

Continued adaptation of A/H2N2 viruses during pandemic circulation in humans

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Abstract

Influenza A viruses of the H2N2 subtype sparked a pandemic in 1957 and circulated in humans until 1968. Because A/H2N2 viruses still circulate in wild birds worldwide and human population immunity is low, the transmissibility of six avian A/H2N2 viruses was investigated in the ferret model. None of the avian A/H2N2 viruses was transmitted between ferrets, suggesting that their pandemic risk may be low. The transmissibility, receptor binding preference and haemagglutinin (HA) stability of human A/H2N2 viruses were also investigated. Human A/H2N2 viruses from 1957 and 1958 bound to human-type $\alpha 2,6$ -linked sialic acid receptors, but the 1958 virus had a more stable HA, indicating adaptation to replication and spread in the new host. This increased stability was caused by a previously unknown stability substitution G205S in the 1958 H2N2 HA, which became fixed in A/H2N2 viruses after 1958. Although individual substitutions were identified that affected the HA receptor binding and stability properties, they were not found to have a substantial effect on transmissibility of A/H2N2 viruses via the air in the ferret model. Our data demonstrate that A/H2N2 viruses continued to adapt during the first year of pandemic circulation in humans, similar to what was previously shown for the A/H1N1pdm09 virus.

DATA AVAILABILITY

All data and materials are available from the corresponding author (S.H.) on reasonable request.

INTRODUCTION

In 1957, a newly emerging influenza A virus of the H2N2 subtype caused significant morbidity and mortality in humans, an event known as the 'Asian influenza' pandemic [1–3]. The pandemic A/H2N2 virus emerged upon reassortment of a previously circulating seasonal human A/H1N1 virus that acquired the H2 haemagglutinin (HA), the N2 neuraminidase (NA) and basic polymerase 1 (PB1) genes of avian origin [4–6]. The A/H2N2 virus was replaced in 1968 by the A/H3N2 'Hong Kong influenza' virus after 11 years of circulation in humans. Today, over 55 years after the last detected A/H2N2 virus infection in humans, immunity against A/H2N2 viruses is low and waning in the human population [7]. Meanwhile, A/H2N2 influenza viruses and viruses containing other viral gene combinations of H2 and N2 segments have been isolated frequently from waterfowl and pigs around the globe [8–13], possibly allowing reintroduction of A/H2N2 viruses into the human population, with or without the need of prior reassortment with other virus subtypes [14].

It was previously shown that a switch in binding preference from avian- to human-type receptors, associated with amino acid substitutions in the HA receptor binding site at positions 226 and 228, contributed to the emergence of the 1957 H2N2 and 1968 H3N2 pandemics [15, 16]. In general, avian A/H2 and A/H3 influenza viruses with 226Q and 228G in HA preferentially

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Abbreviations: dpi, days post-inoculation; HA, haemagglutinin; HEPA, high-efficiency particulate air; hpe, hours post-exposure; hpi, hours post-inoculation; MDCK, Madin-Darby canine kidney; NA, neuraminidase; PB1, basic polymerase 1; RT-PCR, reverse transcriptase PCR; SIA, sialic acid; tMK, tertiary rhesus monkey kidney; TRBC, turkey red blood cell; URT, upper respiratory tract; VCNA, *Vibrio cholerae* NA.

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Three supplementary tables are available with the online version of this article.

bind to α 2,3-linked sialic acids (SIAs, avian-type receptor), whereas human A/H2N2 and A/H3N2 viruses with 226L and 228S in HA preferentially bind α 2,6-linked SIAs (human-type receptor). However, it has been described that A/H2N2 viruses isolated during the first year of the pandemic did not yet have a human-type receptor binding preference [15–17]. Unfortunately, those viruses had been passaged multiple times in eggs, so it could not be excluded that, as a result, these viruses had adapted to binding to avian-type receptors [15–19].

Interestingly, the Q226L and G228S substitutions were also found to be required for the transmission of avian A/H5N1 viruses between ferrets via the air [20, 21]. However, in these studies, a stabilized HA was also a prerequisite for efficient transmission between ferrets. Recently, it was also shown that the HA stability of A/H1N1pdm09 viruses increased during the evolution from precursors in swine, to the early pandemic A/H1N1pdm09 human cases and the later human virus isolates [22]. A possible role of HA stability in the emergence of A/H2N2 and A/H3N2 viruses in humans has not been described to date.

To assess the risk of A/H2N2 viruses that currently circulate in birds to cause a pandemic in humans, the transmissibility of six avian A/H2N2 viruses, isolated between 1999 and 2007, was investigated in the ferret model. In addition, we studied transmissibility via the air in the ferret model of three human A/H2N2 viruses, isolated in 1957, 1958 and 1968, and compared their receptor binding preference and HA acid and temperature stability. We identified a novel amino acid substitution G205S in the HA that emerged during the first year of circulation in humans and that not only stabilized the A/H2N2 HA but also seemed to compensate for the small increase in α 2,3-linked SIA binding induced by G228S. Altogether, our data demonstrate that A/H2N2 viruses continued to adapt to the new host during circulation in humans in the first pandemic year, as previously also described for A/H1N1pdm09 virus [22].

METHODS

Biocontainment

All experiments involving A/H2N2 viruses were conducted at enhanced animal biosafety level 3 (ABSL3+). The ABSL3+ facility of Erasmus MC consists of a negative pressurized (30 Pa) laboratory in which all *in vivo* and *in vitro* experimental work is carried out in class 3 isolators or class 3 biosafety cabinets, which are also negative pressurized (<-200 Pa). Although the laboratory is considered “clean” because all experiments are conducted in closed class 3 cabinets and isolators, special personal protective equipment, including laboratory suits, gloves and FFP3 facemasks is used. Air released from the class 3 units is filtered by high-efficiency particulate air (HEPA) filters and then leaves the facility via a second set of HEPA filters. Only authorized personnel who have received the appropriate training can access the ABSL3+ facility. All personnel working in the facility are vaccinated against seasonal influenza viruses. For animal handling in the facilities, personnel always work in pairs. The facility is secured by procedures recognized as appropriate by the institutional biosafety officers and facility management at Erasmus MC and Dutch and United States government inspectors. Antiviral drugs (oseltamivir and zanamivir) and personnel isolation facilities are directly available to further mitigate risks upon incidents.

Viruses

Avian A/H2N2 virus isolates were obtained from surveillance efforts of wild birds in the Netherlands and Sweden [A/mallard/Netherlands/14/2007 (ACR58574); A/white-fronted goose/Netherlands/22/1999 (AFM82566); A/mallard/Netherlands/31/2006 (ACR58563); A/mallard/Sweden/68735/2007 (AFM82544)]. Avian isolates A/chicken/Jena/4705/1984 (AFJ12827) and A/chicken/New York/Sg-00425/2004 (ACJ69315) were shared within the NIH/NIAID CEIRS network. A/mallard/Sweden/68735/2007 was a kind gift of Jonas Waldenström. These viruses were propagated in the allantoic cavity of embryonated chicken eggs. Human A/H2N2 influenza virus isolates A/Netherlands/M1/1957 (M1/57) and A/Netherlands/M1/1958 (M1/58) were passaged twice in tertiary rhesus monkey kidney (tMK) and twice in Madin-Darby kidney (MDCK) cells, from freeze-dried samples that were collected from individuals presenting between 1957 and 1968 with flu-like symptoms and that tested positive for the presence of influenza virus. A/Netherlands/B1/1968 was first propagated four times in the allantoic cavity of embryonated eggs, followed by three passages in MDCK cells. Gene segments of M1/57, M1/58 and B1/68 were amplified by reverse transcription PCR (RT-PCR) and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 [23]. In addition, the HA gene segment of these three A/H2N2 viruses was cloned in the pCAGG expression plasmid and amino acid substitutions of interest (G205S, G228S, I289L in HA of A/Netherlands/M1/1957 and I289L in HA of A/Netherlands/B1/1968) or a combination thereof were introduced by site-directed mutagenesis. Recombinant A/H2N2 viruses and recombinant viruses (7+1) containing seven gene segments of A/Puerto-Rico/8/1934 and wild-type A/H2N2 HA or mutant HA containing the substitutions G228S, G205S, I289L, G228S+G205S and G228S+I289L were generated upon transfection of HEK293T cells followed by one or two passages in MDCK cells [23]. Recombinant 7+1 viruses were concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore).

