Supplementary Materials for

Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets

Sander Herfst, Eefje J. A. Schrauwen, Martin Linster, Salin Chutinimitkul, Emmie de Wit, Vincent J. Munster, Erin M. Sorrell, Theo M. Bestebroer, David F. Burke, Derek J. Smith, Guus F. Rimmelzwaan, Albert D. M. E. Osterhaus, Ron A. M. Fouchier*

*To whom correspondence should be addressed. E-mail: r.fouchier@erasmusmc.nl

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Risk assessments prior to start of research

The research on transmission of H5N1 virus has been discussed among staff members of the Department of Virology at Erasmus MC since 1998. The work did not commence until much later because we considered the laboratory facilities available at the Rotterdam site at that time inappropriate for this type of work. Between 1998 and 2007, the H5N1 transmission experiments were discussed explicitly and extensively among the staff members of the Department of Virology, followed by discussions with biosafety officers and facility managers of Erasmus MC, as well as with numerous specialists from the influenza and general infectious disease fields around the globe (*61*). Throughout these discussions, and upon site-visits of facilities where research with class 3 and class 4 pathogens is conducted routinely, a plan was drawn to develop adequate research facilities, to obtain permits for working with genetically modified organisms (GMO), and for review of research proposals.

Following a Broad Agency Announcement of the National Institute of Allergy and Infectious Diseases and National Institutes of Health (BAA NIH-NIAID-DMID-07-20) in 2005, the Department of Virology, along with partners in the USA, drafted a research proposal to become an NIAID/NIH Center of Excellence for Influenza Research and Surveillance to support the research agenda of the US Department of Health and Human Services (HHS) Pandemic Influenza Plan in the USA. This proposal was reviewed favorably by NIAID/NIH in consultation with HHS and supported by external expert advisors. Upon signing the research contract, a new GMO permit – explicitly for conducting work with airborne-transmissible H5N1 virus and early pandemic viruses – was obtained from the Dutch Ministry for Infrastructure and the Environment (I&M) in 2007. To this end, I&M was advised by the Committee Genetic Modification (COGEM), which is an independent scientific advisory committee for the Dutch government. I&M and COGEM concluded that the proposed work could be performed with negligible risk to humans and the environment under the conditions realized (permit IG-07-038). The COGEM advice is available on-line (*62*).

In 2007, a “Code of Conduct for Biosecurity” was drafted by the Royal Netherlands Academy of Arts and Sciences (KNAW) upon request of the Dutch Ministry of Education, Culture and

Science (OCW) as required by the Biological and Toxin Weapons Convention (BTWC) ratified in 1972 and the Statement on Biosecurity issued by the InterAcademy Panel (IAP) in 2005 (*33*). As R.A.M. Fouchier was a member of the Biosecurity Focus Group that advised during the conception of this Code of Conduct for Biosecurity, the department of Virology of Erasmus MC was well aware of the Code prior to its publication in 2007, and adhered strictly to this Code at all times. The principles and procedures outlined in the Dutch Code of Conduct are similar to those agreed upon in the USA.

Biosafety and biosecurity measures

All experiments were conducted within the enhanced animal biosafety level 3 (ABSL3+) facility of Erasmus MC that was completed in 2007. The ABSL3+ facility consists of a negative pressurized (-30Pa) 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 (< -200Pa). Air released from the class 3 units is filtered twice by High Efficiency Particulate Air (HEPA) filters and then leaves via the facility ventilation system, again via double 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 ErasmusMC and Dutch and United States government inspectors.

All facilities, procedures, training records, safety drills, inventory records, and logbooks, are subject to inspection and oversight by the institutional biosafety officers of Erasmus MC in close consultation with the facility management. The facilities, personnel, and procedures are further inspected by the US Centers for Disease Control and Prevention (CDC) every 3 years in agreement with the US select agent regulations for oversees laboratories and by the Dutch government (VROM inspection). The most recent CDC inspections took place in February 2011 and March 2012 at which time no shortcomings in biosafety and biosecurity measures were identified.

Occupational health risk

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 and all personnel are offered seasonal and prototype A/H5N1 influenza vaccines with informed consent. Additional immunizations with A/H5N1 vaccine were administered if seroconversion could not be demonstrated. Consent records are held by the Department of Virology at ErasmusMC.

