

Influenza A/H4N2 mallard infection experiments further indicate zanamivir as less prone to induce environmental resistance development than oseltamivir

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Abstract

Neuraminidase inhibitors (NAIs) are the gold standard treatment for influenza A virus (IAV). Oseltamivir is mostly used, followed by zanamivir (ZA). NAIs are not readily degraded in conventional wastewater treatment plants and can be detected in aquatic environments. Waterfowl are natural IAV hosts and replicating IAVs could thus be exposed to NAIs in the environment and develop resistance. Avian IAVs form the genetic basis for new human IAVs, and a resistant IAV with pandemic potential poses a serious public health threat, as NAIs constitute a pandemic preparedness cornerstone. Resistance development in waterfowl IAVs exposed to NAIs in the water environment has previously been investigated in an *in vivo* mallard model and resistance development was demonstrated in several avian IAVs after the exposure of infected ducks to oseltamivir, and in an H1N1 IAV after exposure to ZA. The N1 and N2 types of IAVs have different characteristics and resistance mutations, and so the present study investigated the exposure of an N2-type IAV (H4N2) in infected mallards to 1, 10 and 100 µg l⁻¹ of ZA in the water environment. Two neuraminidase substitutions emerged, H274N (ZA IC₅₀ increased 5.5-fold) and E119G (ZA IC₅₀ increased 110-fold) at 10 and 100 µg l⁻¹ of ZA, respectively. Reversion towards wild-type was observed for both substitutions in experiments with removed drug pressure, indicating reduced fitness of both resistant viruses. These results corroborate previous findings that the development of resistance to ZA in the environment seems less likely to occur than the development of resistance to oseltamivir, adding information that is useful in planning for prudent drug use and pandemic preparedness.

INTRODUCTION

Influenza A viruses (IAVs) can cause disease in animals and in humans. In humans, IAVs give rise to yearly seasonal epidemics and to infrequent pandemic outbreaks with a much more severe clinical disease and a higher mortality. Besides morbidity and mortality in the human population, IAV infections in the poultry industry have a substantial economic impact. Mallards and other waterfowl are the natural hosts

of IAVs [1, 2]. When infected with a low-pathogenic avian influenza A virus (LPAIV), mallards exhibit no or minimal signs of illness [3]. LPAIV is a gastrointestinal infection in mallards [4].

Neuraminidase inhibitors (NAIs) are the gold standard for treatment of human IAV infection [5]. They target the surface glycoprotein neuraminidase (NA), which aids the movement of IAV particles through the mucus of the respiratory tract

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Abbreviations: ELISA, Enzyme-linked immunosorbent assay; EMA, European Medicines Agency; IAV, Influenza A virus; IC₅₀, Half maximal inhibitory concentration; LPAIV, Low-pathogenic avian influenza virus; MDCK, Madin–Darby canine kidney; NA, Neuraminidase; NAI, Neuraminidase inhibitor; OC, Oseltamivir carboxylate; RRT-PCR, Real-time reverse transcription polymerase chain reaction; SVA, Swedish National Veterinary Institute; WHO, World Health Organization; ZA, Zanamivir.

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Supplementary material is available with the online version of this article.

and enables the release of newly formed virions from infected cells [6].

Oseltamivir (Tamiflu) is the most used NAI; due to its convenient oral formulation it is popular for treatment and prophylaxis and in pandemic preparedness stockpiles [7]. However, zanamivir (ZA; Relenza) is increasingly used because of oseltamivir resistance and because its intravenous formulation is suitable for critically ill patients. The i.v. formulation has completed phase 3 clinical trials and has been approved by the European Medicines Agency (EMA) for the treatment of hospitalized patients with influenza [8, 9]. If we experience increasing oseltamivir resistance and/or numerous severely ill patients, it can be expected that ZA use will increase in the future.

IAV resistance to NAIs is rare but occurs. More than 99% of human influenza viruses tested by the World Health Organization (WHO) between May 2015 and May 2016 were susceptible to all NAIs [5]. The most common resistance mutation to oseltamivir is H275Y in H1N1 viruses (H274Y in N2 numbering) [5].

NAIs are mainly excreted via the urine and are not readily degraded in sewage treatment plants [10], and they have been detected in river waters in concentrations of up to 865 ng l⁻¹ for oseltamivir carboxylate (oseltamivir's active metabolite, OC) [11] and 59 ng l⁻¹ for ZA [12], potentially exposing IAVs in wild waterfowl to the drugs and allowing resistance to develop.