Cells

tMK cells were cultured in minimal essential medium with Hank's salts (MEM; Lonza) supplemented with 5% FBS (Greiner or Atlanta Biologicals), 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin mixture (Lonza), 2 mM L-glutamine (Lonza), 1.5 mg

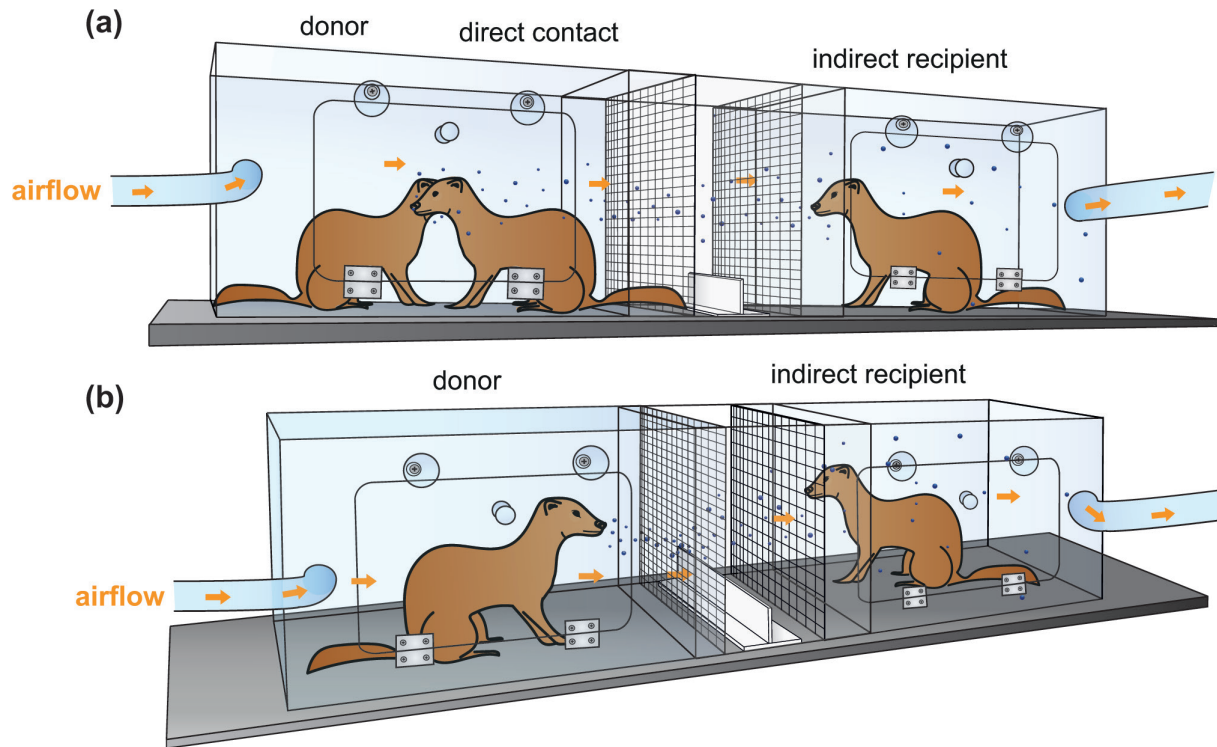


Fig. 1. Experimental transmission set-ups. Schematic representation of the set-ups used to assess the transmissibility of A/H2N2 influenza viruses. (a) Transmission of avian A/H2N2 viruses via direct contact and through the air. Six hours after intranasal inoculation of a donor ferret, a direct contact animal is placed in the same cage as the donor ferret. The next day, an indirect recipient ferret is placed in an opposite cage. To avoid contact transmission with the indirect recipient ferret, the cages are separated by two steel grids, 10 cm apart. In addition, a 5 cm high plate is placed between the cages to avoid spill-over of food, faeces and other large particles. (b) Transmission of human A/H2N2 viruses through the air. A similar procedure was followed as under (a), but the direct contact animal was not included. Orange arrows indicate direction of air flow (100 l min⁻¹). Set-ups were placed in class III isolators in a biosafety level 3+ laboratory.

ml⁻¹ sodium bicarbonate (Lonza) and 10 mM HEPES (Lonza). MDCK cells (ATCC) were cultured in Eagle's minimal essential medium (EMEM; Lonza) supplemented with 10% FBS (Greiner or Atlanta Biologicals), 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin mixture (Lonza), 2 mM L-glutamine (Lonza), 1.5 mg ml⁻¹ sodium bicarbonate (Lonza), 10 mM HEPES (Lonza) and 1× non-essential amino acids (Lonza). 293T cells were cultured in Dulbecco modified Eagle's medium (DMEM; Lonza) supplemented with 10% FBS, 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin mixture (Lonza), 2 mM glutamine, 1 mM sodium pyruvate (Gibco) and non-essential amino acids. Subclone 118 of Vero-WHO (Vero-118) cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Lonza) supplemented with 10% FBS and 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin mixture (Lonza) [24, 25]. All cell lines were maintained at 37°C and 5% CO₂.

Transmission experiments in ferrets

Influenza virus and Aleutian disease virus seronegative 6-month-old female ferrets (*Mustela putorius furo*), weighing 735–1095 g, were obtained from a commercial breeder (TripleF). Animal welfare was monitored on a daily basis. Virus inoculation of ferrets was performed under anaesthesia with a mixture of ketamine/medetomidine (10 and 0.05 mg kg⁻¹ respectively) antagonized by atipamezole (0.25 mg kg⁻¹). Swabs were collected under light anaesthesia using ketamine to minimize animal discomfort. For each experiment, two (avian A/H2N2 viruses) or four (human A/H2N2 viruses) donor ferrets were inoculated intranasally with 10⁶ TCID₅₀ (250 µl instilled dropwise in each nostril) of A/H2N2 virus. For avian A/H2N2 virus isolates, direct contact animals were placed in the same cage as donor animals 6 h post-inoculation (hpi) (Fig. 1a). One day later, for both avian and human A/H2N2 viruses, indirect recipient ferrets were added in an adjacent cage separated by two steel grids, 10 cm apart, to avoid contact transmission (Fig. 1b). The contribution of transmission via fomites was not investigated [26]. Throat and nasal swabs were collected from the ferrets every other day until 7 days post-inoculation (dpi) and 9 days post-exposure (dpe), respectively, to prevent cross-contamination. All swabs were stored at -80°C in virus transport medium consisting of EMEM with Hank's BSS and 25 mM HEPES (Lonza), glycerol 99% (Sigma Aldrich), lactalbumin hydrolysate (Sigma Aldrich), 10 mU polymyxin B sulphate (Sigma Aldrich), 5 mU nystatin (Sigma Aldrich), 50 mg ml⁻¹ gentamicin (Gibco) and 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin mixture (Lonza). Ferrets were killed by exsanguination by heart puncture under anaesthesia.

Virus titrations

Throat and nasal swabs were titrated in MDCK cells. Briefly, confluent cells were inoculated with 10-fold serial dilutions of sample in serum-free EMEM supplemented with 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin mixture (Lonza), 2 mM L-glutamine (Lonza), 1.5 mg ml⁻¹ sodium bicarbonate (Lonza), 10 mM HEPES (Lonza), 1× non-essential amino acids (Lonza) and 20 µg ml⁻¹ trypsin (Lonza). At 1 hpi, the first three dilutions were washed twice with media and 200 µl fresh medium was subsequently added to the whole plate. Supernatants of cell cultures were tested for agglutination activity using turkey erythrocytes 3 days after inoculation. Infectious virus titres (TCID₅₀ ml⁻¹) were calculated from four replicates of each throat and nasal swab using the Spearman–Karber method [27, 28].