All personnel are given basic training in laboratory safety under BSL2 conditions. Employees are trained for a further 3 months under standard BSL3 conditions, supervised by highly experienced personnel. Following initial BSL3 training and a period of independent work under BSL3 conditions, employees are trained for a further 3 months in the ABSL3+ facility, again under the constant supervision of highly experienced personnel with >8 years of research experience. These training programs consist of hands-on work under supervision, following theory components on facilities, procedures and safety drills. Upon completion of the supervised training period, the supervisors judge whether trainees fulfill all requirements for working independently in the facilities. Annual refreshment training sessions on biosafety and biosecurity are provided by the principal investigators, biosafety officers, and facility managers.

All equipment in the facilities is monitored electronically and both acoustic and telephone alarms are employed to ensure that workers do not enter the facilities if equipment is malfunctioning. All personnel have been instructed and trained how to act in case of incidents. All incidents are handled with consultation between a senior staff member of the Virology Department, a clinical microbiologist, the biosafety officers, and the facility management. Antiviral drugs (oseltamivir and zanamivir) are directly available. Erasmus MC has isolation hospital rooms (negative pressure rooms with interlocks) with trained nursing and medical staff to be used in case of serious incidents and to quarantine the infected individual to prevent further dissemination of the pathogen.

Materials and methods

Viruses

Influenza virus A/Indonesia/5/2005 (A/H5N1) was isolated from a human case of HPAI virus infection and passaged once in embryonated chicken eggs followed by a single passage in Madin-Darby Canine Kidney (MDCK) cells. All eight gene segments were amplified by reverse transcription polymerase chain reaction and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 (*63-64*). Mutations of interest (N182K, Q222L, G224S in HA and E627K in PB2) were introduced in reverse genetics vectors using the QuikChange multi-site-directed mutagenesis kit (Aligent, Amstelveen, The Netherlands) according to the instructions of the manufacturer. Recombinant viruses were produced upon transfection of 293T cells and virus stocks were propagated and titrated in MDCK cells as described (*63*).

Cells

MDCK cells were cultured in Eagle's minimal essential medium (EMEM, Lonza Benelux BV, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM Hepes (Lonza), and non-essential amino acids (MP Biomedicals Europe, Illkirch, France). 293T cells were cultured in Dulbecco modified Eagle's medium (DMEM, Lonza) supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2mM glutamine, 1mM sodium pyruvate, and non-essential amino acids.

Virus titration in MDCK cells

Virus titrations were performed as described previously (27). Briefly, MDCK cells were inoculated with tenfold serial dilutions of virus preparations, homogenized tissues, nose swabs, and throat swabs. Cells were washed with PBS one hour after inoculation and

cultured in 200µl of infection media, consisting of EMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5mg/ml sodium bicarbonate, 10mM Hepes, non-essential amino acids, and 20 µg/ml trypsin (Lonza). Three days after inoculation, supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of virus replication in the cells. Infectious virus titers were calculated from four replicates each of the homogenized tissue samples, nose swabs, and throat swabs and for ten replicates of the virus preparations by the method of Spearman-Karber (*65*).

Ferret experiments

An independent animal experimentation ethical review committee, approved by Dutch Government (Stichting DEC Consult) approved all animal studies. All experiments with ferrets were performed under animal biosafety level 3+ conditions in class 3 isolator cages.