Previous *in vivo* experiments using a mallard model have demonstrated that NA substitutions affecting drug susceptibility emerge in response to the exposure of mallards infected with LPAIV to OC and ZA through their water source [13–17]. In a scenario where such substitutions are incorporated via reassortment into pandemic IAVs infecting humans or an avian IAV carrying these substitutions is directly transmitted to humans, the treatment of humans and pandemic preparedness planning may be at risk of failure.

The NAs can be separated into two phylogenetic groups (N1 and N2) based on structural differences at the active site affecting NAI binding [18]. The N1 group includes subtypes N1, N4, N5 and N8. The N2 group includes N2, N3, N6, N7 and N9. Additionally, there is a third group, the IAV-like group 3, including N10 and N11 of bat origin [2].

In previous mallard *in vivo* OC exposure experiments, the H274Y substitution emerged in H1N1 and persisted in the absence of OC, while the R292K substitution emerged in H6N2 but did not persist [13, 14, 19, 20].

Exposing an N1 LPAIV (H1N1) to ZA resulted in the emergence of the R152K and D199G substitutions at 10 µg l⁻¹ of ZA and of the A138V, R152K and T157I substitutions at 100 µg l⁻¹ of ZA. None of the substitutions persisted when drug pressure was removed [17].

Since the N1 and N2 groups behave distinctly different in respect of drug binding, it is crucial to study both phylogenetic NA groups of LPAIVs to be able to properly assess the

risk of environmental resistance development. Therefore, in the present study we performed exposure and persistence experiments with an N2 LPAIV (H4N2) infecting mallards exposed to ZA.

METHODS

Viruses

The LPAIV isolate A/mallard/Sweden/80190/2008 (H4N2), referred to below as 80190/wt, was used in the exposure experiments and originates from a wild mallard sampled at Ottenby Birding Station at Öland Island in Southern Sweden (NA GenBank accession: CY165610 to CY165617). The isolate with the resistance-related substitution H274N that emerged in the exposure experiments is referred to as 80190/H274N. The isolate with the resistance-related substitution E119G that emerged in the exposure experiments is referred to as 80190/E119G.

All isolates were obtained by using 11-day-old specific-pathogen-free embryonated chicken eggs (Valo, Germany). Samples from experimental birds were inoculated in the allantoic cavity, and allantoic fluid was harvested after 2 days. IAV presence was confirmed by haemagglutination using chicken erythrocytes (Lohmann Tierzucht, Cuxhaven, Germany).

Drugs

The ZA used for experiments and the neuraminidase inhibition assay, and ¹³C-¹⁵N₂-labelled ZA for ZA quantification, was obtained from GlaxoSmithKline (London, UK). The OC used for the neuraminidase inhibition assay was obtained from F. Hoffman-La Roche AG (Basel, Switzerland).

Mallard model

Newly hatched mallards (*Anas platyrhynchos*) were purchased from a commercial breeder and reared in captivity to avoid unintended LPAIV exposure. Experiments were performed at the Animal Facility (BSL2) of the Swedish National Veterinary Institute (SVA) under conditions approved by the Ethics Committee on Animal Experiments in Uppsala, Sweden (permits C125/12, C63/13 and C20/14).

Prior to entering the experiments, the absence of previous infection with IAV was confirmed in all birds by serology (FlockCheck, Avian Influenza Virus Antibody Test kit, IDEXX, Hoofddorp, The Netherlands). Additionally, faecal samples were collected prior to inclusion in the experiments to confirm the absence of IAV by real-time PCR as described below.

Birds were introduced to and excluded from the experiment in a consistent pattern throughout all experiments. First, two mallards (generation one) were inoculated esophageally with LPAIV and introduced to the experimental room with a 170 l (1 m²) pool as their single water source. The pool water was changed daily and ZA was added at a specified concentration. Every day, the experiment room was cleaned, but not sterilized. Three days post-inoculation (p.i.), two naive birds

(generation two) were introduced to the room and housed together with generation one until five days p.i., when generation one was euthanized by the administration of sodium pentobarbital [100 mg pentobarbital vet. (100 mg ml⁻¹) kg⁻¹ of body weight] intravenously. Additional generations were added in the same overlapping manner 24 h after the removal of previous generations, allowing natural transmission between the generations and continued viral replication.

Two categories of experiments were performed. First, three exposure experiments, where birds infected with 80190/wt were exposed to 1, 10 or 100 µg l⁻¹ ZA in the water. Each experiment comprised four generations of birds, i.e. eight birds in total. Second, two persistence experiments, where an isolate with a resistance-related substitution that emerged in the exposure experiments was allowed to replicate while the drug pressure was gradually removed (first day with 10 µg l⁻¹ ZA in the water, second and third day with 1 µg l⁻¹, and the remaining 8 days with no ZA), in order to assess the persistence of the substitution. In one experiment 80190/H274N was used, and in the other 80190/E119G. The persistence experiments each comprised three generations, i.e. six birds.