Glycan microarray

Glycan microarray analysis was performed as described previously [29]. As controls, A/Netherlands/213/2003 and A/Indonesia/05/2005 were used. Recombinant A/Puerto-Rico/8/1934(7+1) viruses with A/H2 HA (25 µl) were diluted with PBS-T (PBS+0.1% Tween, 25 µl) and applied to the array surface in the presence of oseltamivir (200 nM) in a humidified chamber for 1 h, followed by successive rinsing with PBS-T, PBS and deionized water (2×) and dried by centrifugation. The virus-bound slide was incubated for 1 h with CR6261 and CR8020 influenza HA stem-specific antibodies (100 µl, 5 µg ml⁻¹ in PBS-T). Quality control of the CR antibodies specificity was performed by incubation of the antibody to the array in the absence of a virus. The slide was then washed again as described above. Subsequently, a secondary goat anti-human Alexa-647 antibody (100 µl, 2 µg ml⁻¹ in PBS-T; Thermo Fisher) was applied, incubated for 60 min in a humidified chamber and washed again as described above. Washed slides were dried by centrifugation and immediately scanned using an Innopsys Innoscan 710 microarray scanner at the appropriate excitation wavelength. To ensure that all signals were in the linear range of the scanner's detector and to avoid any saturation of the signals, various gains and photomultiplier (PMT) values were employed. Images were analysed with Mapix software (version 8.1.0 Innopsys) and processed with an in-house developed Excel macro. The average fluorescence intensity and standard deviation (SD) was measured for each compound after exclusion of the highest and lowest intensities from the spot replicates ($n=4$) and displayed using GraphPad Prism 9.

Modified turkey red blood cell agglutination assay

To assess the receptor binding specificity of recombinant A/Puerto-Rico/8/1934(7+1) viruses with A/H2 HA, a modified turkey red blood cell (TRBC) assay was performed. Briefly, all α 2,3-, α 2,6-, α 2,8- and α 2,9-linked sialic acids were removed from the surface of TRBCs by incubating 62.5 µl of 1% TRBCs in PBS with 50 mU *Vibrio cholerae* NA (VCNA; Roche) in 0.1 mM calcium chloride at 37 °C for 1 h. Removal of sialic acids was confirmed by observation of complete loss of haemagglutination of the TRBCs by the tested influenza A viruses. Subsequently, resialylation was performed using 5 mU α 2,3-(N)-sialyltransferase (Sigma-Aldrich) or 0.5 µg α 2,6-(N)-sialyltransferase (R and D Systems) and 1.5 mM CMP-sialic acid (Merck millipore) at 37 °C in 75 µl for 2 h to produce α 2,3-TRBC and α 2,6-TRBC, respectively. After a washing step, the TRBCs were re-suspended in PBS containing 1% BSA to a final concentration of 0.5% TRBCs. Resialylation was confirmed by haemagglutination of viruses with known receptor specificity: recombinant viruses with seven gene segments of influenza virus A/Puerto Rico/8/1934 and the HA of H3N2 A/Netherlands/213/2003 and the HA of H5N1 A/Indonesia/05/2005. The receptor specificity of recombinant A/Puerto-Rico/8/1934(7+1) viruses with A/H2 HA was tested by performing a standard HA assay with untreated and modified TRBCs. In brief, serial twofold dilutions of virus in PBS were made in a 50 µl volume; 50 µl of 0.5% TRBCs was added, followed by incubation for 1 h at 4 °C before determining the HA titre. Two independent experiments were performed for each assay. Results are shown from one representative experiment.

Fusion assay

Influenza virus HA-induced cell fusion was tested in Vero-118 cells. Approximately 10⁶ Vero-118 cells were transfected in 100 mm Petri dishes (Greiner) with 5 µg pCAGGS-HA using Xtremegene transfection reagent (Roche). One day after transfection, cells were collected using trypsin-EDTA (Gibco) and plated in six-well plates. The next morning, cells were washed and medium was replaced with IMDM supplemented with 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin mixture (Lonza) and 20 µg ml⁻¹ trypsin (Lonza). After 1 h of incubation at 37 °C, cells were washed once with PBS and exposed to PBS at pH 4.8–5.9 in 0.1 increments for 10 min at 37 °C. Subsequently, the PBS was replaced by IMDM supplemented with 10% FBS (Greiner or Atlanta Biologicals) and 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin mixture (Lonza). Eighteen hours after the pH shock, cells were fixed using 80% ice-cold acetone, washed and stained using a 20% Giemsa solution (Merck Millipore) for microscopic analyses. At least two independent experiments were performed for each assay. Results are shown from one representative experiment in Fig. 2 (the results of the individual experiments are shown in Tables S1, S2 and S3, available in the online version of this article).

Temperature stability assay

The HA stability of wild-type and mutant recombinant 7+1 viruses was assessed by exposing the viruses to a temperature gradient. Briefly, viruses were set to similar HA units using PBS. Subsequently, the viruses were incubated in a thermal cycler for 30 min

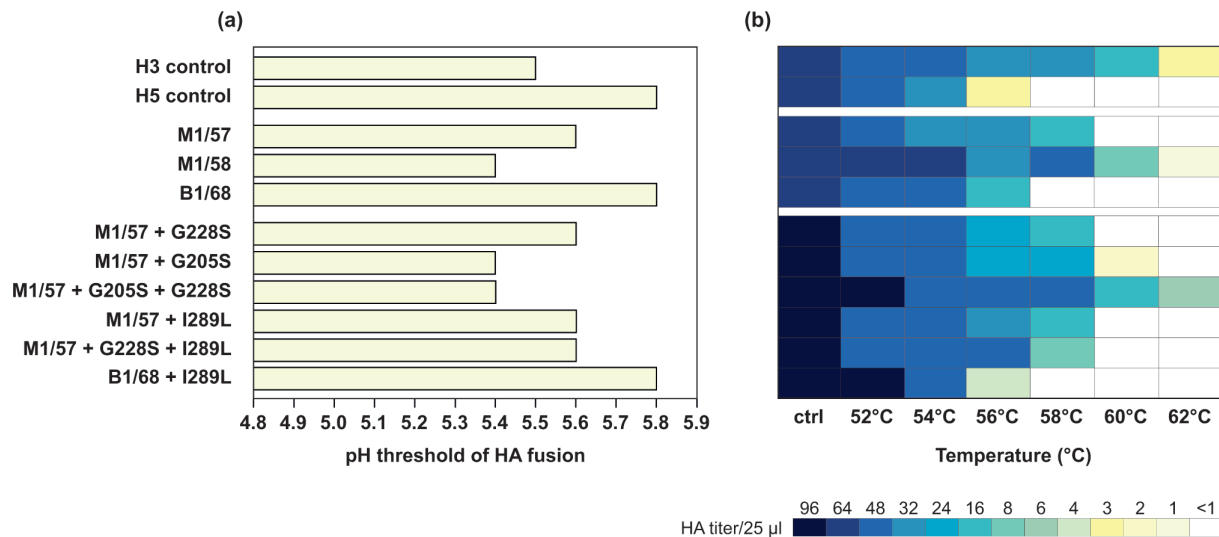


Fig. 2. HA stability of recombinant wild-type and mutant A/H2 viruses. Fusion ability of HAs upon acidification of Vero-118 cells at different pH (a). Vero-118 cells were transfected with wild-type and mutant HA expression plasmids and subsequently exposed to PBS with different pH. The next day, cells were observed for the presence of syncytia (multinucleated cells). Bars indicate the pH threshold at which syncytia formation was detected microscopically. The HAs of A/Netherlands/213/2003 (stable H3) and of A/Indonesia/5/2005 virus (unstable H5) were included as controls. Thermostability of recombinant viruses containing wild-type and mutant HAs (b). A/Puerto-Rico/8/1934(7+1) viruses with A/H2 HA were incubated at different temperatures for 30 min after which the HA titre of viruses was measured for each temperature in an HA assay with TRBCs. As indicated by the colour key, rectangles represent the HA titre per 25 µl of the viruses before (first column, ctrl) and after exposure to various temperatures. A/Netherlands/213/2003 (stable H3) and A/Indonesia/5/2005 virus (unstable H5) were included as controls.

at temperatures ranging from 50 to 62 °C in 2 °C increments. The HA titre was determined by performing an HA assay using turkey erythrocytes, as described above. Two independent experiments were performed for each assay. Results are shown from one representative experiment.

