JOURNAL OF GENERAL VIROLOGY

RESEARCH ARTICLE

Skog *et al.*, *Journal of General Virology* 2023;104:001835
DOI 10.1099/jgv.0.001835

MICROBIOLOGY SOCIETY

OPEN ACCESS

An oseltamivir-resistant avian H1N1 influenza A virus can transmit from mallards to chickens similarly to a wild-type strain: implications for the risk of resistance transmission to humans

Erik Skog[1], Marie Nykvist[2], Mahmoud M. Naguib[2], Michelle Wille[2]†, Caroline Bröjer[3], Viktoria Agarwal[2]‡, Patrik Ellström[1], Gabriel Westman[4], Åke Lundkvist[2] and Josef D. Järhult[1,*]

Abstract

The neuraminidase inhibitor (NAI) oseltamivir is stockpiled globally as part of influenza pandemic preparedness. However, oseltamivir carboxylate (OC) resistance develops in avian influenza virus (AIV) infecting mallards exposed to environmental-like OC concentrations, suggesting that environmental resistance is a real concern. Herein we used an *in vivo* model to investigate if avian influenza H1N1 with the OC-resistant mutation NA-H274Y (51833/H274Y) as compared to the wild-type (wt) strain (51833/wt) could transmit from mallards, which would potentially be exposed to environmentally contaminated environments, to and between chickens, thus posing a potential zoonotic risk of antiviral-resistant AIV. Regardless of whether the virus had the OC-resistant mutation or not, chickens became infected both through experimental infection, and following exposure to infected mallards. We found similar infection patterns between 51833/wt and 51833/H274Y such that, one chicken inoculated with 51833/wt and three chickens inoculated with 51833/H274Y were AIV positive in oropharyngeal samples more than 2 days consecutively, indicating true infection, and one contact chicken exposed to infected mallards was AIV positive in faecal samples for 3 consecutive days (51833/wt) and another contact chicken for 4 consecutive days (51833/H274Y). Importantly, all positive samples from chickens infected with 51833/H274Y retained the NA-H274Y mutation. However, none of the virus strains established sustained transmission in chickens, likely due to insufficient adaptation to the chicken host. Our results demonstrate that an OC-resistant avian influenza virus can transmit from mallards and replicate in chickens. NA-H274Y does not constitute a barrier to interspecies transmission per se, as the resistant virus did not show reduced replicative capacity compared to the wild-type counterpart. Thus, responsible use of oseltamivir and surveillance for resistance development is warranted to limit the risk of an OC-resistant pandemic strain.

INTRODUCTION

Influenza A virus (*Alphainfluenzavirus influenzae*) is a multi-host virus, and in humans comprises both endemic and pandemic strains followed zoonotic spill over events [1]. Three subtypes (H1N1, H2N2 and H3N2) have thus far caused sustained epidemics in humans [2]. Pandemic influenza viruses may evolve through interspecies transmission within the avian reservoir and/or to mammals including pigs and humans. The segmented genome of influenza A virus facilitate the generation of new pandemic viruses. Specifically, novel

Received 10 January 2023; Accepted 26 February 2023; Published 05 April 2023
Author affiliations: [1]Zoonosis Science Center, Department of Medical Sciences, Uppsala University, Uppsala, Sweden; [2]Zoonosis Science Center, Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden; [3]Department of Pathology and Wildlife Disease, Swedish Veterinary Institute, Uppsala, Sweden; [4]Department of Medical Sciences, Section of Infectious Diseases, Uppsala University, Uppsala, Sweden.
***Correspondence:** Josef D. Järhult, josef.jarhult@medsci.uu.se
Keywords: avian influenza; neuraminidase inhibitor; antiviral resistance; chicken; pandemic preparedness; oseltamivir.
Abbreviations: AIV, avian influenza virus; Ct, cycle threshold; days p.i., days post-inclusion; EID, egg infectious dose; F, faecal; IC, inhibitory concentration; IHC, immunohistochemistry; MUNANA, 20-(4-methylumbelliferyl)-α-d-N-acetylneuraminic acid; NA, neuraminidase; NAI, neuraminidase inhibitor; OC, oseltamivir carboxylate; OP, oropharyngeal; RT-PCR, reverse transcription polymerase chain reaction; SPF, specific pathogen free; wt, wild-type.
†Present address: Sydney Institute for Infectious Diseases, School of Life and Environmental Sciences and School of Medical Sciences, The University of Sydney, New South Wales, Sydney, Australia
‡Present address: Institute of Environmental Engineering, ETH, Zürich, Switzerland.
Two supplementary figures and one supplementary data file are available with the online version of this article.

Impact Statement

The neuraminidase inhibitor oseltamivir is stockpiled globally as part of influenza pandemic preparedness. Previous studies show that oseltamivir carboxylate (OC) resistance develops in avian influenza virus infecting mallards exposed to environmental-like OC concentrations suggesting that environmental resistance is a concern. An avian H1N1 strain with the OC resistance mutation NA-H274Y has retained resistance even in an environment without OC, suggesting maintained fitness. Domestic poultry act as an important amplifying host, a source of influenza virus evolution and may transmit influenza virus to humans. Here, we investigated if the oseltamivir-resistant strain can transmit from mallards to chickens and in between chickens and retain the resistance mutation. Our results demonstrate that regardless of the OC resistance mutation, infection was detected in experimentally infected chickens and chickens in contact with infected mallards. Stable transmission between chickens was not established, likely due to poor species adaptation of the virus. Resistant and wild-type strains had similar infection kinetics, and NA-H274Y was retained throughout the experiment. The resistance mutation NA-H274Y is stable in an environment without OC and does not constitute a barrier to interspecies transmission per se, thus demonstrating a risk of an oseltamivir-resistant pandemic virus.

progeny viral strains evolve through reassortment when two different viral strains infect the same cell where the segments can mix to form progeny viruses with one or more novel gene segments [3, 4]. The last four pandemic influenza A viruses (H1N1 1918, H2N2 1957, H3N2 1968 and H1N1 2009) emerged following reassortment events, and importantly comprise genetic material originating from avian influenza viruses (AIVs), but also from swine in at least the three most recent influenza pandemics [1, 5, 6]. Influenza A viruses from animal hosts that have acquired the ability to cross the species barrier can also infect humans by direct transmission, with key examples including highly pathogenic avian influenza H5N1, swine variant viruses in the USA and H7N9 avian influenza viruses causing human cases in China [7–9]. Primary introduction of AIV to poultry mainly occurs from wild birds, particularly waterfowl, with a higher occurrence of infection in poultry with exposure to the outdoor environment [10, 11]. In live poultry markets wild and domestic birds like poultry can commingle facilitating interspecies transmission of AIV [12]. Most of the human H7N9 cases have been associated with exposure to poultry and/or to poultry markets [13, 14].

Neuraminidase inhibitors (NAIs) act against both endemic and pandemic influenza A virus strains. Oseltamivir is the most commonly used NAI and is the standard of care therapy for patients with severe influenza infection. After the emerging cases of highly pathogenic avian influenza virus H5N1 with a case-fatality rate of >50% in humans in 2003 [9], oseltamivir was largely stockpiled as part of pandemic preparedness in high- and middle-income countries [15]. However, resistance development to oseltamivir in influenza A viruses is well documented in humans [16, 17], but also in experimentally infected mammals like cynomolgus macaques and ferrets [18–20]. Oseltamivir is administered as an oral prodrug, oseltamivir phosphate, that is converted to the active metabolite oseltamivir carboxylate (OC). The OC is excreted unchanged in the urine and is not efficiently removed during conventional sewage water treatment [21–23] nor is it readily degraded in the aquatic environment [24, 25]. Thus, OC has been detected in surface river water in concentrations up to 865 ng l^{-1} in Japan [26, 27] and up to 193 ng l^{-1} in the UK [28]. AIVs replicating in the intestines of wild waterfowl can thus get exposed to OC allowing for resistance development. Several *in vivo* studies exposing AIV-infected mallards to NAIs in experimental settings mimicking the natural exposure, support the occurrence of resistance development via this route [29–33]. For example, a low pathogenic AIV H1N1 strain developed oseltamivir resistance through the NA-H274Y (N2 numbering) amino acid substitution in the neuraminidase (NA) gene when exposed to 950 ng l^{-1} OC (the same magnitude as found in river water) in the water source of the mallards [34]. The mutation also persisted when OC exposure was removed, suggesting a negligible fitness cost [35]. When exposed to 80 µg l^{-1} the OC-resistant strain completely outcompeted the wt strain [34].

