N1, den s.k. fågelinfluensan) som cirkulerar bland vilda fåglar utvecklar resistens och dessutom anpassar sig till att spridas mellan människor. I de fall människor drabbas av detta virus är dödligheten över 50% men viruset har hittills inte kunnat sprida sig från

I avhandlingens första arbete beskrivs för första gången fynd av OC i miljön. I Japan används mycket oseltamivir för behandling av säsongsinfluensa och därför undersöktes ett japanskt flodsystem före och under en influensasäsong. Som mest hittades 58 ng/L OC; i en senare japansk studie har upp till 293 ng/L uppmätts i utgående avloppsvatten.


I det tredje arbetet studerades änder från ovan nämnda försök noggrannare vad gäller virusutsöndring och histopatologi. Studien visade att influensainfektionen huvudsakligen är belägen i tarmen samt att den när sitt maximum tidigt – runt 2 dagar efter infektion – både vad gäller virusutsöndring, histopathologiska förändringar och virusantigen påvisbart med immunhistokemi. Vidare verkar infektionen ha ett progressivt förlopp från proximalt till distalt, d.v.s. infektionen är relativt sett mer belägen i tunntarmen tidigt i förloppet och senare i tjocktarmen. Detta mönster sågs både med immunhistokemi och med PCR-detektion av virusets RNA i innehåll från olika delar av tarmen.

Det fjärde arbetet beskriver en analys av 21 influensavirus med en funktionell enzymaktivitetsmetod. Med hjälp av denna metod kan man mäta virusets känslighet för olika läkemedel av typen neuraminidashämmare. Influensaviruset var isolerade från vilda änder från Ottenby Fågelstation på Öland. 11 av virusen hade sedan tidigare kända mutationer som diskuterats vad gäller resistens. Inget virus visade dock nedsatt känslighet jämfört med virus av samma typ utan mutationer. Effekterna av en viss mutation beror dock till stor del på vilken undergrupp av influensavirus den finns i. Resultaten i studien beror sannolikt på att mutationerna fanns i en undergrupp av influensavirus där de inte ger resistens. Studien visar ändå att influensavirus från vilda fåglar innehåller genetiska varianter med resistenspotential; i två
fall dessutom i form av mutationer som tidigare visats ge en synergistisk effekt tillsammans med H274Y.


Det är viktigt att fortsätta studera resistensutveckling i fåglars influensavirus samt att övervaka vilda fåglars virus för att bättre förstå och kunna hantera problemet. Behandling med ozon i reningsverk är en lovande strategi för att minska miljökoncentrationer av OC. Den viktigaste strategin är dock att strikt begränsa användningen av oseltamivir hos i övrigt friska patienter i icke-pandemiska perioder – preparatet har då dessutom begränsad effekt. I ett större perspektiv visar denna avhandling att antivirala läkemedel, liksom antibiotika, är substanser med en begränsad livslängd på grund av resistensutveckling. Livslängden är starkt beroende av läkemedlets användning och mekanismerna för resistensutveckling kan vara komplexa vilket kräver ett brett multidisciplinärt angreppssätt.
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