Supplemental figures and data

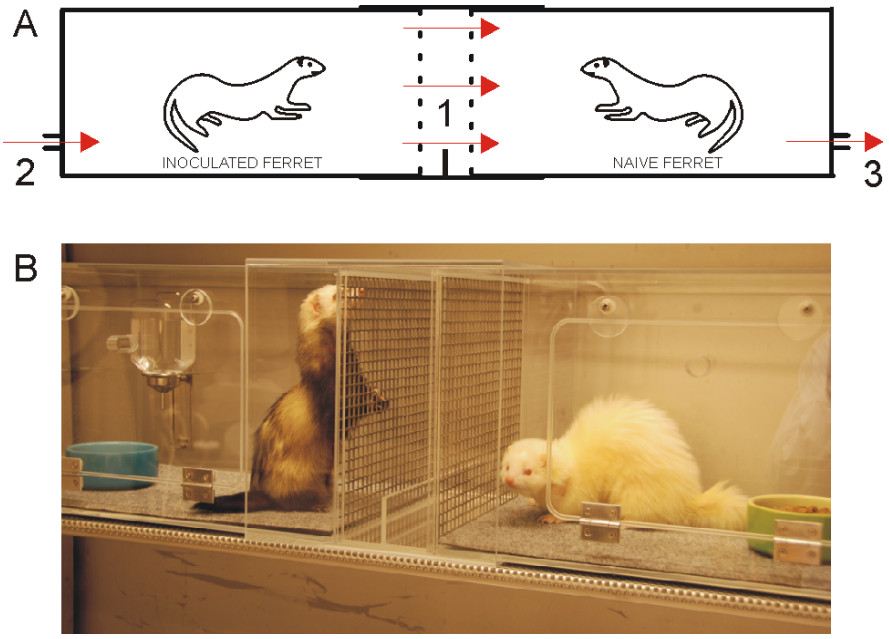

Fig. S1. Schematic presentation (A) and a photograph (B) of paired transmission cages. The transmission cages were specifically designed to allow transmission experiments to be conducted in negatively pressurized isolator cages (1.6m x 1m x 1m) in the ABSL3+ facility. The ferrets are housed in clear Perspex cages, in which each inoculated animal was housed individually next to a naive ferret. Each ferret cage was 30 cm x 30 cm x 55 cm (W x H x L) and the two cages were separated by two stainless steel grids (1), with a grid size of 0.5 cm^2, 10 cm apart. Negative pressure within the isolator cage is used to direct a modest (< 0.1 m/sec) flow of HEPA-filtered air (2) from the inoculated to the naive ferret. The outlet airflow (3) is HEPA filtered to prevent continuous circulation of infectious influenza A virus particles and to prevent cross-contamination. Animals are housed on solid rubber floor tiles, which do not generate dust and avoid unwanted fomite transmission among animals. Arrows indicate airflow.

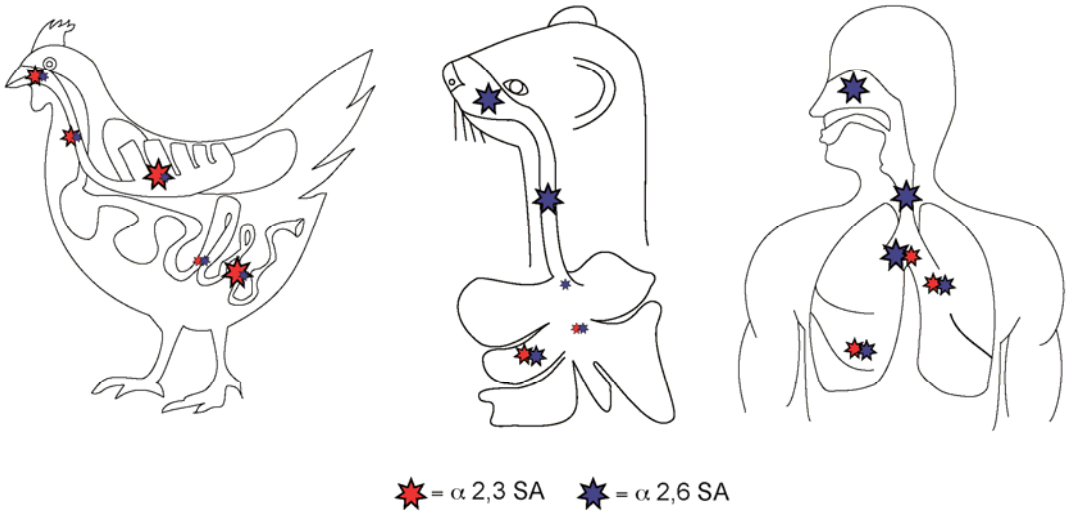

Fig S2. Influenza virus receptor distribution in the respiratory and enteric tract of chickens, and the respiratory tract of ferrets and humans. Avian and human influenza viruses preferentially bind to α-2,3-linked (red) and α-2,6-linked (blue) sialic acid (SA) receptors, respectively. In chicken, although both α-2,3-linked SA and α-2,6-linked SA are present throughout the respiratory tract and gut, α-2,3-linked SA are expressed more abundantly. In contrast, humans and ferrets predominantly express α-2,6-linked SA receptors in the upper respiratory tract (URT) and trachea, and α-2,3-linked SA receptors in the lower respiratory tract (*28*). The size of the red and blue symbols correlates with the relative abundance of SA receptors.

