

Limited airborne transmission of H7N9 influenza A virus between ferrets

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Wild waterfowl form the main reservoir of influenza A viruses, from which transmission occurs directly or indirectly to various secondary hosts, including humans¹. Direct avian-to-human transmission has been observed for viruses of subtypes A(H5N1), A(H7N2), A(H7N3), A(H7N7), A(H9N2) and A(H10N7) upon human exposure to poultry^{2–7}, but a lack of sustained human-to-human transmission has prevented these viruses from causing new pandemics. Recently, avian A(H7N9) viruses were transmitted to humans, causing severe respiratory disease and deaths in China⁸. Because transmission via respiratory droplets and aerosols (hereafter referred to as airborne transmission) is the main route for efficient transmission between humans, it is important to gain an insight into airborne transmission of the A(H7N9) virus. Here we show that although the A/Anhui/1/2013 A(H7N9) virus harbours determinants associated with human adaptation and transmissibility between mammals, its airborne transmissibility in ferrets is limited, and it is intermediate between that of typical human and avian influenza viruses. Multiple A(H7N9) virus genetic variants were transmitted. Upon ferret passage, variants with higher avian receptor binding, higher pH of fusion, and lower thermostability were selected, potentially resulting in reduced transmissibility. This A(H7N9) virus outbreak highlights the need for increased understanding of the determinants of efficient airborne transmission of avian influenza viruses between mammals.

At the end of March 2013, the World Health Organization was notified by the Chinese authorities of three human cases of infection with a novel avian-origin influenza A(H7N9) virus⁹. All three cases developed bilateral pneumonia with progression to acute respiratory distress syndrome and death^{10,11}. As of July 2013, 132 A(H7N9) laboratory-confirmed cases have been reported in ten different provinces of China, including 37 deaths⁸. This novel A(H7N9) virus emerged in humans after reassortment between viruses of poultry and wild bird origin. The haemagglutinin (HA) and neuraminidase (NA) genes are genetically related to H7 and N9 of viruses isolated from wild ducks, whereas the other genes are closely related to A(H9N2) viruses circulating in poultry¹². It is most likely that the new A(H7N9) viruses have circulated undetected in domestic birds because of their low pathogenicity for poultry. A(H7N9) viruses were isolated from specimens at live poultry markets, pointing to domestic birds as a potential source of human infections¹³.

Although A(H7N9) viruses harbour genetic traits associated with human adaptation of avian viruses and increased transmission between mammals, such as the Q217L substitution in HA (H7 numbering, 226 in H3 numbering) conferring a human receptor preference¹⁴ and the E627K substitution in PB2¹⁵, no sustained human-to-human transmission of A(H7N9) viruses has been reported so far. Apart from two confirmed cases that might have arisen from family clusters and for which human-to-human transmission cannot be ruled out, human cases of A(H7N9) infection were epidemiologically unrelated and identified in different parts of China¹¹.

Gaining knowledge about the ability of animal viruses to transmit via the airborne route is crucial to be able to mitigate future pandemics. Recently, the airborne transmissibility of the human isolate A/Shanghai/2/2013 was evaluated in ferrets and in pigs and was found to be less robust than for the 2009 pandemic A(H1N1) (pH1N1) virus¹⁶. Here, we assessed the airborne transmissibility of a different human isolate, A/Anhui/1/2013 (Anhui/1), in the ferret model as described^{17,18}. Both A/Shanghai/2/2013 and A/Anhui/1/2013 possess human receptor specificity and are of particular interest regarding transmission. Four donor ferrets were inoculated intranasally with 10⁶ 50% tissue culture infectious doses (TCID₅₀) of Anhui/1 virus isolate and the following day, four recipient ferrets were placed in adjacent cages, designed to prevent direct contact between animals but to allow transmission via the airborne route. Throat and nasal swabs were collected at 1, 3, 5 and 7 days post-inoculation (d.p.i.) from the donor ferrets and at 1, 3, 5, 7 and 9 days post-exposure (d.p.e.) from the recipient ferrets. Virus shedding from the donor ferrets was detected from 1 d.p.i. onwards with infectious virus titres up to 10^{6.5} TCID₅₀ ml⁻¹ (Fig. 1a–d). Anhui/1 virus was transmitted to three out of four recipient ferrets (F1, F2 and F3). Transmission was detected at 3 d.p.e. for two ferrets (F2 and F3) and at 5 d.p.e. for one ferret (F1) with infectious virus titres in respiratory swabs up to 10⁶ TCID₅₀ ml⁻¹. All three animals infected via the airborne route seroconverted by 2 weeks after exposure, whereas recipient ferret F4 did not seroconvert. Using the same experimental set-up and protocol, we previously tested the transmissibility of the human pH1N1 virus, seasonal human A(H1N1) virus, and avian influenza A(H5N1) virus. Whereas A(H5N1) virus was not transmitted between ferrets via the airborne route, human influenza viruses were transmitted in all donor-recipient pairs^{17,18}. Replication in donor ferrets inoculated with the Anhui/1 virus and pandemic and seasonal A(H1N1) viruses was comparable, but virus shedding from recipient ferrets was less abundant and delayed for the Anhui/1 virus as compared to the A(H1N1) viruses.