RESULTS

Avian A/H2N2 viruses were not transmitted between ferrets through direct contact or via the air

To assess the threat of avian A/H2N2 influenza viruses to mammals, we investigated the transmissibility of the A/H2N2 viruses A/chicken/Jena/4705/1984, A/chicken/New York/Sg-00425/2004, A/mallard/Netherlands/14/2007, A/mallard/Netherlands/31/2006, A/mallard/Sweden/68735/2007 and A/white-fronted goose/Netherlands/22/1999 in a ferret transmission model. For each virus, two individually housed donor ferrets were inoculated intranasally and after 6 h direct contact animals were added to each of the cages. One day after inoculation, indirect recipient ferrets were added to adjacent cages that were separated from the donor cages by two steel grids, 10 cm apart, allowing viruses to be transmitted only via the air (Fig. 1a). All donor ferrets were productively infected, as shown by successful virus isolation from the throat and nose swabs from all animals (Fig. 3). Overall, virus titres were higher in throat swabs than in nose swabs. Peak virus titres reached up to $5.0 \log_{10} \text{TCID}_{50} \text{ ml}^{-1}$ in the throat and $3.0 \log_{10} \text{TCID}_{50} \text{ ml}^{-1}$ in the nose, with no substantial differences between the viruses. No infectious virus was recovered from nose or throat swabs obtained from direct contact and indirect recipient ferrets. These animals did also not seroconvert, confirming the lack of transmission of these viruses between ferrets (data not shown).

A/H2N2 virus from 1958, but not from 1957 and 1968, was transmitted via the air between ferrets

To compare the transmissibility of early and late human A/H2N2 virus isolates via the air, transmission experiments were performed in the ferret model with three human A/H2N2 viruses isolated in 1957 (the start of the pandemic), 1958 and 1968 (the last year that A/H2N2 viruses were detected in humans). One day after intranasal inoculation of four donor animals with either A/Netherlands/M1/1957 (M1/57), A/Netherlands/M1/1958 (M1/58) or A/Netherlands/B1/1968 (B1/68), indirect recipient ferrets were placed in a cage adjacent to the donor ferret. Again, both cages were 10 cm apart and separated by steel grids (Fig. 1b). All three viruses replicated well in the upper respiratory tract (URT) of donor ferrets, as shown by the successful virus isolation from throat and nose swabs of all ferrets on multiple days (Fig. 4). B1/68 displayed a delay in replication with low or undetectable virus titres at 1 dpi, and peak titres only at 3 or 5 dpi, as compared to M1/57 and M1/58 replication of which immediately peaked at 1 dpi. Peak virus titres reached up to $5.75 \log_{10} \text{TCID}_{50} \text{ ml}^{-1}$ in the throat and $5.0 \log_{10} \text{TCID}_{50} \text{ ml}^{-1}$ in the nose, with no substantial differences between the viruses. Of the three viruses tested, only M1/58 was transmitted via the air to indirect recipient ferrets,

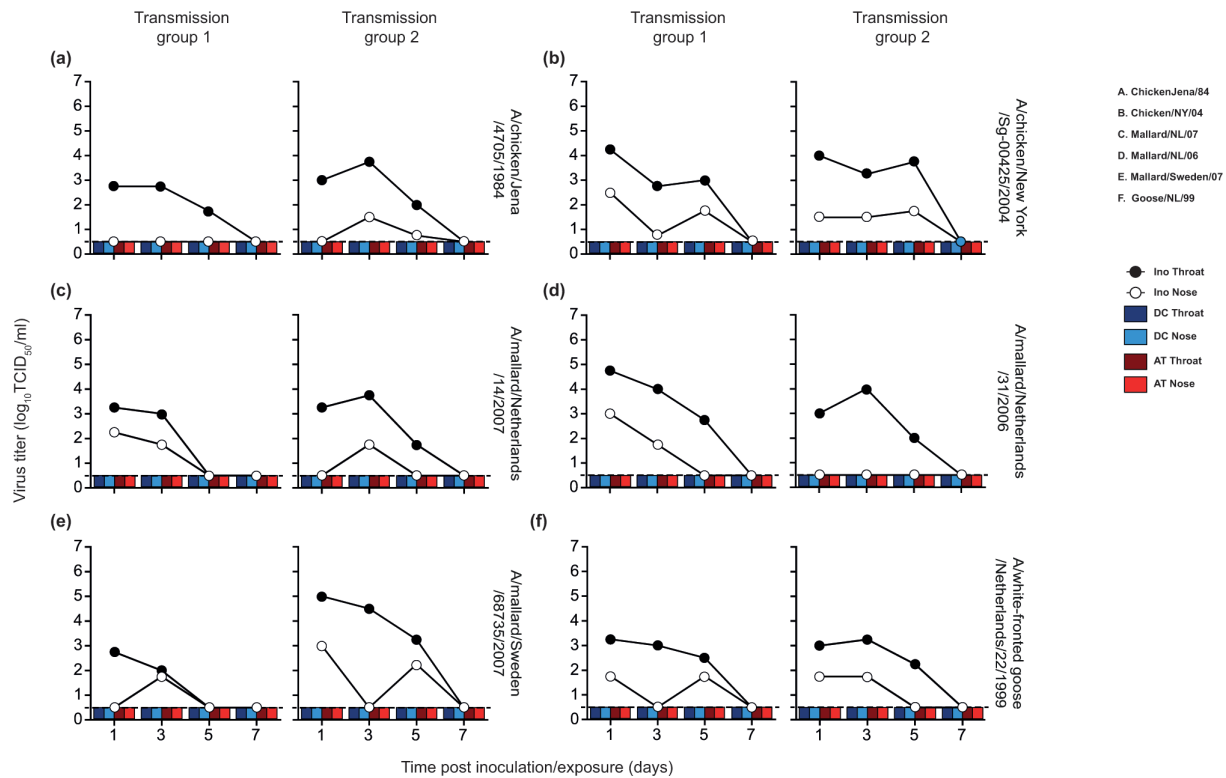


Fig. 3. Lack of transmission of avian A/H2N2 viruses via direct contact or air between ferrets. Transmission experiments are shown for six avian A/H2N2 virus isolates in two individual ferret groups: A/chicken/Jena/4705/1984 (a), A/chicken/New York/Sg-00425/2004 (b), A/mallard/Netherlands/14/2007 (c), A/mallard/Netherlands/31/2006 (d), A/mallard/Sweden/68735/2007 (e) and A/white-fronted goose/Netherlands/22/1999 (f). Black and white circles indicate virus titres in throat and nose swabs of donor ferrets, respectively. Dark and light blue, and dark and light red bars each represent virus titres in throat and nose swabs of direct contact and indirect recipient animals, respectively. Dotted lines indicate detection limit of virus titrations.

as demonstrated by virus isolation from throat and nose swabs from all four indirect recipient animals, between 3 and 9 dpe (Fig. 3b). The lack of transmission of M1/57 and B1/68 was confirmed by the absence of seroconversion in all indirect recipient ferrets as determined by an HA inhibition (HI) assay (data not shown).

The double substitution Q226L/G228S did not facilitate a complete switch to human-type receptor specificity of A/H2 viruses

Sequence analysis of the HA consensus of the human A/H2N2 virus stocks showed three amino acid differences in M1/58 HA as compared to M1/57 HA: G205S (trimer interface), G228S (receptor binding site) and I289L (on the surface of the HA head, but outside of the receptor binding site, Table 1). The absence of 228S in M1/57 HA suggests that this HA was not yet fully adapted to binding to human-type receptors. The phenotypic effect of the G205S and I289L substitutions detected in the HA of M1/58 has not been described in the literature and thus their potential effect on functional properties is unknown. In the HAs from all A/H2 viruses that were sequenced after 1958, so including the B1/68 HA, the I289L substitution had reverted to 289I, whereas 205S and 228S were present in most of these viruses (Table 1). Furthermore, the B1/68 HA carried 25 additional substitutions as compared to the M1/58 HA.