Faecal sampling

Individual fresh faecal swab samples were collected daily from all birds following defecation in single-use cardboard boxes. On the few occasions when a bird did not defecate while in the box, cloacal swabs were collected. The swabs were placed in viral transport media and the samples were stored at -70 °C within 2 hours of collection.

Detection of virus and sequencing of the NA gene

RNA from faecal samples and isolates was extracted in a Maxwell 16 Research Instrument (Promega, Fitchburg, WI, USA) using a Maxwell 16 Viral Total Nucleic Acid Purification kit according to the manufacturer's instructions.

Detection and semi-quantification of IAV from extracted RNA in the samples and isolates were performed in a Corbett Rotor-Gene 2000 thermo cycler (Qiagen, Hilden, Germany) using the iScript one-step reverse transcriptase PCR kit for probes (Bio-Rad, Hercules, CA, USA) in a real-time reverse transcriptase PCR targeting the matrix gene [21]. Samples with a cycle threshold value below 40 were considered positive.

The NA gene in IAV-positive samples was amplified in a one-step reverse transcriptase PCR using a Superscript III One-step RT-PCR kit with Platinum *Taq* High Fidelity DNA polymerase (Life technologies/Thermo Fisher Scientific, Waltham, MA, USA) and in-house primers (Table S1, available in the online version of this article). Reaction volumes of 25 µl contained 12.5 µl of reaction buffer, forward and reverse primers to a final concentration of 200 nM each, 1.25 units of the Platinum *Taq* High Fidelity enzyme, 5 µl RNA extract and RNase-free water. Thermocycling conditions were 55 °C for 30 min and 94 °C for 5 min for reverse transcription, followed by 35 amplification cycles at 94 °C for 1 min, 57.4 °C for 1 min and 68 °C for 1 min, and a final extension at 68 °C for 5 min.

PCR products were purified by enzymatic treatment with ExoSAP-IT (Affymetrix, Inc., CA, USA) and sequenced at Macrogen, Inc. (Seoul, Republic of Korea). Sequence results were analysed using SeqScape software, version 2.7 (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA), using the 80190/wt sequence as a reference. A sequence result was considered reliable if there were at least two high-quality electropherogram sequences at a given nucleotide position. If not, the sequence result was referred to as 'no sequence acquired'. The genotype was determined as 'mixed' if two peaks representing different nucleotides were visible at a single position in the electropherograms, and 'dominant' if only one peak was visible.

Neuraminidase inhibition testing

Phenotypic susceptibility to NAIs in selected isolates from the experiments was assessed by the NA activity and inhibition assay using the fluorogenic 2-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA) substrate (Sigma-Aldrich, St Louis, MI, USA). Before the inhibition assay was performed, the genotype consistency between the isolate and the experimental sample was confirmed by resequencing of isolate NA gene. The isolates were subjected to NA inhibition by OC and ZA in duplicate samples in 96-well plates, following the protocol of the Respiratory Virus Unit, Health Protection Agency, London, UK [22]. Fluorescence was measured in an Infinite M1000 PRO micro plate reader (Tecan Group Ltd, Zürich, Switzerland), and the IC₅₀s were determined from the best-fit dose-response curves using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Statistical analysis

Hypothesis testing of the equality of IC₅₀ results and the NA activity of mutant to wild-type isolates was performed using the Mann-Whitney non-parametric U test (Prism 6 software, GraphPad).

Experimental water concentrations

The measured concentrations of ZA in exposure experiments correlated well with the intended concentrations. In persistence experiments, some deviation was seen in the E119G experiment, where the first day exposure was 6 µg l⁻¹ and the following 2 days of exposure was 0.7 µg l⁻¹, whereas in the H274N persistence experiment, the intended and measured concentrations correlated well. The concentration deviation in the E119G persistence experiment does not change the general conclusion of the paper. Detailed methods and results regarding experimental water concentrations can be found in the Supplementary material.