Sanger sequencing was used to determine the consensus genome sequence of the three airborne-transmitted Anhui/1 viruses isolated from recipient ferrets F1, F2 and F3 (Table 1), and substitutions were detected in all gene segments. Several of these were already present in the inoculum, demonstrating the presence of a mixture of viruses in the Anhui/1 virus isolate. Consultation with other laboratories revealed that heterogeneous virus mixtures were present in A(H7N9) virus isolates shipped to other laboratories also. Viruses with different genotypes were transmitted (Table 1). Only two substitutions, N123D and N149D in HA, were consistently found in all three airborne-transmitted viruses. These substitutions are not part of potential N-linked glycosylation sites. Virus recovered from the recipient ferret F1 at 7 d.p.e., which contained the lowest number of substitutions compared to the Anhui/1 virus isolate and had a high virus load, was used to inoculate four additional ferrets. One day later, these animals were paired with four recipient ferrets in transmission cages (Fig. 1e–h), of which one became infected (F5).

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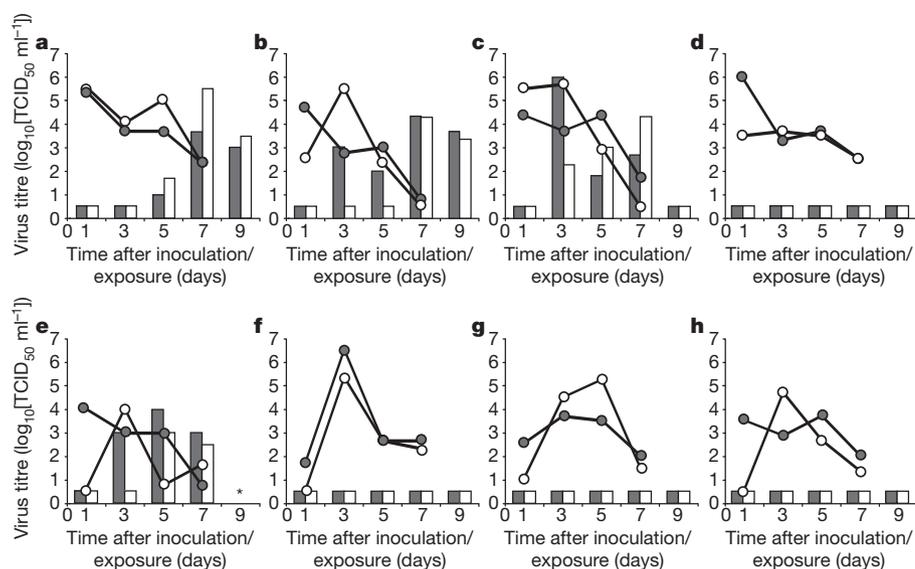


Figure 1 | Airborne transmission of Anhui/1 virus between ferrets. a–d, Transmission experiments are shown for Anhui/1 virus isolate in four ferret pairs (F1–F4). e–h, A nose swab sample from the recipient ferret F1 at 7 d.p.e. was used for the transmission experiments in four ferret pairs shown (F5–F8). Data for individual transmission experiments are shown in each panel, with virus shedding in inoculated and airborne virus-exposed animals shown as lines and bars, respectively. Black circles and bars represent shedding from the throat; white circles and bars represent shedding from the nose. The asterisk indicates the lack of swab collection at 9 d.p.e. for the recipient ferret euthanized at 8 d.p.e. The lower limit of detection is $0.5 \log_{10} \text{TCID}_{50} \text{ml}^{-1}$.

None of the donor ferrets developed clinical signs upon intranasal inoculation. Two recipient ferrets were also without clinical signs, but one recipient ferret (F2) showed loss of appetite, ruffled fur, lethargy and breathing difficulties from 7 d.p.e. onwards. Recipient ferret F5 became moribund and was euthanized at 8 d.p.e. Infectious virus titres up to $10^{4.9} \text{TCID}_{50} \text{g}^{-1}$ in the lungs and $10^{5.8} \text{TCID}_{50} \text{g}^{-1}$ in nasal turbinates were detected in ferret F5. On the basis of pathological analysis, this animal was suffering from a moderate multifocal suppurative rhinitis with associated virus antigen expression in the nasal respiratory and olfactory epithelium (Supplementary Table 4). No lesions or virus antigen expression were seen in the other parts of the respiratory tract, liver or brain that could explain the lethargic state of the ferret.

Consensus virus genome sequences as determined by Sanger sequencing of the airborne-transmitted viruses recovered from recipient ferrets F1 and F5 were identical (Table 1). Substitutions N123D and N149D in HA and M523I in the basic polymerase 1 (PB1) were found consistently in two subsequent transmission experiments. Because mixed populations were detected in the inoculum using Sanger sequencing, next-generation sequencing was performed with respiratory samples of the donor–recipient pairs for which airborne transmission was observed. The entire HA gene and the PB1 part that contained the M523I substitution (nucleotide positions 1126–1616) were sequenced using a 454

sequencing platform (Roche). None of the airborne-transmitted viruses possessed consensus genome sequences identical to that of the Anhui/1 virus isolate (Table 2). Interestingly, an L217Q substitution, conferring a receptor switch from human to avian specificity ($\alpha 2.6$ - to $\alpha 2.3$ -linked sialic acids, respectively), was detected in donor ferrets F1, F2 and F3 in 9.1%, 15.7% and 24.5% of the total number of reads, respectively, but this substitution was not detected in any of the viruses isolated from the ferrets upon airborne exposure. By setting the detection threshold at 1%, we confirmed that the number of variable nucleotide positions in the genome of the Anhui/1 virus isolate was high, and that a rapid gain in clonality occurred after two transmission experiments (Table 2 and Supplementary Table 1). The double substitution N123D/N149D in HA appeared to be selected in most airborne-transmitted viruses and constituted the main viral population after two subsequent transmission experiments (Supplementary Table 2). Although there appeared to be selection of N123D and N149D, it was not possible to determine whether the selection occurred at the level of individual or double substitutions. However, the rapid selection of substitutions in the HA and PB1 genes and the gain in clonality did not change transmission substantially enough to be detectable with the current group size of four ferrets.