While the main reservoir for AIVs is waterfowl, such as mallards (*Anas platyrhynchos*), domestic poultry is an enormous amplifying host and source of viral evolution [36, 37]. There are some indications of the oseltamivir-resistant mutation NA-H274Y in chickens in Iran [38] and in few NA sequences submitted to GISAID (see Discussion). We aimed to better understand the putative pathway for oseltamivir resistance to enter the avian reservoir, particularly poultry. We have previously demonstrated high environmental contamination levels of OC in waterways [39] and have demonstrated the evolution and maintenance of OC resistance in AIVs in mallards, which are potentially using these waterways. Particularly in cold climates large number of mallards gather in the warm nutrient-rich waste water treatment plant effluent that is ice-free year-round where influenza infected mallards can be exposed to OC [21]. Occasional high levels of OC could create an advantage for an oseltamivir-resistant strain to be established in the mallard population [34]. A study with a NAI-resistant H5N6 strain has demonstrated equal or higher replicative capacity in primary chicken kidney cells and embryonated hen's eggs as compared to the corresponding wild-type strain, indicating that NAI resistance per se does not lower replicative fitness in chicken cells [40].

To investigate the ability of oseltamivir-resistant AIV to cross the wild bird (mallard)–domestic poultry (chicken) species barrier, we conducted *in vivo* experiments using an AIV H1N1 strain isolated from a wild mallard, and an isogenic strain carrying the resistance substitution NA-H274Y. An oseltamivir-resistant AIV strain adapted to chicken could potentially increase the risk

for a human-adapted virus with pandemic potential evolving through reassortment or direct transmission. This would make the oseltamivir stockpiles useless and would resemble the problematic situation with the SARS-CoV-2 pandemic, with limited options for treatment and prophylaxis before effective vaccines are widely distributed [6].

METHODS

Viruses

Two AIV isolates were used in this study; one wild-type H1N1 isolate A/mallard/Sweden/51833/2006 (hereafter referred to as 51833/wt) and a corresponding oseltamivir-resistant H1N1 isolate containing the resistance substitution NA-H274Y (hereafter referred to as 51833/H274Y). The wild-type virus, 51833/wt, was sampled from a wild mallard at Ottenby Bird Station, Öland, Sweden (GenBank accession number of the NA gene, AEA02276) [41]. The resistant isolate 51833/H274Y has evolved in previous experiments [34] when 51833/wt was exposed to OC in a similar bird model as used in the experiments in the present study.

Both isolates were propagated in specific-pathogen-free embryonated chicken eggs (ValoBioMedia, Osterholz-Scharmbeck, Germany) by the allantoic route. Harvested fluid was titrated using the Reed and Muench method [42] in embryonated chicken eggs to determine the median egg infectious dose (EID_{50}) and the neuraminidase gene was Sanger sequenced. The viral stocks of the isolates were stored at a final titre of $10^{7.84}$ EID_{50} ml^{-1}.

Experimental setup

Male mallards and female white leghorn chickens (*Gallus gallus domesticus*) were purchased from a commercial breeder and raised indoors in separate rooms at the animal facility at the National Veterinary Institute of Sweden. Housing, animal welfare and all experiments were done in accordance with regulations by the Swedish Board of Agriculture, and the studies were reviewed and approved by the Ethical Committee on Animal Experiments in Uppsala, Sweden (ethical permit no C61/15 and 5.8.18-14748/2017). Prior to infection experiments, all birds were tested for AIV infection as described below. Mallards used in the experiments were 3 months of age and chickens 5–7 weeks of age at inclusion. This avian model used allows for continuous transmission of influenza virus to influenza-naïve birds under controlled conditions, and has been used in previous AIV studies [34]. In the experimental room of the transmission experiment (described below) we used a separate cage with openings big enough to let the chickens but not the mallards in. The rooms contained a common water pool for swimming and drinking and feed *ad libitum* in both experiments. At predetermined endpoints, birds were euthanized using 100 mg kg^{-1} sodium pentobarbital veterinary (100 mg ml^{-1}) intravenously. After euthanasia, necropsies were performed on all chickens, and relevant organs were placed in formalin.

Transmission experiment

Two mallards (gen. 1) each were placed in separate experimental rooms, and were infected artificially by the oesophageal route with 1 ml (EID_{50} $10^{7,84}$) of 51833/wt and 51833/H274Y, respectively. Two additional influenza-naïve mallards (gen. 2) were introduced 3 days after the start of the experiment to each room and they were kept together for 2 days. Gen. 1 was euthanized 5 days after inclusion. The mallards were used to mimic natural transmission of influenza A virus. The primary endpoint of the study was to investigate the viral infection in chickens. Six influenza-naïve chickens (gen. 3) were introduced on experimental day 6 and co-housed with the gen. 2 mallards in each room. The mallards (gen. 2) were euthanized on experimental day 8. An additional six chickens were introduced (gen. 4, second generation of chickens) on experimental day 9 and gen. 3 (the first generation of chickens) was euthanized on the experimental day 11. The last generation of six chickens (gen. 5) was introduced on experimental day 12 and gen. 4 was euthanized on experimental day 14. The last generation of chickens (gen. 5) was euthanized on experimental day 17 (Fig. 1a).

Inoculation experiment

Nine chickens (gen. 1) each were placed in two separate experimental rooms and inoculated artificially oculo-nasally with 1 ml (EID_{50} $10^{7,84}$) of 51833/wt and 51833/H274Y, respectively. We selected this inoculation method because low pathogenic avian influenza A virus predominately infect respiratory epithelial cells in poultry [43]. Three chickens were euthanized 2 days p.i. (days post-inclusion) in order to investigate histopathological and immunohistochemical signs of influenza infection. On the same day, nine additional influenza-naïve chickens were introduced (gen. 2). On the experimental day 4 three chickens from gen. 2 were euthanized. The remaining six chickens from gen. 1 were euthanized on experimental day 5 and the remaining chickens from gen. 2 were euthanized on experimental day 7 (Fig. 2a).

Sampling, viral detection and NA genotyping

Serum samples from all birds were tested for anti-NP antibodies using the Avian Influenza Virus Antibody test kit (IDEXX Laboratories Europe, The Netherlands) prior to the infection experiment.