RESULTS

Prior to inclusion in the experiment, all birds tested negative for IAV by enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription polymerase chain reaction (RRT-PCR). The shedding kinetics of IAV (Fig. 1) was

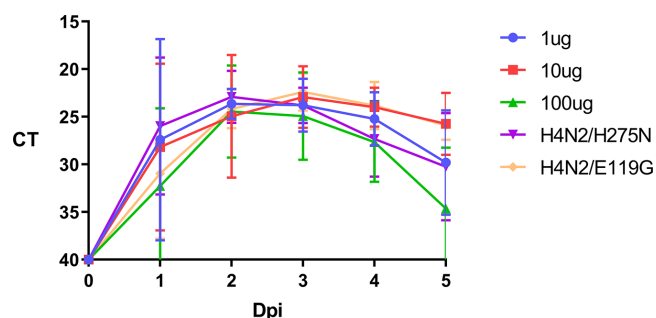


Fig. 1. IAV shedding. C_T cycle threshold value. p.i., post-inoculation/ introduction. 1, 10 and 100 $\mu\text{g l}^{-1}$ = zanamivir exposure experiments (including eight birds each). H4N2/H274N and H4N2/E119G = persistence experiments using 80190/H274N and 80190/E119G, respectively (including six birds each). Note that all samples from the persistence experiments do not necessarily contain H274Y/E119G; e.g. most H4N2/E119G samples are wild-type (see also Fig. 3). The C_T values are means of all the birds in the given experiment at the given day and the error bars denote standard deviation. Negative samples ($C_T \geq 40$) were designated a C_T value of 40.

similar across all experiments and similar to previous studies using this model system [14, 15, 19, 20].

Exposure experiments

Three exposure experiments were performed, exposing mallards infected with 80190/wt to 1, 10 or 100 $\mu\text{g l}^{-1}$ of ZA in the pool water. No substitution increasing the ZA IC_{50} was detected in the 1 $\mu\text{g l}^{-1}$ experiment, whereas two NA substitutions (H274N and E119G) that increased the ZA IC_{50} emerged in the 10 and 100 $\mu\text{g l}^{-1}$ experiments, respectively (Fig. 2). In the 100 $\mu\text{g l}^{-1}$ experiment, in a fairly large proportion of the samples, although positive in RRT-PCR, the NA gene could not be sequenced.

Two NA substitutions that did not increase the ZA IC_{50} emerged; S316G in the 1 $\mu\text{g l}^{-1}$ experiment and D141N in the 100 $\mu\text{g l}^{-1}$ experiment. The S316G substitution was present as mixed genotype from 1 day p.i. throughout the 1 $\mu\text{g l}^{-1}$ experiment, and as the most prevalent genotype in two samples in the second generation. No isolates with only S316G as a genotype were obtained, but the substitution in the mixed genotype ($n=4$) had no impact on the ZA or OC IC_{50} (mean ZA $\text{IC}_{50}=0.72$ nM, $P=0.1143$, mean OC $\text{IC}_{50}=0.21$ nM, $P=0.2000$). The isolates containing S316G are hence considered as wild-type with regard to IC_{50} and are thus not further specified in the following tables or results.

The D141N substitution was detected together with E119G in two samples as a mixed genotype in the first generation in the 100 $\mu\text{g l}^{-1}$ experiment and as a dominant genotype in three faecal samples in the last generation. One isolate (ZA $\text{IC}_{50}=92$ nM, OC $\text{IC}_{50}=0.84$ nM) with both the E119G and D141N substitutions as dominant genotypes, and two isolates with only E119G as a dominant genotype (mean ZA $\text{IC}_{50}=130$ nM, $\text{SD}=40$, mean OC $\text{IC}_{50}=1.0$ nM, $\text{SD}=0.30$), were obtained. Thus, the D141N substitution does not seem to

increase the ZA IC_{50} , although the sample size is limited. The D141N substitution emerged in another mallard experiment and was found to not increase the ZA or OC IC_{50} and to lack any compensatory function in the persistence of substitutions lowering IC_{50} [14, 20]. Hence, the D141N substitution is considered as wild-type with regard to the ZA and OC IC_{50} and is therefore not presented separately in the figures and tables. As discussed below, another isolate with both E119G and D141N had a distinctly different phenotype from the other isolates.

Both the H274N and E119G substitution increased the ZA IC_{50} (Table 1), but E119G to a larger extent; E119G met the WHO criteria for highly reduced inhibition (>100 -fold increase) [23]. The H274N substitution had a small reducing effect on the OC IC_{50} ; it was statistically significant but only ~ 0.4 -fold and thus of limited biological relevance.

The last (13 days p.i.) E119G isolate from the 100 $\mu\text{g l}^{-1}$ experiment displayed a distinctly different phenotype with an increased OC IC_{50} (26 nM, fold change=87). The IC_{50} for ZA was not further increased (68 nM, fold change=71). The NA genotype was identical to that for earlier isolates from the 100 $\mu\text{g l}^{-1}$ experiment. To rule out methodological errors, the neuraminidase inhibition assay was repeated in duplicates on two different occasions, and the isolate was passaged once more in embryonated chicken eggs and once again subjected to the neuraminidase inhibition assay. All these analyses yielded similar results concerning genotype and NAI IC_{50} s.