Residues N123 and N149 are adjacent to the receptor binding site but do not interact directly with $\alpha 2.3$ - and $\alpha 2.6$ -linked sialic acids

Table 1 | Sanger sequence analysis of full viral genomes of the Anhui/1 virus inoculum and airborne-transmitted viruses

Segment	nt position	nt WT	nt mut.	Amino acid position	Amino acid WT	Amino acid mut.	Inoculum	Transmission				
								Transmission 1		Transmission 2		
								F1	F2	F3	F5	
PB2	411	A	G	128	Gly	S				X		
PB2	1017	C	T	330	Phe	S	X*			X		
PB2	1309	C	T	428	Leu	S		X				X
PB2	1846	C	T	607	Leu	S	X*			X		
PB1	1593	G	A	523	Met	Ile		X				X
PB1	2055	G	A	678	Ser	Asn				X		
PA	1070	A	G	349	Glu	Gly				X		
PA	1167	C	T	380	Asp	S				X		
PA	1380	C	T	452	His	S					X	
PA	1616	G	A	531	Arg	Lys					X	
PA	1674	G	T	550	Leu	S				X*		
PA	1776	C	T	584	Cys	S				X*		
HA	442	A	G	123	Asn	Asp	X*	X		X*	X	X
HA	520	A	G	149	Asn	Asp	X*	X		X*	X	X
HA	704	C	A	210	Ala	Glu				X*		
NP	718	A	C	225	Ile	Leu				X*		
NA	46	C	T	10	Thr	Ile	X*			X*	X*	
M	652	T	C	225	Ala	S				X*		
NS	180	C	T	52	Leu	S					X*	

Substitutions in bold were found in two subsequent transmission experiments and were phenotypically characterized. nt, nucleotides; S, silent substitution; WT, wild type.

*Mixture of wild-type and mutant nucleotides.

Table 2 | Amino acid substitutions in the HA gene and the PB1 gene in Anhui/1 viruses before and after transmission in ferrets

Gene	nt position	nt WT	nt mut.	Amino acid position	Amino acid WT	Amino acid mut.	Inoculum* (%/no. reads)	Donor* F1 (%/no. reads)	Recipient* F1 (%/no. reads)	Donor* F2 (%/no. reads)	Recipient* F2 (%/no. reads)	Donor* F3 (%/no. reads)	Recipient* F3 (%/no. reads)	Donor* F5 (%/no. reads)	Recipient* F5 (%/no. reads)
HA	117	C	T	14	Thr	S	19.5/6,629	–	–	12.5/7,954	20.3/10,349	7.2/4,763	9.6/5,364	–	–
HA	257	C	T	61	Thr	Ile	5.6/8,142	–	–	–	–	–	–	–	–
HA†	442†	A†	G†	123†	Asn†	Asp†	48.7/8,648†	14.4/9,183†	99.8/6,224†	37.4/11,196†	28.5/12,139†	36.5/6,810†	90.1/8,417†	99.8/11,576†	99.9/10,182†
HA	448	G	A	125	Ala	Thr	1.8/7,740	–	–	14.7/10,044	20.5/10,733	7.6/6,088	10.4/7,250	–	–
HA†	520†	A†	G†	149†	Asn†	Asp†	62.5/6,163†	17.9/6,737†	99.9/3,622†	50.4/8,447†	28.6/7,818†	61.2/5,266†	90.4/6,840†	99.7/9,229†	99.7/9,047†
HA	704	C	A	210	Ala	Glu	1.8/3,903	–	–	28.4/4,804	28.6/4,109	56.4/3,359	–	–	–
HA‡	725‡	T‡	A‡	217‡	Leu‡	Gln‡	–	9.1/3,831‡	–	15.7/4,749‡	–	24.5/3,322‡	–	–	–
HA	1032	G	T	319	Lys	Asn	–	–	–	8.3/6,447	–	–	–	–	–
HA	1218	C	T	381	Asn	S	–	5.2/2,102	–	–	–	23.4/1,963	–	–	–
HA	1396	G	A	441	Glu	Lys	–	–	–	–	14.5/2,299	–	–	–	–
HA	1422	G	A	449	Glu	S	–	–	–	–	–	12.6/4,027	–	–	–
HA	1575	C	T	500	Ser	S	6.1/6,532	–	–	–	15.5/2,245	–	7.8/2,387	–	–
HA	1706	T	C	NCR	–	–	–	–	93.0/470	–	–	–	–	–	–
PB1§	1404	G	A	460	Gln	S	6.8/603	–	–	–	–	11.9/1,024	–	–	–
PB1†	1593†	G†	A†	523†	Met†	Ile†	–	–	100/256†	–	–	–	–	82.2/1,795†	99.9/1,064†

NCR, non-coding region; S, silent substitution.

*Percentage variant present with a detection threshold of 5% and number of reads is shown.

†Substitutions were found in two subsequent transmission experiments and were phenotypically characterized.

‡Substitution (L217Q) corresponds to the receptor switch from α 2,6- to α 2,3-linked sialic acids preference.

§The PB1 gene was partially sequenced (nucleotide positions 1126–1616).