To investigate if G228S, G205S and I289L affect the receptor binding preference of the M1/57 and M1/58 HAs and hence may explain the difference in transmissibility between M1/57 and M1/58, the three single amino acid substitutions or a combination thereof were introduced into the HA of M1/57. As 228S and 205S were already present in the wild-type B1/68 HA, only the I289L substitution was introduced in this HA to investigate if this substitution affected receptor binding and would explain the lack of transmission of this virus isolate.

Viruses harbouring a wild-type or mutant H2 HA were generated in the attenuated A/Puerto Rico/8/1934 (PR8) backbone and glycan microarray analyses were performed with 12 different linear and bi-antennary N-glycans [29]. The linear glycans consisted of three galactose-N-acetylglucosamine (LacNAc) repeats, whereas biantennary glycans contained one to three LacNAc repeats. All glycans were either non-sialylated (#1–4), serving as controls, or capped with α 2,3- (#5–8) or α 2,6-linked (#9–12)

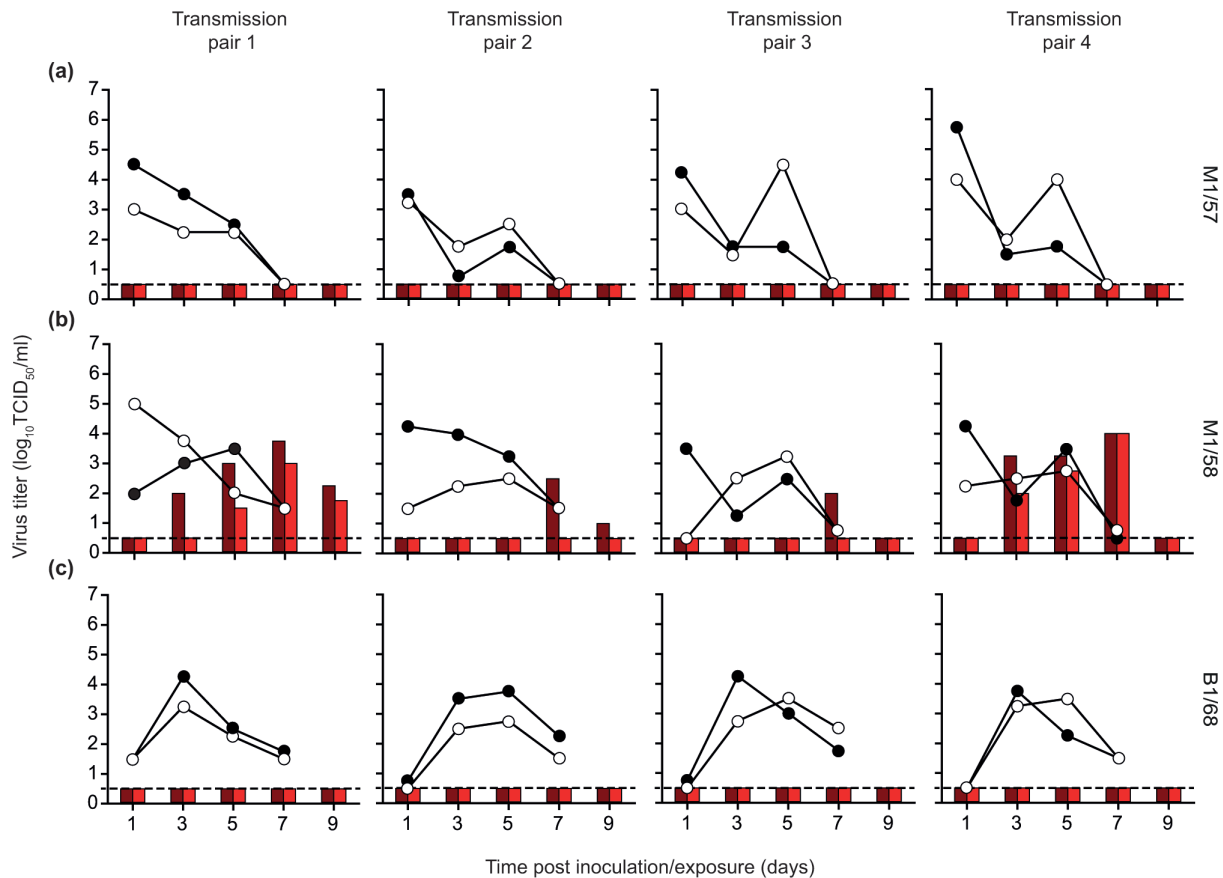


Fig. 4. Transmission of human A/H2N2 viruses between ferrets via aerosol or respiratory droplets. Transmission experiments are shown for three human A/H2N2 virus isolates: A/Netherlands/M1/1957 (M1/57) (a), A/Netherlands/M1/1958 (M1/58) (b) and A/Netherlands/B1/1968 (B1/68) (c). An individual donor–recipient pair is shown in each panel. Black and white circles represent virus titres in throat and nose swabs of donor ferrets, respectively. Dark and light red bars each represent virus titres in throat and nose swabs of indirect recipient ferrets. Dotted lines indicate detection limit of virus titrations.

SIAs (Fig. 4a). A/Netherlands/213/2003 (human H3) and A/Indonesia/5/2005 (avian H5) were used as controls as these viruses selectively bind to $\alpha 2,6$ - and $\alpha 2,3$ -linked SIAs, respectively. All three wild-type A/H2 viruses bound to $\alpha 2,6$ receptors. However, whereas M1/57 and M1/58 only showed low levels of binding to $\alpha 2,3$ receptors, B1/68 showed dual receptor binding preference with substantial binding to $\alpha 2,3$ receptors (Fig. 4b). Introduction of G228S in M1/57 HA resulted in an increase in $\alpha 2,3$ -linked SIA binding. Interestingly, although introduction of G205S alone in M1/57 HA did not change the receptor binding phenotype, the addition of G205S to G228S seemed to reverse the effect of G228S, resulting in low binding to $\alpha 2,3$ -linked SIAs, similar to wild-type M1/57 and M1/58 HAs. Furthermore, the double substitution G205S and G228S in M1/57 increased binding to the linear glycan with $\alpha 2,6$ -linked SIAs (#9). Amino acid substitution I289L in the M1/57 HA, either in the presence or absence of 228S, did not affect the receptor binding preference of these HAs. The I289L substitution also did not affect the receptor binding preference of the B1/68 HA. To investigate the binding properties in a different assay, the binding specificity of the A/H2N2 HAs was also assessed in a modified red blood cell assay. In brief, TRBCs were treated with VCNA to remove SIAs followed by resialylation with solely $\alpha 2,3$ - or $\alpha 2,6$ -linked SIAs. The glycans that are present on the turkey erythrocytes are heterogenic and consist primarily of biantennary glycans containing one or two LacNAc repeats [30]. Consequently, the results of this assay are expected to be most comparable with the shorter glycan structures on the array, but not entirely. As expected, the human A/H3 and avian A/H5 control viruses bound exclusively to $\alpha 2,6$ - or $\alpha 2,3$ -linked SIAs, respectively (Table 2). In agreement with the glycan array analysis, introduction of 228S in M1/57 overall led to a slight increase in binding to $\alpha 2,3$ receptors, while maintaining binding to $\alpha 2,6$ receptors. In addition, also in this assay, the B1/68 HA showed a dual binding preference to both $\alpha 2,6$ - and $\alpha 2,3$ -linked SIAs.