Persistence experiments

In the persistence experiments, mallards infected with an 80190/H274N or an 80190/E119G isolate were exposed to initially decreasing concentrations of ZA in the water for 3 days, followed by 8 days without drug pressure. The H274N substitution was detected throughout the three generations comprising the experiment (Fig. 3), but it was found as a mixed genotype on days 8 and 10 of the experiment.

The E119G substitution was only detected in the first generation. The isolates from the second and third generations of mallards only showed the wild-type genotype. One isolate of the first generation was detected as a mixed genotype (day 5). The D141N substitution was detected throughout the whole experiment, but, as mentioned previously, it did not affect the IC_{50} value and is therefore considered to be wild-type.

The H274N isolates displayed an increased IC_{50} in the same magnitude as the exposure experiments (Table 2). The one E119G isolate had an increased OC IC_{50} of the same magnitude as the 13 days p.i. E119G isolate from the exposure experiment.

DISCUSSION

To complement previous studies regarding the development of resistance among LPAIVs infecting the natural host while exposed to ZA, we performed several experiments in an *in vivo* mallard model using an H4N2 LPAIV. In three separate

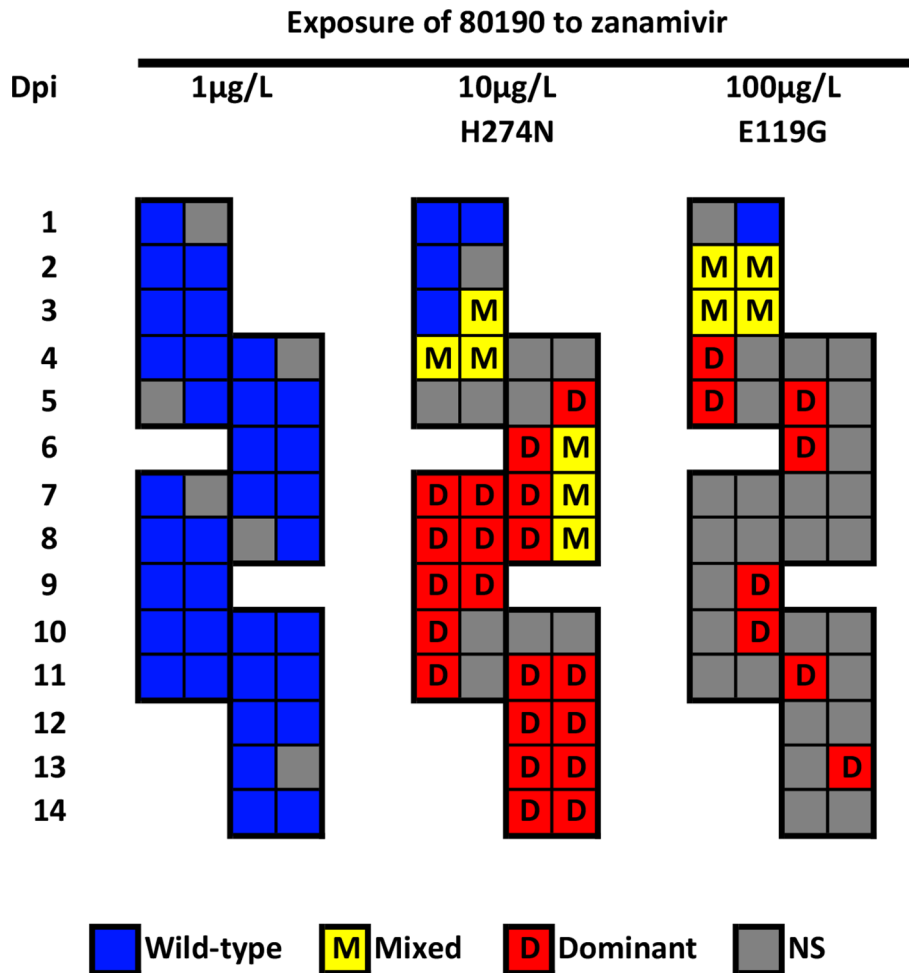


Fig. 2. NA genotype results in the three exposure experiments (1, 10 and 100 µg l⁻¹ of ZA). NA substitutions that increase the ZA or OC IC₅₀ are presented in the figure. The NA substitutions H274N and E119G emerged in the 10 and 100 µg l⁻¹ experiments, respectively. p.i., post-inoculation/introduction. Each experiment comprised four generations of two birds each (a total of eight birds in each experiment). In the figure 'Wild-type' only indicates wild-type observed at the amino acid residue, 'Mixed' indicates mixed genotype containing wild-type and relevant substitutions in various proportions and 'Dominant' only indicates relevant substitutions observed at the residue. 'NS' indicates no NA sequence acquired.