(Fig. 2). Recombinant viruses were generated based on seven gene segments of A/Puerto Rico/8/1934 (Puerto Rico/8) with the wild-type Anhui/1 HA and the Anhui/1 HA with amino acid substitutions N123D (Anhui/1(N123D)), N149D (Anhui/1(N149D)) and N123D plus N149D (Anhui/1(N123D/N149D)). Binding to α 2,3-linked sialic acids of Anhui/1(N123D), Anhui/1(N149D) and Anhui/1(N123D/N149D) viruses as assessed using a modified turkey red blood cell (TRBC) assay¹⁹ was increased slightly by two- to fourfold compared to Anhui/1 virus (Supplementary Table 3).

It was recently proposed that stability of HA in an acidic environment—such as mammalian nasal mucosa—is a determinant for airborne transmissibility of influenza viruses²⁰. It has also been noted that

viruses that fuse at low pH have higher thermostability than those fusing at higher pH²¹. We observed that fusion for Anhui/1 HA occurred at pH 5.6, a higher pH than reported previously for human viruses²². Neither the single N123D and N149D substitutions nor the double N123D/N149D substitution reduced the threshold pH for HA-mediated membrane fusion compared to the wild-type Anhui/1 HA (Supplementary Fig. 1). Both single substitutions N123D and N149D and the double substitution N123D/N149D decreased the temperature stability compared to Anhui/1 (Supplementary Fig. 2).

We also assessed the effect of the M523I substitution on the polymerase complex activity in mammalian cells using a minigenome assay at 33 °C, the temperature of the mammalian upper respiratory tract,

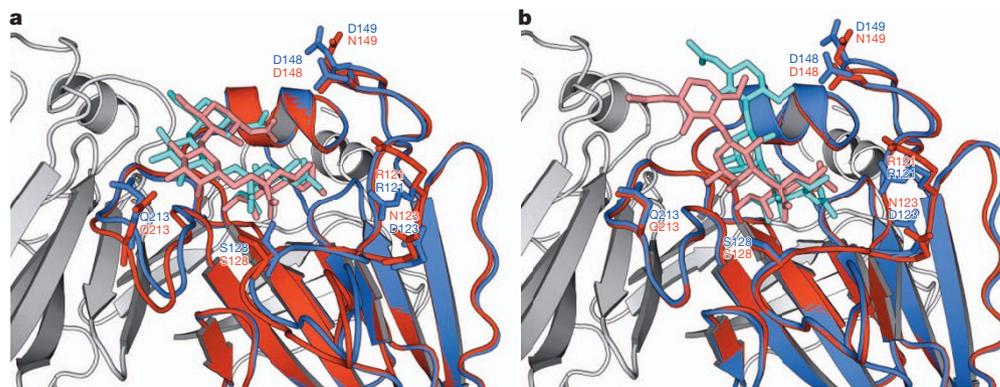


Figure 2 | Cartoon representation of a model of the trimer structure of HA of Anhui/1 (red) and Anhui/1(N123D,N149D) (blue) bound to α 2,6- (a) and α 2,3-linked sialic acids (b). The structures of the three-sugar glycans NeuAc α 2,6Gal β 1-4GlcNAc (a) and NeuAc α 2,3Gal β 1-4GlcNAc (b) were docked into the H7 receptor binding site. The glycans and the amino acid substitutions discussed in the text are shown as sticks. Amino acids N123 and N149 are adjacent to the receptor binding site and, in Anhui/1, do not interact directly with the three-sugar glycans that are depicted in the figure. The mutations cause small changes in the position of some of the residues around

the receptor binding site, notably R121 and D148, and additionally for the α 2,6-linked glycan, residues S128 and Q213. The D123 mutant can form stronger interactions with the side chain of R121, restricting the movement and orientating its side chain to point towards the receptor binding site and interact with the glycan. In Anhui/1, N149 interacts with the neighbouring residue, D148, restricting its orientation. The D149 mutant allows the side chain of D148 to rearrange and interact with the glycan. These changes allow both the α 2,6- and α 2,3-linked glycans to alter position and form more interactions with the HA. All residues are labelled in H7 numbering.

and at 37 °C as previously described²³. No differences in polymerase activity were observed for the polymerase complex with or without the M523I substitution in PB1 (Supplementary Fig. 3).

Here we report that airborne transmission of Anhui/1, as for A/Shanghai/2/2013, can occur between ferrets. Keeping in mind that quantifying transmission in our current experimental model is difficult, and that any transmission may not be directly extrapolated to transmission between humans, these data suggest that Anhui/1 transmission is more efficient than for other avian influenza viruses, which are not airborne transmitted in ferrets, but less robust—with fewer animals becoming infected, and less and delayed virus shedding—as compared to seasonal and pandemic A(H1N1) virus transmission¹⁷. Despite efficient virus replication in ferrets (Fig. 1) and attachment to α 2.6-linked sialic acids (Supplementary Table 3), we speculate that the residual binding to α 2.3-linked sialic acids (Supplementary Table 3), the fusion occurring at a relatively high pH (Supplementary Fig. 1), and instability of HA (Supplementary Fig. 2) may be responsible, at least in part, for limited transmission of Anhui/1 A(H7N9) virus. Contrary to what was observed for A(H5N1) virus^{18,20}, the substitutions selected upon ferret passage and transmission of A(H7N9) virus—N123D and N149D—increased binding to α 2.3-linked sialic acids, increased the pH threshold for membrane fusion, and decreased the thermostability of HA, thus not contributing to increased virus transmission.

Influenza viruses carrying human adaptation markers can arise in poultry. This seems to be the case for the newly emerged A(H7N9) viruses, but also during the 2003 A(H7N7) outbreak in the Netherlands²⁴ and for A(H5N1) viruses currently circulating in poultry²⁵. Fortunately, additional changes required to, for example, further tune receptor preference, lower the pH for HA fusion, and increase HA stability, may be needed for the A(H7N9) viruses to transmit efficiently in mammals^{18,20}. Increased understanding of the mechanisms and molecular determinants that facilitate crossing the species barrier and airborne transmission of avian influenza viruses between mammals is urgently needed.