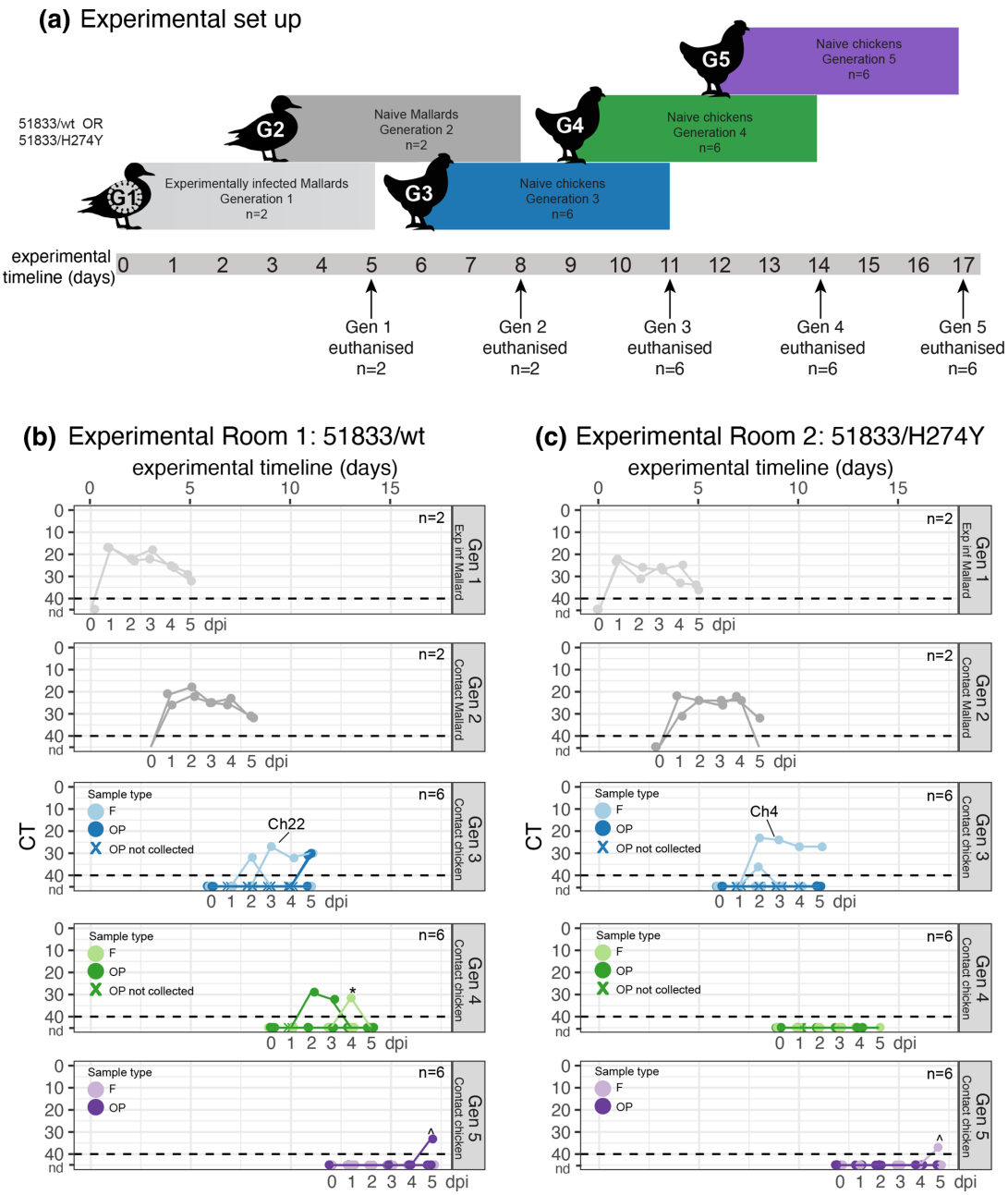

Fig. 1. Transmission experiment. (a) Experimental set up with overlapping bird generations. This same experimental set up was used in two experimental rooms, each with a different virus. Experimental room 1 with 51833/wt and experimental room 2 with 51833/H274Y. The first two generations of the experiment are mallards (gen. 1–gen. 2), followed by three chicken generations (gen. 3–gen. 5). Bird silhouettes indicate day of introduction into the experiment. Experimentally infected mallards are indicated by a duck silhouette comprising a virus silhouette. Gen. 1–gen. 5 within the bird silhouettes indicate the generation number, and the coloured box indicates the generation number, immunological status of birds and number of birds introduced. Time of euthanasia is indicated by arrows. Viral shedding results for concurrent experiments comprising the (b) 51833/wt infection in experimental room 1 and (c) 51833/H274Y infection in experimental room 2. Each panel comprises a different generation, named gen. 1–gen. 5, and sample sizes are included in each panel. Each point is the Ct value, connected with lines. The first two rows comprise mallard generation of which we only collected faecal samples and comprised only two birds each. For each of the chicken generations there were six birds and we collected both faecal (F) and oropharyngeal (OP) samples. For Gen. 3, we did not collect oropharyngeal samples for experimental days 7–10, and for gen. 4 we did not collect oropharyngeal samples for experimental day 10, as indicated by coloured X's. The Ct values are presented in reverse order, where the lowest Ct value indicates the highest viral shedding. 'n.d' indicates no virus was detected by real-time RT-PCR – a dashed line at 40 demonstrates the detection limit. Points with a '*' indicate that samples were positive in matrix PCR in one of two duplicate samples, but negative in NA-PCR. Points with a '^' indicate that samples were positive in the matrix PCR, but negative in NA-PCR. Individuals with notable results have been highlighted with animal numbers (ChX). Separate plots for each individual and sample type are presented in Fig. S1, available in the online version of this article. 'days p.i.' indicates days post-inclusion and Ct indicates cycle threshold. Bird silhouettes generated by M. Wille.