Experiment 1

To evaluate the effect of receptor binding site (RBS) mutations on A/H5N1 virus replication in the ferret respiratory tract, four groups of six influenza virus seronegative female ferrets (*Mustela putorius furo*) were inoculated intranasally with 1 x 10^6 $TCID_{50}$ of $A/H5N1_{wildtype}$, $A/H5N1_{HA\ N182K}$, $A/H5N1_{HA\ Q222L,G224S,}$ or $A/H5N1_{HA\ N182K,Q222L,G224S}$ (2 × 250 µL, divided over both nostrils), respectively (Fig. S3). Nose and throat swabs were collected daily and immediately suspended in 1 ml of virus transport medium (VTM) containing glycerol and antibiotics. Three animals of each group were euthanized at 3 and 7 days post inoculation (dpi), and nasal turbinates (narrow and curled bone shelves that stick out into the breathing passage of the nose), trachea and lungs were collected, homogenized in 3 ml of VTM, after which the supernatant was collected and stored at -80°C. Virus titers in swabs and respiratory tissues were determined by end-point titration in MDCK cells as described above.

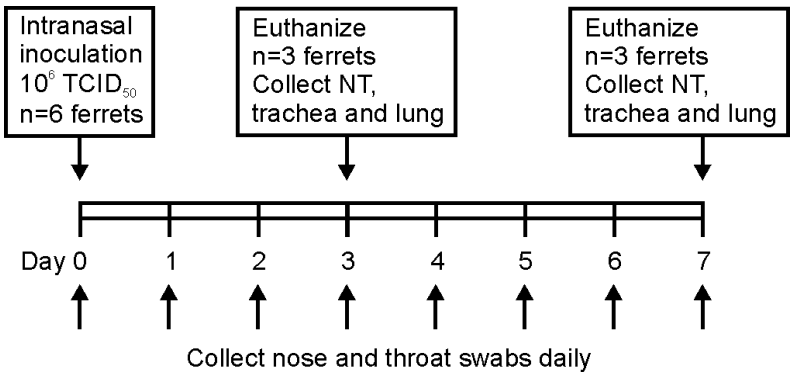

Fig. S3. Schematic overview of experiment 1 to evaluate replication of $A/H5N1_{wildtype}$, $A/H5N1_{HA\ N182K}$, $A/H5N1_{HA\ Q222L,G224S,}$ or $A/H5N1_{HA\ N182K,Q222L,G224S}$ in ferrets. NT; nasal turbinates.

Experiment 2

Amino acid substitution E672K in PB2 is one of the most consistent host-range determinants of influenza viruses. To test its effect on airborne-transmission of an A/H5N1 virus with specificity for the human α2,6-linked SA receptor, we introduced this E627K mutations in virus A/H5N1$_{HA\ Q222L,G224S}$ that was used in experiment 1. Airborne transmission experiments were performed as described previously (*27*). In short, four female adult ferrets were inoculated intranasally with 1 x 10^6 $TCID_{50}$ of A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ by applying 250µL of virus suspension to each nostril. Each donor ferret was then placed in a transmission cage (Fig. S1). One day after inoculation, one naïve recipient ferret was placed opposite each inoculated ferret. Each transmission pair was housed in a separate transmission cage designed to prevent direct contact between the inoculated and naïve ferrets but allowing airflow from the donor to the recipient ferret. Nose and throat swabs were collected on 1, 3, 5, and 7 dpi for donor ferrets and on 1, 3, 5, 7, 9 and 11 days post exposure (dpe) for the recipient ferrets as described for experiment 1. Virus titers in swabs were determined by end-point titration in MDCK cells. If virus shedding was detected in the recipient ferrets upon exposure to the donor ferrets, this was judged as evidence for airborne transmission.