METHODS SUMMARY

Viruses. Influenza virus Anhui/1 was isolated from a human case of infection and passaged three times in embryonated chicken eggs and once in Madin–Darby canine kidney (MDCK) cells. The virus was provided by the Chinese CDC via the WHO collaborating centre in the UK under the WHO PIP framework. A synthetic construct of the Anhui/1 HA gene segment was provided by R. Webby. The HA gene segment was cloned in the pCAGGs expression plasmid. The PB2, PB1, PA and NP gene segments were cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000^{26,27}. Mutations of interest (M523I in PB1, D123N and D149N in HA) were introduced in constructs using the QuikChange multi-site-directed mutagenesis kit (Stratagene). Recombinant viruses containing seven gene segments of A/Puerto-Rico/8/1934 and wild-type or mutant Anhui/1 HA were produced upon transfection of 293T cells. Virus stocks were propagated and titrated in MDCK cells as described²⁶.

Ferret models. Transmission experiments were performed as described^{17,18}. In short, four female seronegative ferrets, 1–2 years of age, were inoculated intranasally with 10^6 TCID₅₀ of virus. Each donor ferret was then placed in a transmission cage. One day after inoculation, one naive recipient ferret was placed opposite each donor ferret. Each transmission pair was housed in a separate transmission cage designed to prevent direct contact but allowing airflow from donor to recipient. Nose and throat swabs were collected on 1, 3, 5 and 7 d.p.i. for donor ferrets and on 1, 3, 5, 7 and 9 d.p.e. for recipient ferrets. Virus titres in swabs were determined by end-point titration in MDCK cells. A nose swab sample of recipient ferret F1 at 7 d.p.e. was used for the second transmission experiment, with a final dose of approximately 10^4 TCID₅₀ for each ferret.

Full Methods and any associated references are available in the online version of the paper.

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1. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**, 152–179 (1992).

2. Beigel, J. H. *et al.* Avian influenza A (H5N1) infection in humans. *N. Engl. J. Med.* **353**, 1374–1385 (2005).
3. Hirst, M. *et al.* Novel avian influenza H7N3 strain outbreak, British Columbia. *Emerg. Infect. Dis.* **10**, 2192–2195 (2004).
4. Fouchier, R. A. *et al.* Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc. Natl Acad. Sci. USA* **101**, 1356–1361 (2004).
5. Ostrowsky, B. *et al.* Low pathogenic avian influenza A (H7N2) virus infection in immunocompromised adult, New York, USA, 2003. *Emerg. Infect. Dis.* **18**, 1128–1131 (2012).
6. Chen, Y. *et al.* Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. *Lancet* **381**, 1916–1925 (2013).
7. Arzey, G. G. *et al.* Influenza virus A (H10N7) in chickens and poultry abattoir workers, Australia. *Emerg. Infect. Dis.* **18**, 814–816 (2012).
8. WHO. Avian Influenza A(H7N9) Virus http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/index.html (2013).
9. WHO. Human Infection with Influenza A(H7N9) Virus in China http://www.who.int/csr/don/2013_04_01/en/index.html (1 April 2013).
10. Gao, R. *et al.* Human infection with a novel avian-origin influenza A (H7N9) virus. *N. Engl. J. Med.* **368**, 1888–1897 (2013).
11. Li, Q. *et al.* Preliminary report: Epidemiology of the avian influenza A (H7N9) outbreak in China. *N. Engl. J. Med.* <http://dx.doi.org/10.1056/NEJMoa1304617> (2013).
12. Liu, D. *et al.* Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. *Lancet* **381**, 1926–1932 (2013).
13. Cowling, B. J. *et al.* Comparative epidemiology of human infections with avian influenza A H7N9 and H5N1 viruses in China: a population-based study of laboratory-confirmed cases. *Lancet* **382**, 129–137 (2013).
14. Xiong, X. *et al.* Receptor binding by an H7N9 influenza virus from humans. *Nature* <http://dx.doi.org/10.1038/nature12372> (20 June 2013).
15. Steel, J., Lowen, A. C., Mubareka, S. & Palese, P. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. *PLoS Pathog.* **5**, e1000252 (2009).
16. Zhu, H. *et al.* Infectivity, transmission, and pathology of human H7N9 influenza in ferrets and pigs. *Science* **341**, 183–186 (2013).
17. Munster, V. J. *et al.* Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. *Science* **325**, 481–483 (2009).
18. Herfst, S. *et al.* Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* **336**, 1534–1541 (2012).
19. Chutinimitkul, S. *et al.* *In vitro* assessment of attachment pattern and replication efficiency of H5N1 influenza A viruses with altered receptor specificity. *J. Virol.* **84**, 6825–6833 (2010).
20. Imai, M. *et al.* Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* **486**, 420–428 (2012).
21. Ruigrok, R. W. *et al.* Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. *Virology* **155**, 484–497 (1986).
22. Galloway, S. E., Reed, M. L., Russell, C. J. & Steinhauer, D. A. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. *PLoS Pathog.* **9**, e1003151 (2013).
23. Subbarao, E. K., London, W. & Murphy, B. R. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J. Virol.* **67**, 1761–1764 (1993).
24. Jonges, M. *et al.* Comparative analysis of avian influenza virus diversity in poultry and humans during a highly pathogenic avian influenza A (H7N7) virus outbreak. *J. Virol.* **85**, 10598–10604 (2011).
25. Russell, C. A. *et al.* The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. *Science* **336**, 1541–1547 (2012).
26. de Wit, E. *et al.* Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments. *Virus Res.* **103**, 155–161 (2004).
27. Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G. & Webster, R. G. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl Acad. Sci. USA* **97**, 6108–6113 (2000).