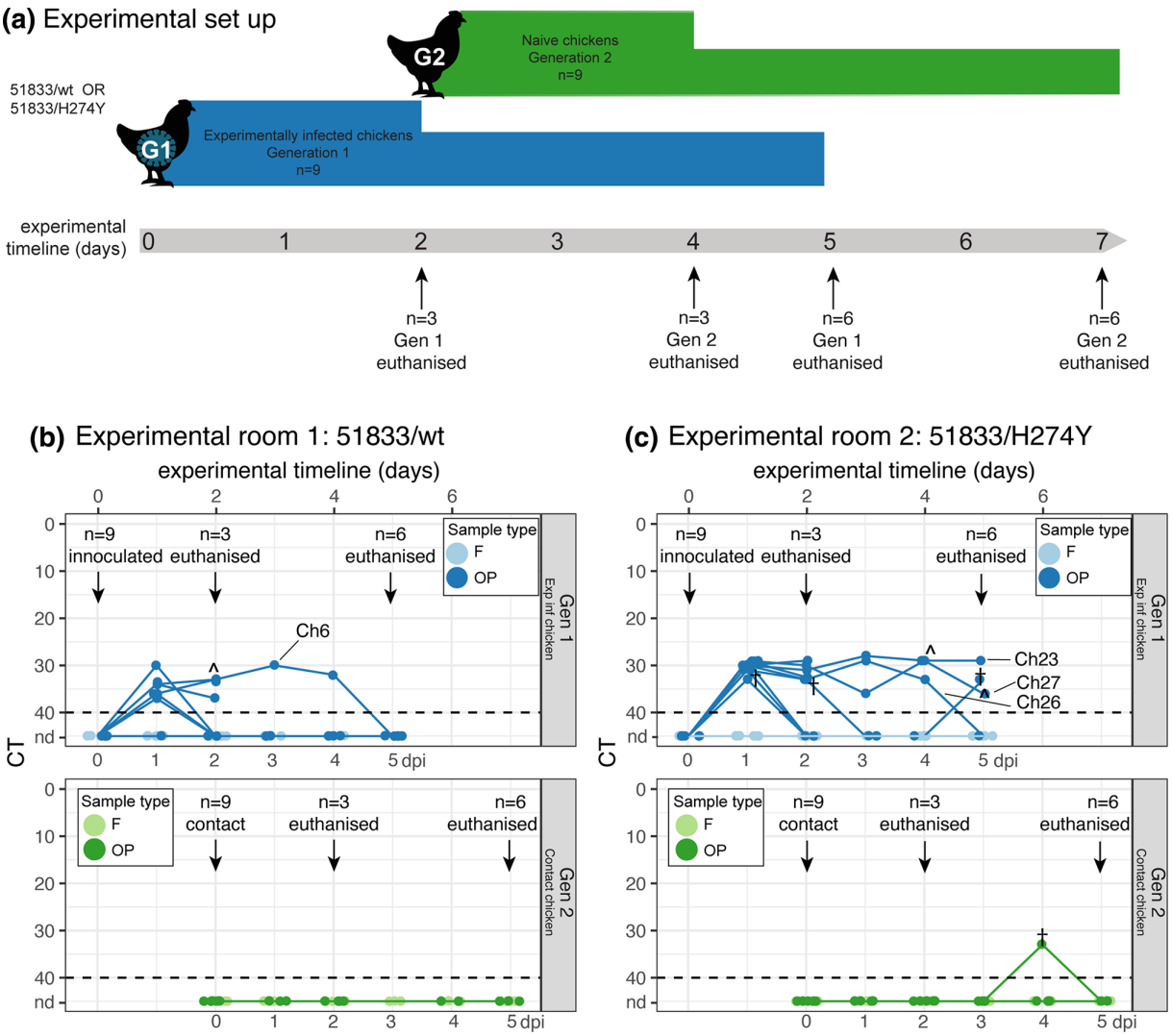

Fig. 2. Inoculation experiment. (a) Experimental set up with overlapping bird generations. This same experimental set up was used in two experimental rooms, each with a different virus. Experimental room 1 with 51833/wt and experimental room 2 with 51833/H274Y. In both experiments, the first generation comprised nine experimentally infected chickens, with the experiment undertaken with 51833/wt and 51833/H274Y, respectively. The second generation comprised nine immunologically naïve contact chickens. Chicken silhouettes indicate the day of introduction to the experimental room. Chickens were euthanized at two time points within each generation: generation 1 was euthanized at 2 days p.i. (*n*=3) and 5 days p.i. (*n*=6), and generation 2 was euthanized experimental day 4 at 2 days p.i. (*n*=3) and experimental day 7 at 5 days p.i. (*n*=9), as indicated by arrows. Viral shedding results for the experiment comprising the (b) 51833/wt infection in experimental room 1 and (c) 51833/H274Y infection in experimental room 2. Numbers of birds introduced and euthanized at different time points for each room are shown. Each panel comprises a different generation, named gen. 1–gen. 2. Each point is the Ct value, connected with lines. Both faecal (F) and oropharyngeal (OP) samples were collected from each individual on each day. The Ct values are presented in reverse order, where the lowest Ct value indicates the highest viral shedding. 'n.d' indicates no virus was detected by rRT-PCR – a dashed line at 40 is used to demonstrate the detection limit. Points with a '^' indicate that samples were positive in the matrix PCR, but negative in NA-PCR. Points with a '†' indicate that samples were positive in matrix and NA-PCR, but no sequence was obtained. Individuals with notable results have been highlighted with bird numbers (ChX). Separate plots for each individual and sample type are presented in Fig. S2, available in the online version of the article. 'days p.i.' indicates days post-inclusion and Ct indicates cycle threshold. Bird silhouettes generated by M. Wille.

Faecal (F) and/or oropharyngeal (OP) samples were collected daily from all birds using a sterile tipped applicator, and placed into viral transport media. The faecal samples comprised droppings of birds which were placed in separate clean boxes. All samples were frozen immediately in −70 °C before processing.

RNA was extracted with a Maxwell 16 Viral Total Nucleic Acid Purification Kit in a Maxwell 16 Instrument Extraction Robot (Promega Biotech, Wisconsin, USA). A real time RT-PCR targeting the conserved influenza matrix gene was run in a RotorGene-6000 (Qiagen, The Netherlands), reaction volume 20 µl using an iTaq Universal One-Step reverse transcriptase PCR kit (Bio-Rad,

Table 1. Primers for NA amplification and Sanger sequencing

Primer	Sequence (5'–3')	Location (5'–3')	Application
FwY_N1_51833_AG	TGGAATAGCCAGTTTGATGTTACA	51–74	Amplification and sequencing
RevY_N1_51833_AG_IC	AAGACCAACCCACAGTGTCACTAT	1346–1369	Amplification and sequencing
51785_GAP601_FW	CTCATGCTCCCACTTGGAAT	366–385	Sequencing
RevI_N1_51833_AG_IC	ATCATTGGGGCGTGGATTGT	971–990	Sequencing

Hercules, CA, USA) according to protocol by Spackman *et al.* [44] with the modified temperature profile 50 °C for 30 min, 94 °C for 10 min, 50 cycles of 94 °C for 10 s and 58 °C for 20 s. Samples with a Ct-value >40 were considered negative.

Conventional PCR targeting the NA gene was run on all real-time RT-PCR positive samples using a Superscript III One-Step RT-PCR Platinum *Taq* HiFi kit (Thermo Fisher Scientific, Waltham, MA, USA) and in-house primers (Table 1). A reaction volume of 25 µl containing 0.25 µl enzyme mix, final primer concentrations of 400 nM each and 5 µl RNA sample was used. The temperature profile was 55 °C for 30 min, 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 68 °C for 1 min and a final extension of 68 °C for 5 min. PCR product was confirmed with gel electrophoresis. PCR products were cleaned with Illustra ExoProStar 1-step reagent (GE Healthcare, Chicago, IL, USA) and sent for Sanger sequencing to Macrogen, Amsterdam, Holland using in-house primers (Table 1).

Sequences were analysed using SeqScape v2.7 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with the 51833/wt sequence as a reference. At least two high-quality electropherograms were required in order to consider the sequence reliable at any given nucleotide position.

Egg propagation and neuraminidase inhibition testing

Three to four faecal samples from a chicken that excreted the virus more than two consecutive days from the 51833/wt and 51833/H274Y group, respectively, of the transmission experiment were propagated in specific pathogen free (SPF) embryonated chicken eggs (Valo, Germany). In the inoculation experiment three to four oropharyngeal samples from chickens who excreted the virus for more than 2 days consecutively (one chicken in the 51833/wt group and three chickens in the 51833/H274Y group) were propagated in SPF embryonated chicken eggs. Allantoic fluids of the eggs were assessed for influenza A virus presence using standard hemagglutination test and real-time RT-PCR targeting the matrix gene.

The egg propagated faecal samples from the transmission experiment were assessed for phenotypic NAI resistance. NA activity and inhibition was assessed using the fluorescence assay with the substrate 20-(4-methylumbelliferyl)-α-D-N-acetylneuramic acid (MUNANA) (Sigma-Aldrich, St Louis, MI, USA). NA inhibition by OC was tested in duplicate samples following the protocol of the Respiratory Virus Unit, Health Protection Agency, London, UK [45]. Fluorescence was measured using a micro plate reader Infinite M1000 PRO (Tecan Group, Zürich, Switzerland). IC_{50} for OC were determined from the best-fit dose-response curves using the protocol [46].

Histopathology and immunohistochemistry

In the transmission experiment, organ samples from chickens that excreted virus for consecutive days were analysed with histopathology and immunohistochemistry (IHC) staining of AIV nucleoprotein in cloacal bursa, lung and bronchus, duodenum, jejunum, ileum, caecum and colon. In the inoculation experiment, organ samples from two positive chickens from each room euthanized at 2 days p.i. were analysed with histopathology and IHC using the same organs as in the transmission experiment with the addition of nasal concha, brain, spleen and liver. Tissues were embedded in paraffin wax and four-micron-thick sections were prepared and stained with haematoxylin and eosin (H and E). Duplicate sections were processed for IHC using a commercial anti-influenza A nucleoprotein primary monoclonal antibody (EBS-1–238, Biologicals LTD) as previously described by [47].

RESULTS

Pre-experiment AIV results

All birds were negative for influenza A virus antibodies prior to the infection experiments. All birds, except one, were negative by real-time RT-PCR for AIV prior to inclusion. Specifically, in the inoculation experiment, one chicken from gen. 2 of the 51833/H274Y group (chicken 29) tested positive in real-time RT-PCR in oropharyngeal sample (Ct value 32–33) but negative in faecal sample before entry to the experimental room. The same chicken tested negative in both faecal and oropharynx the following 2 days before it was euthanized. This, in addition to the fact that no other samples were positive prior to inclusion, strongly indicates that the chicken was not influenza infected before inclusion in the experiment.