exposure experiments, an LPAIV H4N2 virus was inoculated into mallards and exposed to 1, 10 and 100 µg l⁻¹ of ZA via the sole water source of the birds. Subsequently, the persistence of an H274N substitution that emerged at 10 µg l⁻¹ and an E119G substitution that emerged at 100 µg l⁻¹ was assessed in two experiments.

IAV shedding

The shedding patterns were similar in all five experiments, and they were also similar to those in previous observations in the mallard model [14, 15, 19, 20]. Hence, neither exposure to 1–100 µg l⁻¹ of ZA nor the presence of the H274N or E119G substitutions appear to impact on IAV shedding. Thus, the shedding data do not suggest reduced replication capacity of the H4N2 LPAIV under the tested levels of ZA drug pressure or when the H274N or E119G substitution is present.

Effects of ZA exposure

Similar to what has been observed for H1N1 (17), exposing H4N2 to 1 µg l⁻¹ ZA did not result in resistance-related NA substitutions, but exposure to 10 and 100 µg l⁻¹ did. H274N and E119G emerged at 10 and 100 µg l⁻¹, respectively. This is in contrast to the exposure of H1N1 to OC, where the H274Y substitution had already emerged at 1 µg l⁻¹ [13], but similar to the exposure of H6N2 to OC, where resistance emerged at 12 µg l⁻¹ [13].

The H274N substitution was first detected in a mixed genotype in the first generation of birds and dominated the population completely throughout the last (third) generation. The 274 residue is a well-known resistance hotspot; the H274Y substitution is well described in N1 viruses and confers high-level resistance to OC [5, 13]. H274N has been detected in

Table 1. Neuraminidase inhibitor susceptibility of isolates from exposure experiments

Experiment	NA substitution	<i>n</i>	ZA IC ₅₀ ^a (nM) [fold change ^b]	<i>P</i> ^c	OC IC ₅₀ ^a (nM) (fold change ^b)	<i>P</i> ^c
1 µg l ⁻¹	WT	5	0.72 [0.75]	ref	0.21 (0.70)	
10 µg l ⁻¹	WT	1	0.76 [0.79]	ref	0.23 (0.77)	ref
	H274N	5	5.3 [5.5]	0.0016*	0.17 (0.57)	0.038*
100 µg l ⁻¹	WT	2	1.0 [1.0]	ref	0.25 (0.83)	ref
	E119G (4–10 days p.i.)	5	106 [110]	0.0016*	0.79 (2.6)	0.0016*
	E119G (13 days p.i.)	1	68 [71]	–	26 (87)	–

Isolates with substitutions in the dominant genotype are included in the table. The E119G isolate from 13 days p.i. is displayed separately due to its distinct phenotype.

a, IC₅₀ is the 50% inhibitory concentration, all IC₅₀s are means of *n* isolates with that genotype

b, Fold change indicates change of IC₅₀ in relation to 80190/wt (isolate IC₅₀/80190/wt IC₅₀).

c, Statistical testing of IC₅₀s of isolates with respective NA substitutions vs the collated eight wild-type isolates (marked as ref in the table) using the Mann–Whitney U test.

WT, wild-type; pi, post-inoculation; *P*, *P*-value; *, statistically significant at the *P*<0.05 level.

human H3N2, exhibiting an elevated ZA IC₅₀ (15.12 nM) and has been found to confer reduced ZA sensitivity in H1N1 [24, 25]. The ZA IC₅₀ increase (5.5-fold) in our experiment did not meet the WHO criteria for reduced inhibition (>10-fold) [23]. A minor decrease in the OC IC₅₀ was also observed for the H274N-containing isolates. This is in line with previous

observations in H1N1, where replacing the amino acid His (H) with the small side-chain residue Asn (N) causes higher or unchanged sensitivity to OC [25].