Supplementary Information is available in the online version of the paper.

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METHODS

Biocontainment. All experiments were conducted within the enhanced animal biosafety level 3 (ABSL3+) facility of Erasmus MC. The ABSL3+ facility 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 via the facility ventilation system, again via HEPA filters. Only authorized personnel that have received the appropriate training can access the ABSL3+ facility. 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) are directly available¹⁸.

Viruses. Influenza virus A/Anhui/1/2013 (Anhui/1) was isolated from a human case of infection and passed three times in embryonated chicken eggs and once in Madin-Darby Canine Kidney (MDCK) cells. The virus was provided by the Chinese CDC via the WHO collaborating centre in the UK in the context of the WHO PIP framework. A synthetic construct of the Anhui/1 HA gene segment was provided by R. Webby. The PB2, PB1, PA and NP gene segments were amplified by reverse transcription polymerase chain reaction (RT-PCR) from the Anhui/1 virus isolate and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000^{26,27}. In addition, the HA gene segment was cloned in pCAGGs expression plasmid. Mutations of interest (M523I in PB1, D123N and D149N in HA) were introduced in reverse genetics and pCAGGs vectors using the QuikChange multi-site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer. Recombinant viruses containing seven gene segments of A/Puerto Rico/8/1934 and wild-type Anhui/1 HA or Anhui/1 HA containing the mutations N123D and N149D were produced upon transfection of 293T cells. Virus stocks were propagated and titrated in MDCK cells as described previously²⁶.

Cells. MDCK cells were cultured in Eagle's minimal essential medium (EMEM, Lonza) supplemented with 10% fetal bovine serum (FBS), 100 U ml^{−1} penicillin (Lonza), 100 U ml^{−1} streptomycin (Lonza), 2 mM glutamine (Lonza), 1.5 mg ml^{−1} sodium bicarbonate (Lonza), 10 mM HEPES (Lonza), and non-essential amino acids (MP Biomedicals Europe). 293T cells were cultured in Dulbecco modified Eagle's medium (DMEM, Lonza) supplemented with 10% FBS, 100 U ml^{−1} penicillin, 100 U ml^{−1} streptomycin, 2 mM glutamine, 1 mM sodium pyruvate (Gibco), and non-essential amino acids. Vero cells were cultured in Iscove's modified Dulbecco's medium plus L-glutamine (IMDM, Lonza) supplemented with 10% FBS, 100 U ml^{−1} penicillin, 100 mg ml^{−1} streptomycin and 2 mM glutamine.

Virus titration in MDCK cells. Virus titrations were performed as described previously¹⁷. Briefly, MDCK cells were inoculated with tenfold serial dilutions of virus stocks, nose swabs, throat swabs and homogenized tissue samples. Cells were washed with PBS 1 h after inoculation and cultured in 200 µl of infection media, consisting of EMEM supplemented with 100 U ml^{−1} penicillin, 100 U ml^{−1} streptomycin, 2 mM glutamine, 1.5 mg ml^{−1} sodium bicarbonate, 10 mM HEPES, non-essential amino acids, and 20 µg ml^{−1} trypsin (Lonza). Three days after inoculation, supernatants of cell cultures were tested for haemagglutinating activity using turkey erythrocytes as an indicator of virus replication in the cells. Infectious virus titres were calculated from four replicates each of the homogenized tissue samples, nose swabs, and throat swabs and for ten replicates of the virus stocks by the method of ref. 28.

Ferret models. An independent animal experimentation ethical review committee approved all animal studies. The Animal Experiments Committee (Dier Experimenten Commissie, DEC) judges ethical aspects of projects in which animals are involved. Research projects or educational projects involving laboratory animals can only be executed if they are approved by the DEC. The DEC considers the application and pays careful attention to the effects of the intervention on the animal, its discomfort, and weighs this against the social and scientific benefit to humans or animals. The researcher is required to keep the effects of the intervention to a minimum, based on the following: refinement, replacement, reduction. All experiments with ferrets were performed under animal biosafety level 3+ conditions in class 3 isolator cages. No method of randomization was used to determine how animals were divided to the experimental groups. The investigator was not blinded to the group allocation during the experiments or when assessing the outcome.

Airborne transmission experiments were performed as described previously^{17,18}. In short, four female influenza virus and Aleutian disease virus seronegative adult ferrets, 1–2 years of age, were inoculated intranasally with 10⁶ TCID₅₀ of virus by applying 250 µl of virus suspension to each nostril. The sample size of four is based

on earlier calculations for this type of experiment²⁹. Each donor ferret was then placed in a transmission cage. One day after inoculation, one naive recipient ferret was placed opposite each donor ferret. Each transmission pair was housed in a separate transmission cage designed to prevent direct contact but allowing airflow from the donor to the recipient ferret. Nose and throat swabs were collected on 1, 3, 5 and 7 d.p.i. for donor ferrets and on 1, 3, 5, 7 and 9 d.p.e. for the recipient ferrets. Virus titres in swabs were determined by end-point titration in MDCK cells. A nose swab sample of recipient ferret F1 at 7 d.p.e. was used for the second transmission experiment, with a final dose of approximately 10⁴ TCID₅₀ for each ferret. All animals were monitored daily for clinical signs. Necropsy was performed on one ferret that was moribund and due to ethical reasons was removed before the end of the experiment. Nasal turbinates, trachea, lungs, brain and liver were collected, homogenized in 3 ml of virus transport medium, after which the supernatant was collected and stored at −80 °C. Virus titres in the supernatant were determined by end-point titration in MDCK cells. Duplicate samples of these tissues were fixed in 10% neutral-buffered formalin for pathological analysis.