Transmission experiment

In the transmission experiment we aimed to determine whether chickens could be infected from co-housed mallards, and whether this virus would propagate through multiple generations of chickens. Following oesophageal inoculation, all mallards were infected starting at 1 day p.i. and followed similar shedding pattern as seen in previous experiments using the same bird model [29–32, 34, 35]. These experimentally infected mallards successfully infected the contact mallards (gen. 2) (Fig. 1b, c in Methods). We utilized a second generation of mallards as it is well established that AIV-infection patterns of experimentally infected birds may differ from naturally infected birds, and we wanted to test whether this virus was able to transmit from 'naturally' infected mallards to chickens. In the experiment using 51833/wt, chicken 22 (gen. 3) shed virus in faeces for 3 consecutive days and the last day this bird was also positive in OP swabs (Fig. 1b in Methods). In the experiment using 51833/H274Y, chicken 4 (gen. 3) shed the virus for 4 consecutive days in faeces (Fig. 1c in Methods). In both groups, one individual (chicken 23, and chicken 2, respectively), were positive for only a single day (faecal sample Ct 32 and 36, respectively) (Fig. 1b, c in Methods and Fig. S1). Due to technical reasons oropharyngeal samples were not collected the first 4 days p.i. in gen. 3 (the first chicken generation) and the first day p.i. in gen. 4 (the second chicken generation) in any of the experimental rooms. One bird in gen. 4 (chicken 25) in the 51833/wt group was positive in oropharynx 2 days in a row (2–3 days p.i.). The same chicken was negative in all faecal samples (Fig. 1b in Methods).

Sanger sequencing of the neuraminidase gene confirmed that all positive faecal samples (n=24) from the 51833/H274Y group had maintained resistance substitution (NA-H274Y) and had no other substitutions in the neuraminidase gene.

Inoculation experiment

In the inoculation experiment we aimed to determine the shedding pattern of an OC-resistant H1N1 strain in chickens when experimentally inoculated oculo-nasally. All faecal samples collected over the duration of the experiment, regardless of whether we used 51833/wt or 51833/H274Y were negative. One chicken out of nine shed virus oropharyngeally for more than 2 consecutive days when infected with 51833/wt (chicken 6) (1–4 days p.i.) (Fig. 2b in Methods). In the 51833/H274Y group, three out of nine chickens shed the virus for more than 2 consecutive days in OP (chicken 23, 26 and 27, from day 1 to day 3–5 p.i.) (Fig. 2c in Methods). In the 51833/wt group an additional two chickens were positive in OP 2 consecutive days (chicken 2 and 3) and in the 51833/H274Y group an additional three chickens (chickens 19, 20 and 22) were positive in OP 2 consecutive days (one sample was positive in matrix and NA-PCR but no sequence could be obtained, chicken 19) in gen. 1 (Fig S2). In gen. 2 one sample in the 51833/H274Y group was positive in real-time RT-PCR but negative in NA-PCR (chicken 34) (Fig. 2c in Methods). All other samples were negative in real-time RT-PCR. All positive samples in the 51833/H274Y group where a sequence could be obtained (n=20) maintained NA-H274Y and had no other amino acid substitutions in the neuraminidase gene.

Egg propagation and neuraminidase inhibition testing

From the transmission experiment (Fig. 1 in Methods), positive faecal samples from 3 consecutive days from one chicken in the 51833/wt group (chicken 22) and from 4 consecutive days from one chicken in the 51833/H274Y group (chicken 4) were successfully propagated in SPF embryonated chicken eggs (n=7). From the inoculation experiment (Fig. 2 in Methods) positive OP samples from 3 consecutive days from one bird (chicken 6) of the 51833/wt group and positive OP samples from 3 to 4 consecutive days from three birds (chickens 23, 26 and 27) of the 51833/H274Y group (n=14) were all successfully propagated in SPF embryonated chicken eggs apart from one sample (chicken 27 at 5 days p.i.). Real-time RT-PCR targeting the matrix gene on egg isolates showed lower Ct values than the corresponding experimental sample.

In order to investigate phenotypic resistance egg propagated samples from the transmission experiment were tested for IC_{50} for OC. The mean value for tested samples from the 51833/wt room was 1.2 nM and 387.7 nM for tested samples from the 51833/H274Y room, a 320-fold lower sensitivity to OC (Table 2). The IC_{50} for the inoculated 51833/wt isolate was 0.8 nM as compared to 405 nM for the inoculated 51833/H274Y isolate, a difference in sensitivity to OC of the same magnitude.

Histopathology and immunohistochemistry

In the transmission experiment with the 51833/wt, chicken 22 that shed the virus for 3 consecutive days (Fig. 1b in Methods, Fig S1A) showed no positive staining in the cytoplasm or nuclei of epithelial cells in any of the analysed organs at 5 days p.i., but positive staining particulate matter was present in the lumen of the caecum and colon, as well as associated to superficial epithelial cells (Fig. 3). There were also a few positive cells in the lamina propria.

In the 51833/H274Y group, chicken 4 that shed the virus for 4 days consecutively (Fig. 1c in Methods, Fig S1B) had positive staining particulate matter in the lumen of the caecum and the colon (Fig. 4). No positive cells were observed with IHC. No histopathological lesions or signs of influenza antigen were observed in chickens analysed from the inoculation experiment.

Table 2. Neuraminidase inhibition susceptibility (IC_{50}) for OC of isolates from positive faecal samples from chickens that shed virus for consecutive days in the transmission experiment. One isolate per day p.i. and experiemntal group was tested. 'days p.i.' indicates days post-inclusion

Days p.i.	51833/wt (nM)	51833/H274Y (nM)
0	–	–
1	–	–
2	–	404.9
3	0.9	409.2
4	1.3	404.1
5	1.4	332.4
Mean	**1.2**	**387.7**

DISCUSSION

In order to assess the interspecies transmission capacity of an oseltamivir-resistant influenza virus, a crucial step in formation of pandemic viruses, we performed *in vivo* transmission experiments between mallards and chickens as well as chicken inoculation experiments. We demonstrated limited interspecies transmission, with no differences between wild-type and resistant virus.

In the transmission experiment, one chicken out of six in the 51833/wt group and one chicken out of six in the 51833/H274Y group became contact infected. AIV replication was supported by 3 consecutive days of positive faecal samples (3–5 days p.i.) and a positive OP sample for the wild-type virus, and by 4 consecutive days of positive faecal samples (2–5 days p.i.) for the 51833/H274Y virus. In addition, Ct values were stable over time and virus could be cultured from faecal samples from both chickens, further supporting virus replication rather than contamination or ingestion of virus. The resistance substitution NA-H274Y was retained in all positive samples from both mallards and chickens in the transmission experiment utilizing 51833/H274Y as the inoculum, despite the absence of OC. Resistance against oseltamivir of the same level, as has been described previously for this amino acid substitution, was confirmed phenotypically for all tested samples [34]. No definite signs of virus infection were observed with histopathology and IHC with only a few influenza positive cells in the colon of the 51833/wt exposed chicken. The positively staining particulate matter that was seen in both chickens in the lumen of the caecum and colon as well as directly