A newly identified substitution G205S stabilized the HA of A/H2 viruses

Upon attachment to SIA receptors and receptor-mediated endocytosis, the HA protein is exposed to a low pH in the endosome which triggers a conformational change that mediates fusion of the viral and endosomal membranes, resulting in the release

Table 1. Amino acids at positions 205, 226, 228, and 289 in the HA of human A/H2N2 viruses

	Amino acid position in HA				Passage history	GenBank accession no.
	205	226	228	289		
A/El Salvador/2/1957 [17]	G	Q	G	I	Egg ₂ MDCK _X	HM204767
A/El Salvador/2/1957* [17]	G	L	G	I	MDCK _X	HM204775
A/Netherlands/M1/1957 (M1/57)	G	L	G	I	tMK ₂ MDCK ₂	KM402801
A/Netherlands/M2/1957	G	L	G	I	MDCK ₃	KM885170
A/Singapore/1/1957	G	L	S	I	E _X MDCK ₁	CY125894
A/Netherlands/M1/1958 (M1/58)	S	L	S	L	tMK ₂ MDCK ₂	CY077741
A/Albany/6/1958 [17]	G	L	S	I	E ₁₀	AF270723
A/Netherlands/N1/1959	V	L	G	I	tMK ₂ MDCK ₂	CY077904
A/Netherlands/H1/1960	S	L	S	I	E ₁ tMK ₁ MDCK ₃	CY077786
A/Netherlands/K1/1963	S	L	S	I	E ₁ tMK ₁ MDCK ₂	CY077733
A/England/12/1964	S	L	S	I	MK ₁ E ₁₁ MDCK ₃	AY209967
A/Sydney/2/1964	G	L	S	I	E ₇ MDCK ₃	KP412320
A/Taiwan/1/1964	S	L	S	I	YMDCK ₂	DQ508881
A/England/1/66	S	L	S	I	MK ₁ E ₁ A ₁₆ MDCK ₂	KP412318
A/England/10/67	S	L	S	I	E ₂ MDCK ₃	AY209980
A/Tokyo/3/67	S	L	S	I	YMDCK ₃	AY209987
A/Netherlands/B1/68 (B1/68)	S	L	S	I	E ₄ MDCK ₃	KM402809
A/Netherlands/B2/68	S	L	S	I	E _X MDCK ₄	KM885174

Viruses marked in grey were used in the current study. A, amniotic cavity; E, allantoic cavity; MDCK, Madin-Darby kidney cells; MK, monkey kidney cells; tMK, tertiary monkey kidney cells; X, unknown passage number; Y, unknown passage history in eggs.

*This variant of A/El Salvador/2/1957 was plaque-purified from the nasal wash of a ferret during transmission experiments with A/El Salvador/2/1957 [17].

of the viral genome in the cytoplasm [31]. Previous work has shown that the HAs of influenza A viruses that are transmissible via the air between mammals are comparatively stable, as demonstrated in fusion and temperature stability assays [20, 21, 32]. To assess if the HA acid stability of A/H2N2 viruses had changed during circulation in humans, fusion assays were performed. For this, Vero-118 cells were transfected with HA-expression plasmids and exposed to trypsin to cleave and activate the HA, followed by exposure to a pH gradient to induce membrane fusion. Cell cultures were subsequently inspected visually using a light microscope for the presence of syncytia (multinucleated cells) to determine the pH threshold triggering the conformational change and subsequent membrane fusion (Fig. 5a). Human H3 (stable) and avian H5 (unstable) HAs were included as controls, for which the pH threshold for fusion was 5.5 and 5.8, respectively. The H2 HA of M1/57, M1/58 and B1/68 required a pH for fusion of 5.6, 5.4 and 5.8, respectively, demonstrating that the H2 HA stability had changed during circulation in humans. Introduction of G228S in the M1/57 HA did not change the pH of fusion. In contrast, introduction of G205S alone or in combination with G228S increased the acid stability of the M1/57 HA from 5.6 to 5.4, comparable to that of M1/58. The substitution I289L did not affect the acid stability of the M1/57 and B1/68 HA, suggesting that one or more of the 25 additional substitutions may be responsible for the decreased stability of the B1/68 HA.

The switch of influenza virus HA from a metastable non-fusogenic to a stable fusogenic conformation can also be triggered at neutral pH when the HA is exposed to increasing temperature. This conformational change of HA is biochemically indistinguishable from the change triggered by low pH and results in a loss of the ability to bind the receptor [33]. To assess the thermostability of the H2 HAs, viruses were exposed for 30 min to increasing temperatures ranging from 52 to 62 °C at neutral pH. Subsequently, the HA titre of each A/H2 virus was recorded (Fig. 5b). In agreement with the fusion assay, M1/57 HA was less stable than M1/58 HA but more stable than B1/68 HA. Introduction of G228S did not affect the thermostability of M1/57, whereas introduction of G205S was found to stabilize the M1/57 HA. This stability was further increased when G228S was added to G205S in the M1/57 HA. Introduction of I289L alone or in combination with G228S did not affect the thermostability of M1/57 HA, neither

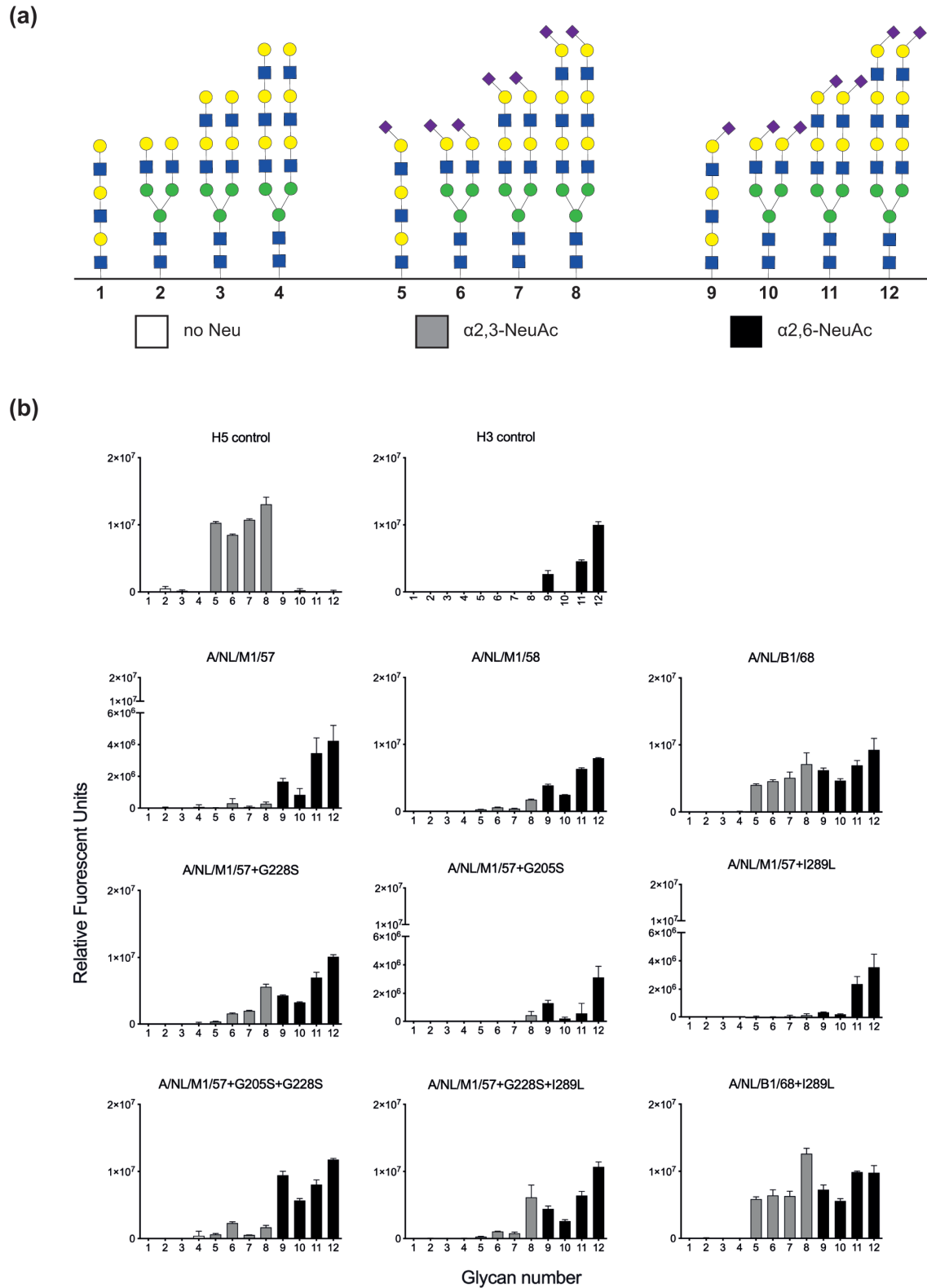


Fig. 5. Binding specificity of human A/H2 viruses to selected glycans. Synthesized glycans (a) were used to assess the binding specificity of A/H2 viruses to $\alpha 2,3$ - and $\alpha 2,6$ -linked SIAs on a glycan microarray (b). Glycans 1–4 are non-sialylated controls (white), while structures 5–8 (grey) and 9–12 (black) represent $\alpha 2,3$ - and $\alpha 2,6$ -linked glycans, respectively. Human A/Netherlands/213/2003 (H3 control, $\alpha 2,6$ -linked SIA specificity) and avian A/Indonesia/5/2005 virus (H5 control, $\alpha 2,3$ -linked SIA specificity) were used as controls. The mean intensity and standard deviation (SD) were calculated from four independent replicates. Neu, neuraminic acid; NeuAc, N-acetyl neuraminic acid.