Serology. The exposure of recipient ferrets to Anhui/1 viruses was confirmed by an haemagglutination inhibition assay using standard procedures³⁰. Briefly, blood of the recipient ferrets was collected 12–14 d.p.e. Antisera were pre-treated overnight with receptor-destroying enzyme (*Vibrio cholerae* neuraminidase) at 37 °C and incubated at 56 °C for 1 h the next day. Twofold serial dilutions of the antisera, starting at a 1:20 dilution, were mixed with 25 µl of a virus stock containing 4 haemagglutinating units and were incubated at 37 °C for 30 min. Subsequently, 25 µl 1% turkey erythrocytes were added and the mixture was incubated at 4 °C for 1 h. Haemagglutination inhibition was expressed as the reciprocal value of the highest dilution of the serum that completely inhibited agglutination of virus and erythrocytes.

Sequencing. Viral RNA was extracted from respiratory swab samples collected from the ferrets that were infected via the airborne route and the virus inoculum, using the High Pure RNA Isolation kit (Roche). All eight gene segments of the influenza viruses were amplified by RT-PCR using eight primer sets that cover the full viral genome which specifically amplify each gene segment³¹, and sequenced using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and a 3130XL genetic analyser (Applied Biosystems), according to the instructions of the manufacturer. The consensus sequence was determined for viruses isolated from the following samples: virus inoculum obtained after three egg passages and one MDCK passage; recipient F1, nose swab 7 d.p.e.; recipient F2, throat swab 5 d.p.e.; recipient F3, nose swab 5 d.p.e.; and recipient F5, nose swab 5 d.p.e. Primer sequences are available upon request. Sequences were compared to reference sequences obtained from the GISAID EpifluTM database (accession numbers EPI439503 through to EPI439510).

Viral RNA was extracted from the virus inoculum and respiratory swabs of ferrets using the High Pure RNA Isolation kit (Roche). RNA was subjected to RT-PCR, using 5 primer sets (for HA: set 1 5'-AGCAAAAGCAGGGGATACAA-3' and 5'-GTATGACTTAGTCATCTGCGG-3'; set 2 5'-GGCGGAATTGACAAGGAAGC-3' and 5'-CCACTATGATAGCAATCTCCTTCAC-3'; set 3 5'-GTGACTTTCAGTTTCAATGGGGC-3' and 5'-GATTCTCCATTGCTACCAAGA GTTC-3'; set 4 5'-CTAACCAACAATTGTAGTTAATAGAC-3' and 5'-AGTAAACAAGGGTGTTTT-3'; for PB1: 5'-CAGCGGAAATGCTCGCAAAT-3' and 5'-TTGAGCTGTTGCTGGTCCAA-3') that amplify the region containing the PB1 M523I mutation and the complete HA gene segment. These fragments, approximately 500–600 nucleotides in length, were sequenced using the Roche 454 GS Junior sequencing platform. The fragment library was created for each sample according to the manufacturer's protocol without DNA fragmentation (GS FLX Titanium Rapid Library Preparation, Roche). The emulsion PCR (Amplification Method Lib-L) and GS Junior sequencing run were performed according to instructions of the manufacturer (Roche). Sequence reads from the GS Junior sequencing data were sorted by barcode and aligned to reference sequence A/Anhui/1/2013 using CLC Genomics software 6.0.2. Primers used to amplify the fragments were trimmed at the 3' and 5' ends of the sequence reads. For quality control, sequence reads were trimmed at the 3' end for Phred scores less than 30. The threshold for the detection of single nucleotide polymorphisms was manually set at 1% and 5%.

Model generation. A model of the structure of the HA of Anhui/1 was built by using MODELLER³² based upon the crystal structure of HA of H7N3 virus A/turkey/Italy/02 (Protein Data Bank code 1T18). The N123D and N149D mutations were introduced into the structure using the program Andante³³. Three-sugar glycans NeuAc α 2,6Gal β 1-4GlcNAc and NeuAc α 2,3Gal β 1-3GlcNAc were docked into the binding site of the Anhui/1 HA structure and that of the Anhui/1 (N123D/N149D) HA structure. Several strategies were then used to explore the docking of the glycan within the binding pocket. Alternative glycan conformations were produced by altering the phi angle of the glycosidic bond between the second and third sugars, and the exploration of alternative side-chain conformations of

amino acids within the binding pocket was performed by using a rotamer search³³. Lower energy structures were produced iteratively, until no further energy changes were seen. All simulations were performed using the University of Cambridge CAMGRID computing cluster³⁴.