In the 100 µg l⁻¹ experiment, the E119G substitution was detected 2 day p.i. in the mixed genotype and in the dominant

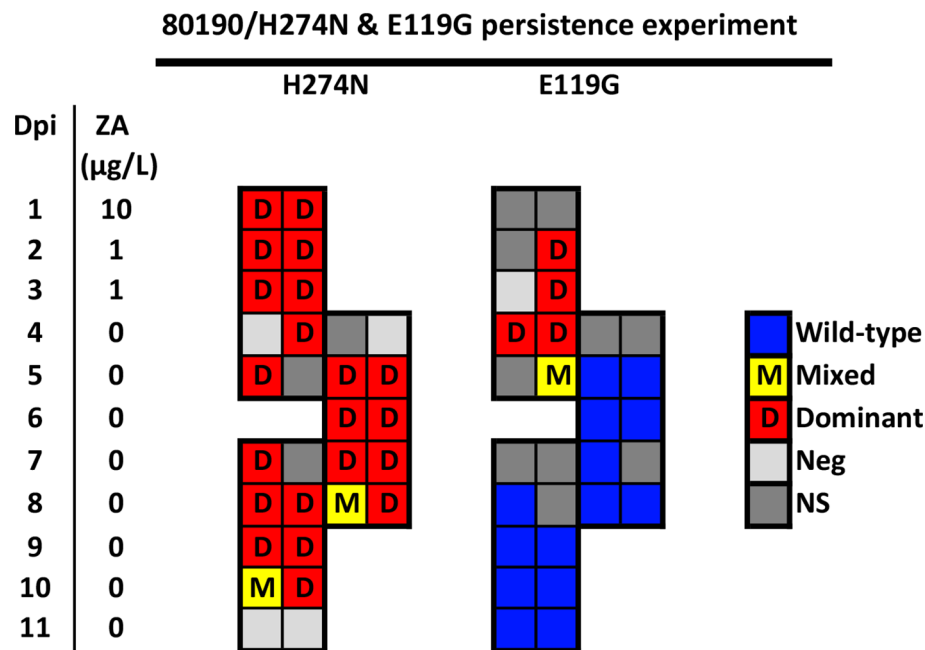


Fig. 3. NA genotype results in the persistence experiments. p.i., post-inoculation/introduction. ZA, zanamivir. Each experiment comprised three generations of two birds, i.e. a total of six birds. In the figure 'Wild-type' only indicates wild-type observed at the amino acid residue, 'Mixed' indicates mixed genotype containing wild-type and relevant substitution in various proportions and 'Dominant' only indicates relevant substitution observed at the residue. 'Neg' indicates negative in NA PCR reaction and 'NS' indicates no sequence acquired.

Table 2. Neuraminidase inhibitor susceptibility of isolates from persistence experiments

Experiment	NA substitution	<i>n</i>	IC ₅₀ ^a (nM)	
			ZA (fold change ^b)	OC (fold change ^b)
H4N2/H274N	H274N	5	9.96 (11.45)	0.18 (–0.10)
H4N2/E119G	WT	2	0.80 (0.00)	0.35 (0.75)
	E119G	1	207.7 (258.6)	62.4 (311)

IC₅₀ values of isolates from the persistence experiments with a dominant genotype.

a, IC₅₀ is the 50% inhibitory concentration, all IC₅₀s are means of *n* isolates with that genotype.

b, Fold change indicates change of IC₅₀ in relation to 80190/wt (isolate IC₅₀/80190/wt IC₅₀). WT, wild-type.

genotype from 3 days p.i. onwards. The highly reduced inhibition to ZA (110-fold increase in IC₅₀) renders the therapeutic effects of ZA treatment of such a virus less likely, although direct comparisons between IC₅₀s and clinical effect are difficult. The E119G substitution that emerged in an H4N2 of avian origin replicating in Madin–Darby canine kidney (MDCK) cell culture under ZA pressure has been reported to confer a 200- to 700-fold increase in ZA IC₅₀ [26, 27]. In an highly pathogenic avian H5N1 virus, the substitution was detected in ZA-selective MDCK cell culture and caused a 1400-fold increase in ZA IC₅₀ [28]. In human pandemic H1N1 virus, the substitution has been reported both *in vivo* in an immunocompromised child treated with ZA and OC and in *in vitro* recombination experiments, with 440- and 1500-fold increases in the ZA IC₅₀, respectively [29, 30].

E119G has also been detected in N9 [31]. Other substitutions at the 119 residue have been repeatedly reported previously. The E119V substitution has emerged in N2 viruses in response to OC treatment in humans and is associated with OC resistance [32–34]. The E119D substitution has been obtained *in vitro* and *in vivo* in both N1 and N2 by ZA selective pressure [26, 35, 36]. Both E119D and E119G were detected in phase II adult and paediatric studies, respectively [37, 38]. The E119A substitution has been reported in N1 and N2 viruses [26, 35, 39]. Thus, the acquisition of E119G in response to exposure to ZA and the magnitude of the IC₅₀ increase seen in our study are in line with previous data.