Table 2. Binding specificity of human A/H2 viruses in a modified turkey red blood cell assay

Virus	HA titre (HAU* per 25 µl)		
	Untreated TRBCs†	α2,3 Resialylated	α2,6 Resialylated
A/Netherlands/213/2003	24	–	48
A/Indonesia/05/2005	32	24	–
M1/57	24	0.5	24
M1/58	48	6	64
B1/68	48	16	64
M1/57+G228S	32	6	32
M1/57+G205S	48	–	24
M1/57+G205S + G228S	48	8	64
M1/57+I289L	32	0.5	32
M1/57+G228S + I289L	32	3	32
B1/68+I289L	48	6	64

*Haemagglutination units.

†Turkey red blood cells.

did introduction of I289L in the B1/68 HA. Overall, the assay results on acid stability and temperature stability yielded mirror images for all viruses and amino acid substitutions.

A stabilized HA and human receptor binding did not promote robust airborne transmission of A/H2N2 viruses between ferrets

To assess whether the substitution G228S alone was sufficient, or the addition of the stabilizing substitution G205S to G228S was required for the airborne transmissibility of M1/58, recombinant viruses M1/57, M1/58 and M1/57 with G228S, or G205S and G228S in HA were generated and tested in the ferret transmission model. Donor ferrets inoculated with each virus were productively infected, as shown by the successful isolation of infectious virus until 5 or 7 dpi, and all four viruses replicated equally well, with peak titres reaching up to $6.25 \log_{10} \text{TCID}_{50} \text{ ml}^{-1}$ in ferrets inoculated with M1/57 (Fig. 6). M1/57 and M1/57 harbouring the G228S substitution were transmitted to one out of four indirect recipient ferrets, whereas M1/58 and M1/57 containing the double substitution G205S/G228S were transmitted to two out of four indirect recipient ferrets. The lack of transmission in the A/H2N2 virus-negative indirect recipient animals was confirmed by the absence of seroconversion (data not shown). Transmission of recombinant M1/57 was not expected based on the previous transmission experiments with the virus isolate (Fig. 4) and M1/57 was also only detected from 7 dpe onwards in one recipient ferret. To investigate if the M1/57 virus had acquired substitutions that favoured transmissibility, as previously observed in another A/H2N2 transmission study [17], viruses from the throat and nose samples of the M1/57-positive indirect recipient ferret were sequenced. However, no mutations were detected in any of the HA sequences (data not shown).

DISCUSSION

As a contribution to the risk assessment of A/H2N2 influenza viruses for humans, we here evaluated the transmissibility of avian A/H2N2 viruses, isolated from various bird species, in the ferret model. The six avian A/H2N2 virus isolates tested, replicated in the URT of ferrets, but did not transmit to recipient animals either through direct contact or via the air. Similar results were previously obtained in a study by Jones *et al.*, in which the transmissibility of nine avian H2N2 isolates was assessed in the ferret model [34]. Of these, five virus isolates replicated in the URT of donor animals of which three isolates were transmitted to one or two out of two direct contact animals. In this study, the isolate A/chicken/Jena/4705/1984, which was also used in the present study, replicated in donor animals, but just as described here, was not transmitted via direct contact. None of the five A/H2N2 isolates were transmitted between ferrets via the air. Moreover, in a study by Pappas *et al.* a chicken A/H2N2 virus was transmitted to two out of three direct contact animals, but was also not transmitted between ferrets via the air [35]. This indicates that despite limited transmission via direct contact in the ferret model, the risk of avian A/H2N2 viruses without prior reassortment or adaptation is low for mammals.

Of the three human A/H2N2 viruses tested, only the A/H2N2 virus isolated in 1958 was transmitted via the air between ferrets, but not the viruses isolated in 1957 and 1968. To investigate the observed difference in transmissibility of the 1957 and 1958

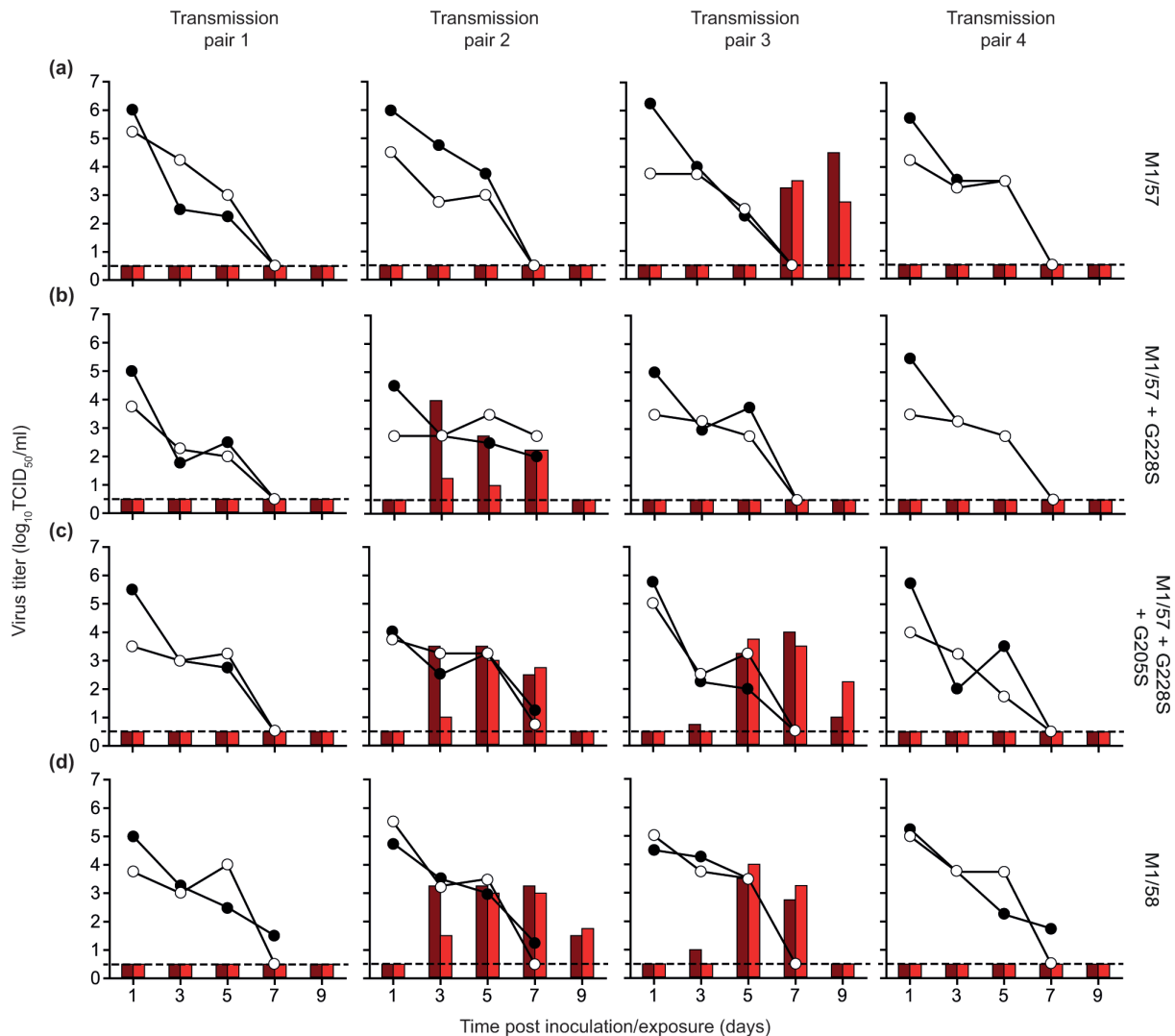


Fig. 6. Limited transmission of recombinant human wild-type and mutant A/H2N2 viruses via aerosol or respiratory droplets. Transmission experiments are shown for four viruses: M1/57 (a), M1/57+G228S (b), M1/57+G205S+G228S (c) and M1/58 (d). An individual donor–recipient pair is shown in each panel. Black and white circles represent virus titres in throat and nose swabs of donor ferrets, respectively. Dark and light red bars each represent virus titres in throat and nose swabs of indirect recipient ferrets. Dotted lines indicate detection limit of virus titrations.