Modified TRBC haemagglutination assay. Modified TRBC assays were performed as described previously³⁵. 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 μ l of 1% TRBCs in PBS with 50 mU *Vibrio cholerae* NA (VCNA; Roche) in 8 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 control influenza A viruses. Subsequently, resialylation was performed using 0.25 mU of α 2,3-(N)-sialyltransferase (COSMOBIO, bio-connect) or 12 mU of α 2,6-(N)-sialyltransferase (COSMOBIO, bio-connect) and 1.5 mM CMP-sialic acid (Sigma-Aldrich) at 37 °C in 75 μ l 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% bovine serum albumin to a final concentration of 0.5% TRBCs. Resialylation was confirmed by haemagglutination of viruses with known receptor specificity; recombinant viruses with six or seven gene segments of influenza virus A/Puerto Rico/8/1934 and the HA and NA of A/Vietnam/11/2004 H5N1 without the basic cleavage site or the HA of A/Netherlands/213/2003 H3N2. The receptor specificity of mutant viruses (recombinant viruses with seven gene segments of influenza virus A/Puerto Rico/8/1934 and the HA of Anhui/1 with or without the substitutions D123N and/or D149N) was tested by performing a standard haemagglutination assay with the modified TRBCs. In brief, serial twofold dilutions of virus in PBS were made in a 50 μ l volume; 50 μ l of 0.5% TRBCs were added, followed by incubation for 1 h at 4 °C before determining the haemagglutination titre.

Fusion assay. Influenza virus HA-induced cell fusion was tested in Vero-118 cells transfected with 5 μ g of pCAGGs-HA using Xtremegene transfection reagent (Roche). One day after transfection, cells were collected using trypsin-EDTA and plated in 6-well plates. The next morning, cells were washed and medium was replaced with IMDM medium containing 10 μ g ml⁻¹ of trypsin. After 1 h, cells were washed with PBS and exposed to PBS at pH 5.0, 5.2, 5.4, 5.6, 5.8, or 6.0 for 10 min at 37 °C. Subsequently, the PBS was replaced by IMDM supplemented with 10% FBS. Eighteen hours after the pH shock, cells were fixed using 80% ice-cold acetone, washed, and stained using a 20% Giemsa solution (Merck Millipore).

HA stability assay. The stability of HAs from the mutant viruses (recombinant viruses with seven gene segments of influenza virus A/Puerto Rico/8/1934 and the HA of Anhui/1 with or without the substitutions N123D and/or N149D and the HA of H5N1 A/Indonesia/5/2005 (INDO) with and without the substitution T318I) was evaluated by performing a thermostability assay. In short, viruses were diluted to 64 HA units per 25 μ l using PBS. The samples were incubated in a thermal cycler for 30 min at a temperature of 50 °C, 52 °C, 54 °C, 56 °C and 58 °C. Subsequently, the HA titre was determined by performing a haemagglutination assay using turkey erythrocytes.

Minigenome assay. A model viral RNA (vRNA), consisting of the firefly luciferase open reading frame flanked by the non-coding regions (NCRs) of segment 8 of influenza A virus, under the control of a T7 RNA polymerase promoter was used for minigenome assay³⁶. The reporter plasmid (0.5 μ g) was transfected into 293T cells in 6-well plates, along with 0.5 μ g of each of the pHW2000 plasmids encoding PB2, PB1, PA and NP, and 1 μ g of pAR3132 expressing T7 RNA polymerase and 0.02 μ g of the *Renilla* luciferase expression plasmid pRL (Promega) as an internal control. At 48 h after transfection, luminescence was measured using the Dual-Glo Luciferase Assay System (Promega) according to instructions of the manufacturer in a TECAN Infinite F200 machine (Tecan). Relative light units (RLU) were calculated as the ratio of Firefly and *Renilla* luciferase luminescences.

Pathology and immunohistochemistry. After fixation in 10% neutral-buffered formalin, tissues were embedded in paraffin, sectioned at 3 μ m, and stained with haematoxylin and eosin for the detection of histological lesions by light microscopy. For the detection of virus antigen by immunohistochemistry, tissues were stained with a monoclonal antibody against influenza A virus nucleoprotein as the primary antibody as described previously³⁷. After determining the cell types expressing viral antigen, the percentage of positively staining cells per tissue was estimated and ranked on an ordinal scale: 0, 0% of cells; 1, 1–25%; 2, 25–50%; 3, >50%.

28. Reed, L. J. & Munch, H. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 493–497 (1938).
29. Nishiura, H., Yen, H. L. & Cowling, B. J. Sample size considerations for one-to-one animal transmission studies of the influenza A viruses. *PLoS ONE* **8**, e55358 (2013).
30. Hirst, G. K. Studies of antigenic differences among strains of influenza A by means of red cell agglutination. *J. Exp. Med.* **78**, 407–423 (1943).
31. Hoffmann, E., Stech, J., Guan, Y., Webster, R. G. & Perez, D. R. Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol.* **146**, 2275–2289 (2001).
32. Šali, A. & Blundell, T. L. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815 (1993).
33. Smith, R. E., Lovell, S. C., Burke, D. F., Montalvo, R. W. & Blundell, T. L. Andante: reducing side-chain rotamer search space during comparative modeling using environment-specific substitution probabilities. *Bioinformatics* **23**, 1099–1105 (2007).
34. Calleja, M. *et al.* *Grid Tool Integration Within the eMinerals Project* (Proc. UK e-Science All Hands Meeting, 2004).
35. Nobusawa, E., Ishihara, H., Morishita, T., Sato, K. & Nakajima, K. Change in receptor-binding specificity of recent human influenza A viruses (H3N2): a single amino acid change in hemagglutinin altered its recognition of sialyloligosaccharides. *Virology* **278**, 587–596 (2000).
36. de Wit, E. *et al.* Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host. *J. Virol.* **84**, 1597–1606 (2010).
37. van Riel, D., Rimmelzwaan, G. F., van Amerongen, G., Osterhaus, A. D. & Kuiken, T. Highly pathogenic avian influenza virus H7N7 isolated from a fatal human case causes respiratory disease in cats but does not spread systemically. *Am. J. Pathol.* **177**, 2185–2190 (2010).