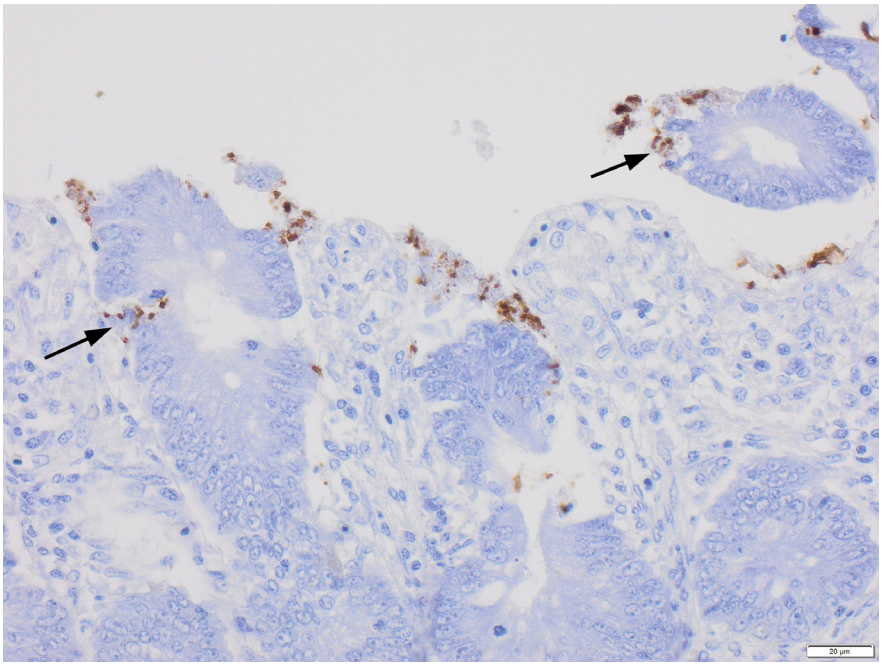

Fig. 3. Caecum, contact chicken (Ch 22) at 5 days p.i. in the transmission experiment where mallards were inoculated with 51833/wt. Positive staining particulate matter in lumen and associated to epithelial cells (arrows). Immunohistochemistry with anti-avian influenza nucleoprotein antibody. ''days p.i.' indicates days post-inclusion.

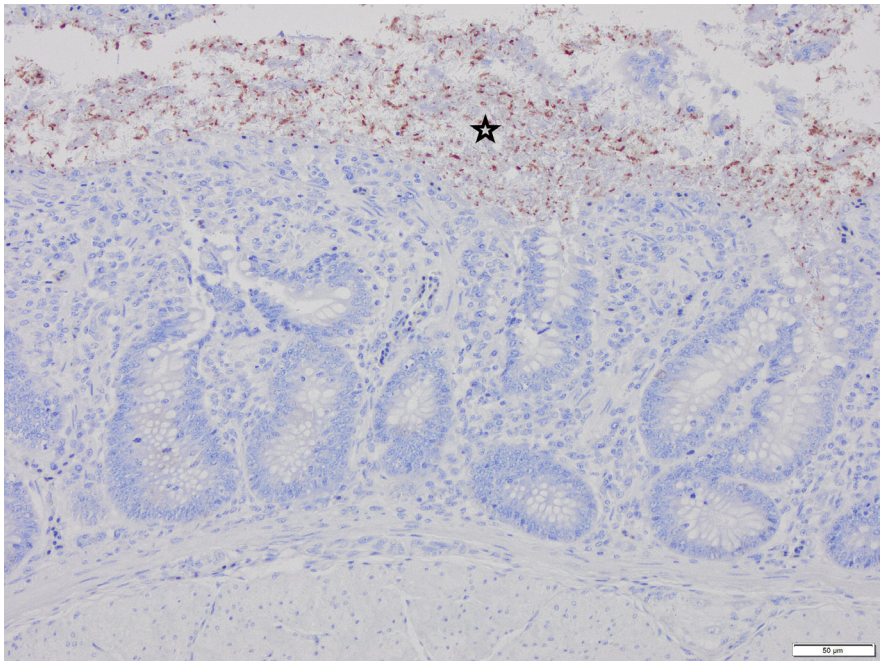

Fig. 4. Colon, contact chicken (Ch 4) at 5 days p.i. in the transmission experiment where mallards were inoculated with 51833/H274Y. Positive staining particulate matter in lumen (star). Immunohistochemistry with anti-avian influenza nucleoprotein antibody. 'days p.i.' indicates days post-inclusion.

associated with epithelial cells, could be remains from apoptotic or sloughed cells infected with avian influenza virus. Unspecific staining can, however, not be ruled out. No histopathological signs of infection were observed in the lungs in any of the birds at 5 days p.i. It is possible that signs of respiratory infection would have been observed if necropsies had been performed closer to exposure. In a previous study where white leghorn chickens were intranasally and intratracheally inoculated with four different influenza subtypes, virus was detected in trachea and lung tissue at 1 day p.i., particularly in subtypes H4N8 and H9N2, indicating early local replication [48]. Overall, since Ct values in the experiment were relatively high and IHC only was analysed in duplicate sections of organs there is a chance that a low-grade infection could have been undetected with IHC. In gen. 4 of the transmission experiment (the second generation of chickens), one chicken in the 51833/wt group was positive in oropharyngeal samples for 2 consecutive days but shed no virus in the faeces. Positive OP samples might be due to transmission of the wild-type virus between chickens, but contamination at sampling or ingestion of virus cannot be excluded because positivity was only seen for 2 consecutive days and no other positive samples were observed in the second or third generation of chickens in any of the groups.

In the inoculation experiment, we used a route of inoculation more suited to imitating respiratory infections (oculo-nasal infection), as influenza virus predominately causes respiratory infection in poultry, pigs and humans as opposed to gastrointestinal infection, which is more common in mallards [3, 36, 49]. We found no positive faecal samples in any birds in the experiments, but positive OP samples on more than 2 consecutive days in one out of nine chickens in the 51833/wt group (1–4 days p.i.) and three out of nine in the 51833/H274Y group (from day 1 to day 3–5 p.i.) with stable Ct values over time. Virus could be cultured from OP samples in chicken eggs resulting in isolates with lower Ct values than corresponding experimental samples, demonstrating infectious virus and supporting respiratory tract infection. In each group, an additional two chickens were positive for 2 consecutive days but were euthanized at 2 days p.i. Hence, we cannot exclude that these chickens would have been positive for additional days if they had not been euthanized. Six out of nine chickens of the first generation in the 51833/wt group and all chickens of the first generation in the 51833/H274Y group were positive at 1 day p.i. In chickens that were positive only 1 or 2 consecutive days, it cannot be excluded that this is due to contamination at sampling or ingestion and not viral replication even though earlier studies support early respiratory tract infection at 1 day p.i. in chickens [48]. The resistance substitution NA-H274Y was retained in all positive samples from the 51833/H274Y group where sequence could be obtained (n=20). No signs of infection were observed with histopathology or IHC, probably due to the limited infection with no PCR-positive faecal samples and generally high Ct values for OP samples.

Limitations of our study include the unavailability of OP samples the first 4 days after chickens were introduced in the transmission experiment, which makes it difficult to evaluate respiratory infection during that time. The infected chicken in the 51833/wt group (chicken 22) was positive also in OP sample 5 days p.i. It cannot be ruled out that additional OP samples earlier in the experiment would have been positive given the results of the inoculation experiment where the infection was predominately

respiratory. Furthermore, infection in chickens was limited in all groups and experiments of this study. This is likely due to insufficient adaptation of the H1N1 mallard strain to chickens [50].

In conclusion, the results of this study demonstrate that an oseltamivir-resistant avian influenza A H1N1 virus strain can transmit from mallards and replicate in chickens, in an experimental setting mimicking conditions suitable for natural transmission, with no difference to corresponding wild-type virus. The resistance mutation NA-H274Y does not constitute a barrier to interspecies transmission per se as the resistant virus did not show reduced replicative capacity compared to the wild-type counterpart in infected chickens. However, neither of the two virus strains were able to establish a sustained transmission in chickens in the two different experiments.