viruses, phenotypic assays were performed to study the HA receptor binding preference and the HA stability, two phenotypes that were previously shown to affect transmissibility [32]. Both viruses were found to preferentially bind α 2,6-linked SIAs, without an additive effect of the G228S substitution that was present in M1/58 but not in M1/57. Furthermore, M1/58 had a more stable HA as the result of a G205S substitution that was absent in M1/57. However, when M1/57 recombinant viruses harbouring HAs with substitutions from M1/58 were tested in transmission experiments, no major differences in transmissibility were observed. The transmission results hinted that those viruses harbouring both G205S and G228S (M1/57 with both substitutions and M1/58) may be transmitted slightly more efficiently than the viruses without these substitutions (M1/57 and G228S mutant), but the small number of animals used in this study and the limited overall power of experiments in ferrets do not allow a statistically sound conclusion (Fig. 6). An alternative possibility is that other substitutions in the genome of M1/58, particularly the avian-origin NA and PB1 segments, may explain the difference in transmissibility.

Previously, Pappas *et al.* described a similar analysis to that shown here, in which the human A/H2N2 strain A/Albany/6/1958 was transmitted via the air between ferrets, whereas the human A/H2N2 strain A/El Salvador/2/1957 was only transmitted via the air in one out of six ferret pairs, late during the experiment. Sequence analysis of a nasal wash sample collected from the indirect contact ferret showed that the transmission event was associated with a natural variant containing the Q226L substitution in HA, similar to M1/57 used in the current study. The Q226L substitution in A/El Salvador/2/1957 was found to change the receptor binding preference from α 2,3-linked to α 2,6-linked SIAs [17].

Based on the transmission data from multiple avian and human influenza viruses collected over the past years, transmission of a virus with a predominant $\alpha 2,3$ -linked SIA binding preference between mammals is highly unlikely. In addition, it was previously shown that passaging of human influenza viruses in eggs may result in egg-adaptation and the ability to bind to $\alpha 2,3$ -linked SIAs [18, 19]. Therefore, it may be possible that due to the eight passages in eggs, the binding preference of A/El Salvador/2/1957 shifted to $\alpha 2,3$ -linked SIAs. In contrast, M1/57 that was used in our study has only been passaged in mammalian cells (two passages in tertiary monkey kidney cells and two in MDCK cells, Table 1), and may therefore better represent the viruses that circulated during the first year of the A/H2N2 pandemic. Although the possibility that $\alpha 2,3$ -linked SIA binding A/H2N2 viruses were circulating early in the pandemic cannot be excluded, our binding data of A/M1/57 suggest that the viruses that started the pandemic had already acquired $\alpha 2,6$ -linked SIA binding.

Introduction of G228S in M1/57 HA did not change the binding to $\alpha 2,6$ -linked SIAs, but an increase in $\alpha 2,3$ -linked SIA binding was observed. This $\alpha 2,3$ -linked SIA binding was also observed for A/El Salvador/2/1957 upon introduction of G228S, and in A/Albany/6/1958 that already contained Q226L and G228S [17]. This effect of G228S in M1/57 was reversed when G205S was also introduced (Fig. 5). Both A/El Salvador/2/1957 and A/Albany/6/1958 did not contain 205S, which may explain the residual $\alpha 2,3$ -linked SIA binding of those HAs.

In addition to preferential binding to $\alpha 2,6$ -linked SIAs, a stable HA is required for transmission of influenza A viruses via the air between mammals, as previously demonstrated for A/H5N1 viruses [21, 22, 32]. While the early A/H2N2 virus M1/57 had a pH threshold for fusion of 5.6, the pH threshold of M1/58 was 5.4 (Fig. 5a). A comparative analysis between M1/57 and M1/58 identified a stabilizing substitution, G205S, which lowered the pH threshold for fusion of the M1/57 HA from 5.6 to 5.4. To our knowledge, this amino acid position has so far not been described to affect the acid or temperature stability of influenza A virus HA of any subtype [36]. Interestingly, this serine at position 205 became fixed, because most A/H2N2 viruses isolated after 1958 had a serine at position 205 (Table 1). This indicates that the increased HA stability was an advantage for the virus for circulation in the human population. Such an increase in stability over time during circulation in humans has previously also been described for A/H1N1pdm09 virus, where early isolates from the 2009 pandemic had a pH threshold for fusion of around 5.5 whereas viruses isolated in later years displayed lower pH threshold values ranging from 5.2 to 5.4 [22].

Surprisingly, however, during the subsequent 10 years of circulation in humans, the H2 HA stability decreased again, as demonstrated by the B1/68 HA with a pH threshold for fusion of 5.8, similar to the unstable A/H5 HA control. In addition, the B1/68 showed a dual receptor binding preference for both $\alpha 2,3$ - and $\alpha 2,6$ -linked SIAs, despite the presence of G205S, Q226L and G228S, and was not transmitted via the air between ferrets. In contrast, a previously studied A/H2N2 virus from 1967 (A/England/10/1967) was shown to be transmitted via the air in three out of three ferret pairs and this virus bound preferentially $\alpha 2,6$ -linked SIAs as shown in a modified TRBC assay [35]. The B1/68 HA carried 25 substitutions as compared to the M1/58 HA, and it cannot be excluded that these substitutions and their potential phenotypic effects were the result of egg adaptation since this virus was propagated four times in eggs, before being passaged in mammalian cells (Table 1).

In conclusion, the data presented here indicate that circulating A/H2N2 avian viruses are not directly transmissible between ferrets. Assuming that ferrets represent a reasonable model for humans, this would suggest that the risk of circulating A/H2N2 viruses to cause a pandemic without prior adaptation or reassortment is low. However, in the absence of immunity against A/H2N2 viruses and a sufficiently large naïve population, the virus may not require to spread efficiently via the air to initiate a pandemic. In the current study, A/H2N2 viruses were less efficiently transmitted via the air between ferrets as compared to other human influenza viruses [32]. This may either indicate that ferrets are a suboptimal model for human-to-human transmission of A/H2N2 viruses or that A/H2N2 viruses may have caused a pandemic despite lower transmissibility compared to other pandemic strains. Nevertheless, continuous surveillance as a component of pandemic risk assessment is warranted. Our data on HA receptor binding, HA stability and transmissibility in the ferret model demonstrated that A/H2N2 viruses continued to adapt to replication and spread in humans during the first years of pandemic circulation, resulting in the acquisition of the hitherto unknown G205S stability substitution in HA.

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Conflicts of interest

The authors declare no competing interests.

Ethical statement

Animals were housed and experiments were performed in strict compliance with the Dutch legislation for the protection of animals used for scientific purposes (2014, implementing EU Directive 2010/63). Research was conducted under a project licence from the Dutch competent authority (licence number AVD101002015340) and the study protocols were approved by the institutional Animal Welfare Body (Erasmus MC permit numbers 122-10-17, 122-11-15 and 15-340-18).

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