Interestingly, the last E119G from the 100 µg l^{–1} exposure experiment and the only obtained E119G isolate from the persistence experiment displayed a distinctly different phenotype compared to other E119G isolates in that the OC IC₅₀ was markedly increased, whereas the ZA IC₅₀ was not obviously affected. The NA sequence was identical to that for earlier E119G isolates, so the genotypic explanation for the phenotypic change must lie elsewhere. Further analysis by e.g. HA or whole-genome sequencing would be needed to assess the reasons in-depth, but this was beyond the scope of this

study. However, since neither the E119G genotype nor the resistant phenotype persisted in the persistence experiment, the question is of limited relevance for environmental resistance accumulation.

In a large proportion of the faecal samples from the 100 µg l^{–1} exposure experiment, NA sequencing failed, despite positive RRT-PCR results. The reason for this is unknown but may include inhibition or alterations at the primer targeting positions. The positive RRT-PCR results and unchanged virus shedding pattern indicate that viral replication was comparable to that in the other experiments. The lower number of NA sequences obtained from this experiment do not affect the overall conclusions of the study. The exposure experiments were only performed in eight birds at each ZA concentration, and in one sense, each experiment can be considered to be one biological replica as the same one viral population was studied, albeit in different birds. Thus, it cannot be precluded that other resistance-related substitutions would have evolved if the experiments had been repeated. However, if any one resistance-related substitution had a clear fitness advantage in this genetic backbone, we would likely have detected it (compare e.g. to H1N1 LPAIV, where H274Y has been detected in two different exposure experiments [13]).

Persistence of H274N and E119G

The persistence of the H274N and E119G substitutions was assessed by withdrawing ZA drug pressure from the water of birds where the H4N2 LPAIV was replicating.

For H274N, the mutated virus dominated the viral population, but by the last day in the second and third generations, the substitution was detected in the mixed genotype with wild-type. This indicates reduced fitness for the substitution in N2 and is in contrast to H274Y in N1, where an H1N1 LPAIV carrying the H274Y substitution has been replicating for multiple generations in the mallard model without any reversion to a mixed genotype [17, 19]. The reduced fitness is in line with previous work on the exposure of a N1 LPAIV to ZA [17] and another N2 LPAIV exposed to OC [20].

It is known that in N1 viruses, the E119G substitution also has impaired fitness [28, 30, 40]. However, N2 and N9 viruses with the substitution have been reported to replicate to equal or greater titres in embryonated chicken eggs, compared to the parent virus [27, 41]. In this study it was shown that the E119G substitution was still seen in the first generation after the drug pressure was removed, although the virus then reverted to wild-type in the second and third generations, where no E119G could be detected. This indicate that the wild-type virus outcompeted the mutant virus in the absence of ZA, which means that E119G does exhibit impaired viral fitness in this H4N2 LPAIV genetic makeup as compared to the corresponding wild-type virus.

Our results suggest that fitness is decreased more by E119G than by H274N in this genetic background (i.e. more rapid reversion to wild-type), which may increase the biological

significance of H274N. On the other hand, the impact of H274N on ZA sensitivity is limited.

Significance

The results in this study are in line with our previous work with H1N1 LPAIV and ZA in the mallard model. As compared to oseltamivir, the environmental resistance potential of ZA seems to be lower, as indicated by the following: (i) NA substitutions related to reduced ZA sensitivity were not observed at ZA concentrations lower than $10\text{ }\mu\text{g l}^{-1}$; (ii) in response to withdrawn drug pressure, no persistence of the substitutions was detected in either N1 or N2; (iii) the substitutions that emerged at lower ZA concentrations ($10\text{ }\mu\text{g l}^{-1}$) only resulted in a minor increase in ZA IC_{50} , likely resulting in low clinical significance; (iv) several substitutions emerged in response to ZA, in contrast to OC exposure, which seems to give rise to one specific dominant substitution. Furthermore, the river levels of ZA measured to date are far lower than the OC levels, decreasing the relative probability of ZA environmental resistance development at the present time.

Therefore, this study, together with previous findings, indicates that the development of resistance to ZA in the environment in response to drug residue pollution is less likely than the development of resistance to OC. In summary, *in vivo* mallard data indicate that ZA rather than OC is preferred from an environmental resistance development point of view, an important piece of information for policymakers and pandemic preparedness planners.

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Conflicts of interest

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Ethical statement

Animal experiments were performed at the Swedish National Veterinary Institute (SVA) under conditions approved by the Swedish Board of Agriculture and the Ethics Committee on Animal Experiments in Uppsala, Sweden (permits C125/12, C63/13 and C20/14).

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