The subtype used in this study (H1N1) is of particular concern since it has been found to cross the species barriers between pigs, humans and poultry [10, 51]. The resistance substitution NA-H274Y has been established in a H1N1 human influenza strain dominating the season 2008–2009 [16, 52] and has also been found in highly pathogenic H5N1 isolates from oseltamivir-treated patients [53, 54]. H5N1 containing NA-H274Y has also been found in backyard chickens in Iran [38]. In that study full sequence analysis for HA and NA was performed on virus samples from dead backyard chicken flocks, but it is not mentioned how many birds were sampled. Phenotypic resistance testing was not performed in that study. A search in the GISAID database for all avian N1 influenza resulted in 10 441 sequences of which only seven contained the H274Y amino acid substitution. All sequences were identified in H5N1 strains. The sequences were obtained from a turkey in New Hampshire (USA) 2022, a great horned owl (*Bubo virginianus*) in Massachusetts (USA), a chicken in Maine (USA) 2022, a mute swan (*Cygnus olor*) in Caspian Sea (Russian Federation) 2006, a chicken in Hong Kong (China) 2002 and two strains from swans in Astrakhan (Russian Federation) 2005. These two strains had identical NA sequences, which might be due to the fact that it is the same virus sampled from two different birds, but it cannot be ruled out that this is a duplicate submission. For none of the strains it has been commented on the resistant substitution and no phenotypic resistant testing has been performed. The same search in GenBank resulted in additionally one strain containing NA-H274Y (GenBank accession number of the NA gene, ADU17040). Interestingly this was a H1N1 strain sampled from a duck in Minto Flats, Interior Alaska (USA) 2007. This is an area of high densities of nesting ducks.

The oseltamivir-resistance substitution NA-H274Y has been found stable even in an environment without oseltamivir in infected mallards, indicating limited or no fitness cost and a possibility to be retained in nature also during periods without OC presence [35]. Thus, our results suggest that an OC-resistant AIV strain evolved in the wild bird population during occasionally high levels of OC in the environment might have the potential to be introduced into the poultry population after adaptation [55]. If NA-H274Y would be established in chicken populations, further transmission to humans as a potentially pandemic virus, either via direct transmission or after reassortment might be possible [2, 56].

A pandemic oseltamivir-resistant influenza A virus would resemble the SARS-CoV-2 pandemic with initially limited options for prophylaxis or treatment, as the influenza pandemic preparedness rely heavily on oseltamivir stockpiles [57]. The new influenza drug baloxavir marboxil is a cap-dependent endonuclease inhibitor [58] and might be a treatment option, but global availability is currently limited and it will most likely be difficult to meet the raising demand of the drug in a pandemic situation. In addition, resistance development for baloxavir marboxil has been observed and might develop further with large-scale use [59, 60]. Hence, responsible use of oseltamivir and surveillance for resistance development is warranted.

Funding information
We acknowledge financial support from The Swedish Research Council Vetenskapsrådet to JJ (grant no 2016-02606), The Swedish Research Council FORMAS to JJ (grant no 2016-00790) and The Family Olinder-Nielsen´s Foundation to ES. The funders had no role in planning, performing or evaluating the study.

Acknowledgements
We gratefully acknowledge the staff, in particular Susanne Bloemberg, at the Animal Facility at Swedish Veterinary Institute for technical assistance and great animal care. We also gratefully acknowledge all data contributors, i.e. the authors and their originating laboratories responsible for obtaining the specimens, and their submitting laboratories for generating the genetic sequence and metadata and sharing via the GISAID, on which some of this research is based. Data supporting this study is based on sequences available on GISAID up to 9 February 2023 - please see the Supplementary Data file, available with the online version of this article.

Author contribution
J.J., E.S., M.N., M.M.N. contributed to conception and design of the study. E.S., M.W. and V.A. performed the lab work. E.S., M.N. and M.M.N. performed animal experiments. Histopathology and immunohistochemistry were made by C.B. Data analysis was made by E.S. and J.J. E.S. wrote the first draft of the manuscript. J.J., M.W. and M.M.N. provided critical revision of the manuscript. Supervision was made by J.J., P.E. and Å.L.
All authors contributed to manuscript revision, read and approved the submitted version.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The studies were reviewed and approved by the Ethical Committee on Animal Experiments in Uppsala, Sweden (ethical permit no C61/15 and 5.8.18-14748/2017).

References

1. Yen HL, Webster RG. Pandemic influenza as a current threat. *Curr Top Microbiol Immunol* 2009;333:3–24.
2. Mostafa A, Abdelwhab EM, Mettenleiter TC, Pleschka S. Zoonotic potential of influenza A viruses: a comprehensive overview. *Viruses* 2018;10:497.
3. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev* 1992;56:152–179.
4. Vijaykrishna D, Mukerji R, Smith GJD. RNA virus reassortment: an evolutionary mechanism for host jumps and immune evasion. *PLoS Pathog* 2015;11:e1004902.
5. Belshe RB. The origins of pandemic influenza-lessons from the 1918 virus. *N Engl J Med* 2005;353:2209–2211.
6. Subbarao K. The critical interspecies transmission barrier at the animal–human interface. *Trop Med Infect Dis* 2019;4:72.
7. Xu X, Subbarao K, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 1999;261:15–19.
8. de Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL. A pandemic warning? *Nature* 1997;389:554.
9. Li KS, Guan Y, Wang J, Smith GJD, Xu KM, *et al*. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 2004;430:209–213.
10. Alexander DJ. An overview of the epidemiology of avian influenza. *Vaccine* 2007;25:5637–5644.
11. Tian H, Zhou S, Dong L, Van Boeckel TP, Cui Y, *et al*. Avian influenza H5N1 viral and bird migration networks in Asia. *Proc Natl Acad Sci* 2015;112:172–177.
12. Wang C, Wang J, Su W, Gao S, Luo J, *et al*. Relationship between domestic and wild birds in live poultry market and a novel human H7N9 virus in China. *J Infect Dis* 2014;209:34–37.
13. Zhou X, Li Y, Wang Y, Edwards J, Guo F, *et al*. The role of live poultry movement and live bird market biosecurity in the epidemiology of influenza A (H7N9): A cross-sectional observational study in four eastern China provinces. *J Infect* 2015;71:470–479.
14. Fan M, Huang B, Wang A, Deng L, Wu D, *et al*. Human influenza A(H7N9) virus infection associated with poultry farm, Northeastern China. *Emerg Infect Dis* 2014;20:1902–1905.
15. Wan Po AL, Farndon P, Palmer N. Maximizing the value of drug stockpiles for pandemic influenza. *Emerg Infect Dis* 2009;15:1686–1687.
16. Hurt AC. The epidemiology and spread of drug resistant human influenza viruses. *Curr Opin Virol* 2014;8:22–29.
17. Takashita E, Daniels RS, Fujisaki S, Gregory V, Gubareva LV, *et al*. Global update on the susceptibilities of human influenza viruses to neuraminidase inhibitors and the cap-dependent endonuclease inhibitor baloxavir, 2017-2018. *Antiviral Res* 2020;175:104718.
18. Marjuki H, Mishin VP, Chesnokov AP, De La Cruz JA, Davis CT, *et al*. Neuraminidase mutations conferring resistance to oseltamivir in influenza A(H7N9) viruses. *J Virol* 2015;89:5419–5426.
19. Kiso M, Iwatsuki-Horimoto K, Yamayoshi S, Uraki R, Ito M, *et al*. Emergence of oseltamivir-resistant H7N9 influenza viruses in immunosuppressed cynomolgus macaques. *J Infect Dis* 2017;216:582–593.
20. Govorkova EA, Ilyushina NA, Boltz DA, Douglas A, Yilmaz N, *et al*. Efficacy of oseltamivir therapy in ferrets inoculated with different clades of H5N1 influenza virus. *Antimicrob Agents Chemother* 2007;51:1414–1424.
21. Fick J, Lindberg RH, Tysklind M, Haemig PD, Waldenström J, *et al*. Antiviral oseltamivir is not removed or degraded in normal sewage water treatment: implications for development of resistance by influenza A virus. *PLoS One* 2007;2:e986.
22. Leknes H, Sturtzel IE, Dye C. Environmental release of oseltamivir from a Norwegian sewage treatment plant during the 2009 influenza A (H1N1) pandemic. *Sci Total Environ* 2012;414:632–638.
23. He G, Massarella J, Ward P. Clinical pharmacokinetics of the prodrug oseltamivir and its active metabolite Ro 64-0802. *Clin Pharmacokinet* 1999;37:471–484.
24. Gonçalves C, Pérez S, Osorio V, Petrovic M, Alpendurada MF, *et al*. Photofate of oseltamivir (Tamiflu) and oseltamivir carboxylate under natural and simulated solar irradiation: kinetics, identification of the transformation products, and environmental occurrence. *Environ Sci Technol* 2011;45:4307–4314.
25. Accinelli C, Saccà ML, Fick J, Mencarelli M, Lindberg R, *et al*. Dissipation and removal of oseltamivir (Tamiflu) in different aquatic environments. *Chemosphere* 2010;79:891–897.
26. Takanami R, Ozaki H, Giri RR, Taniguchi S, Hayashi S. Antiviral drugs zanamivir and oseltamivir found in wastewater and surface water in Osaka, Japan. *J Wat Envir Tech* 2012;10:57–68.
27. Takanami R, Ozaki H, Giri RR, Taniguchi S, Hayashi S. Detection of antiviral drugs oseltamivir phosphate and oseltamivir carboxylate in Neya river, Osaka, Japan. *J Wat Envir Tech* 2010;8:363–372.
28. Singer AC, Järhult JD, Grabic R, Khan GA, Lindberg RH, *et al*. Intra- and inter-pandemic variations of antiviral, antibiotics and decongestants in wastewater treatment plants and receiving rivers. *PLoS One* 2014;9:e108621.
29. Gillman A, Nykvist M, Muradrasoli S, Söderström H, Wille M, *et al*. Influenza A(H7N9) virus acquires resistance-related neuraminidase I222T substitution when infected mallards are exposed to low levels of oseltamivir in water. *Antimicrob Agents Chemother* 2015;59:5196–5202.
30. Gillman A, Muradrasoli S, Söderström H, Nordh J, Bröjer C, *et al*. Resistance mutation R292K is induced in influenza A(H6N2) virus by exposure of infected mallards to low levels of oseltamivir. *PLoS One* 2013;8:e71230.
31. Nykvist M, Gillman A, Söderström Lindström H, Tang C, Fedorova G, *et al*. *In vivo* mallard experiments indicate that zanamivir has less potential for environmental influenza A virus resistance development than oseltamivir. *J Gen Virol* 2017;98:2937–2949.
32. Tepper V, Nykvist M, Gillman A, Skog E, Wille M, *et al*. Influenza A/H4N2 mallard infection experiments further indicate zanamivir as less prone to induce environmental resistance development than oseltamivir. *J Gen Virol* 2020;101:816–824.
33. Achenbach JE, Bowen RA. Effect of oseltamivir carboxylate consumption on emergence of drug-resistant H5N2 avian influenza virus in Mallard ducks. *Antimicrob Agents Chemother* 2013;57:2171–2181.
34. Järhult JD, Muradrasoli S, Wahlgren J, Söderström H, Orozovic G, *et al*. Environmental levels of the antiviral oseltamivir induce development of resistance mutation H274Y in influenza A/H1N1 virus in mallards. *PLoS One* 2011;6:e24742.
35. Gillman A, Muradrasoli S, Söderström H, Holmberg F, Latorre-Margalef N, *et al*. Oseltamivir-resistant influenza A (H1N1) virus strain with an H274Y mutation in neuraminidase persists without drug pressure in infected mallards. *Appl Environ Microbiol* 2015;81:2378–2383.
36. Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus ADME, *et al*. Global patterns of influenza A virus in wild birds. *Science* 2006;312:384–388.
37. Gilbert M, Xiao X, Robinson TP. Intensifying poultry production systems and the emergence of avian influenza in China: a "One Health/Ecohealth" epitome. *Arch Public Health* 2017;75:48.
38. Yegani S, Shoushtari AH, Eshratabadi F, Molouki A. Full sequence analysis of hemagglutinin and neuraminidase genes and proteins of highly pathogenic avian influenza H5N1 virus detected in Iran, 2015. *Trop Anim Health Prod* 2019;51:605–612.
39. Söderström H, Järhult JD, Olsen B, Lindberg RH, Tanaka H, *et al*. Detection of the antiviral drug oseltamivir in aquatic environments. *PLoS One* 2009;4:e6064.
40. Bialy D, Shelton H. Functional neuraminidase inhibitor resistance motifs in avian influenza A(H5Nx) viruses. *Antiviral Res* 2020;182:104886.

41. Latorre-Margalef N, Tolf C, Grosbois V, Avril A, Bengtsson D, *et al*. Long-term variation in influenza A virus prevalence and subtype diversity in migratory mallards in northern Europe. *Proc Biol Sci* 2014;281:20140098.
42. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938;27:493–497.
43. Pantin-Jackwood MJ, Swayne DE. Pathogenesis and pathobiology of avian influenza virus infection in birds. *Rev Sci Tech* 2009;28:113–136.
44. Spackman E, Suarez DL. Type A influenza virus detection and quantitation by real-time RT-PCR. *Methods Mol Biol* 2008;436:19–26.
45. Lackenby A. Respiratory virus unit HPA. influenza MUNANA neuraminidase activity and inhibition assay (flourescent IC50 assay) SOP no V-6815/01-10; (n.d.)
46. Lackenby A. Respiratory Virus Unit HPA; (n.d.). www.isirv.org/site/index.php/methodology/nai-assay-ic50
47. Bröjer C, Agren EO, Uhlhorn H, Bernodt K, Mörner T, *et al*. Pathology of natural highly pathogenic avian influenza H5N1 infection in wild tufted ducks (*Aythya fuligula*). *J Vet Diagn Invest* 2009;21:579–587.
48. Bergervoet SA, Germeraad EA, Alders M, Roose MM, Engelsma MY, *et al*. Susceptibility of chickens to low pathogenic avian influenza (LPAI) viruses of wild bird- and poultry-associated subtypes. *Viruses* 2019;11:1010.
49. Wille M, Bröjer C, Lundkvist Å, Järhult JD. Alternate routes of influenza A virus infection in Mallard (*Anas platyrhynchos*). *Vet Res* 2018;49:110.
50. Swayne DE, Slemons RD. Comparative pathology of a chicken-origin and two duck-origin influenza virus isolates in chickens: the effect of route of inoculation. *Vet Pathol* 1994;31:237–245.
51. Starick E, Fereidouni SR, Lange E, Grund C, Vahlenkamp T, *et al*. Analysis of influenza A viruses of subtype H1 from wild birds, turkeys and pigs in Germany reveals interspecies transmission events. *Influenza Other Respir Viruses* 2011;5:276–284.
52. Moscona A. Global transmission of oseltamivir-resistant influenza. *N Engl J Med* 2009;360:953–956.
53. Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, *et al*. Avian influenza A (H5N1) infection in humans. *N Engl J Med* 2005;353:1374–1385.
54. de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJD, *et al*. Oseltamivir resistance during treatment of influenza A (H5N1) infection. *N Engl J Med* 2005;353:2667–2672.
55. Taubenberger JK, Kash JC. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe* 2010;7:440–451.
56. de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, *et al*. Human cases of influenza at the human-animal interface, January 2015–April 2017. *Wkly Epidemiol Rec* 2017;92:460–475.
57. Nguyen-Van-Tam JS, Openshaw PJM, Nicholson KG. Antivirals for influenza: where now for clinical practice and pandemic preparedness? *Lancet* 2014;384:386–387.
58. Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, *et al*. Baloxavir marboxil for uncomplicated influenza in adults and adolescents. *N Engl J Med* 2018;379:913–923.
59. Uehara T, Hayden FG, Kawaguchi K, Omoto S, Hurt AC, *et al*. Treatment-emergent influenza variant viruses with reduced baloxavir susceptibility: impact on clinical and virologic outcomes in uncomplicated influenza. *J Infect Dis* 2020;221:346–355.
60. Omoto S, Speranzini V, Hashimoto T, Noshi T, Yamaguchi H, *et al*. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. *Sci Rep* 2018;8:9633.