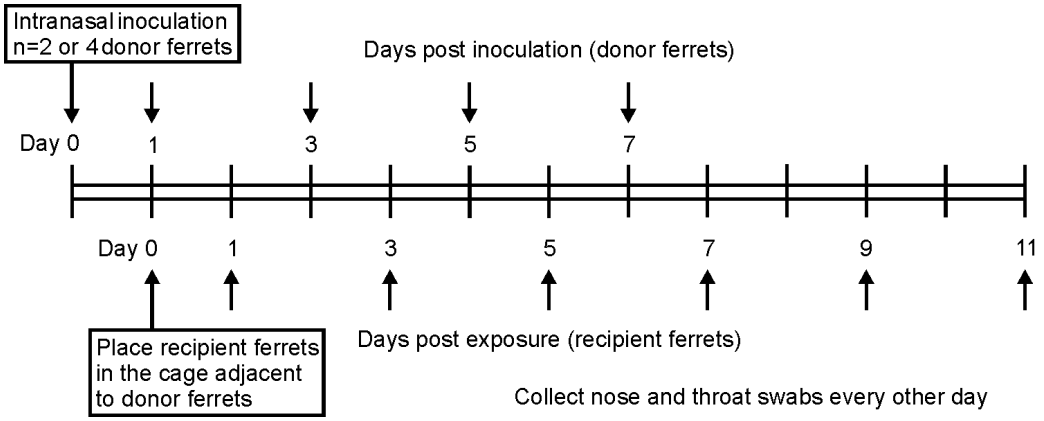

Fig. S4. Schematic overview of experiments to test airborne transmission of influenza virus in experiments 2 and 4.

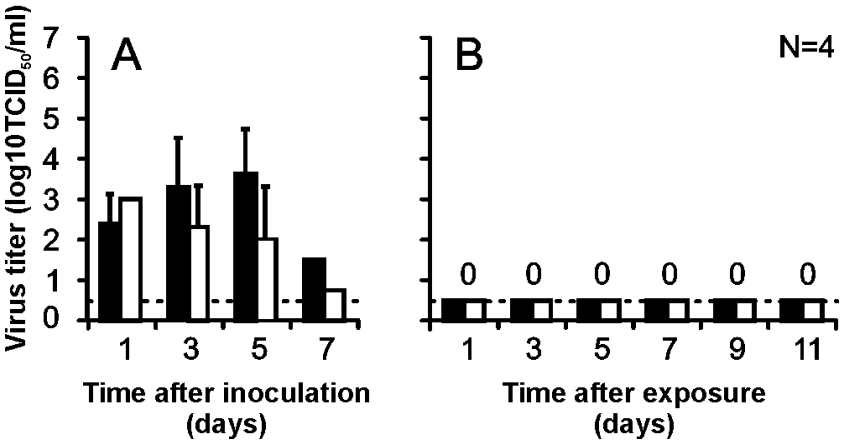

Fig. S5. Shedding of influenza virus A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ in ferrets in transmission experiments (experiment 2). (A) Nose (white bars) and throat swabs (black bars) were collected on days 1, 3, 5, and 7 after inoculation from ferrets inoculated intranasally and (B) on days 1, 3, 5, 7, 9, and 11 after exposure for naïve ferrets in an adjoining cage. Virus titers were determined by end-point titration in MDCK cells. The geometric mean titers of positive samples are displayed, with error bars indicating standard deviations. Numbers in panel B indicate the number of ferrets infected via airborne transmission.

Experiment 3

Since no airborne transmission was observed in experiment 2, A/H5N1$_{wildtype}$ and A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ were serially passaged in ferrets to allow adaptation for efficient replication in mammals. Each virus was inoculated intranasally with 1 x 10^6 $TCID_{50}$ in one ferret (2 x 250 µl, divided over both nostrils). Nose and throat swabs were collected daily, as described for experiment 1. Animals were euthanized at 4 dpi and nasal turbinates and lungs were collected as described for experiment 1. Nasal turbinates were homogenized in VTM, and this homogenate was used to inoculate the next ferret intranasally, resulting in passage 2 (Fig. 2, Fig. S6). Subsequent passages 3 - 6 were performed in the same way. From passage six onwards, nasal washes were collected at 3 dpi in addition to the nasal swabs. To this end, one milliliter of PBS was delivered drop wise to the nostrils of the ferrets, thereby inducing sneezing. Approximately 200 µl of the 'sneeze' was collected in a Petri dish, and PBS was added to a final volume of 2 ml. For passage 7 through 10, the nasal wash sample was used for the following passages in ferrets. The passage-10 nasal washes of the ferrets inoculated with A/H5N1$_{wildtype}$ and A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ were used for sequence analyses using the 454/Roche GS-FLX sequencing platform, as well as for transmission experiments (experiment 4).

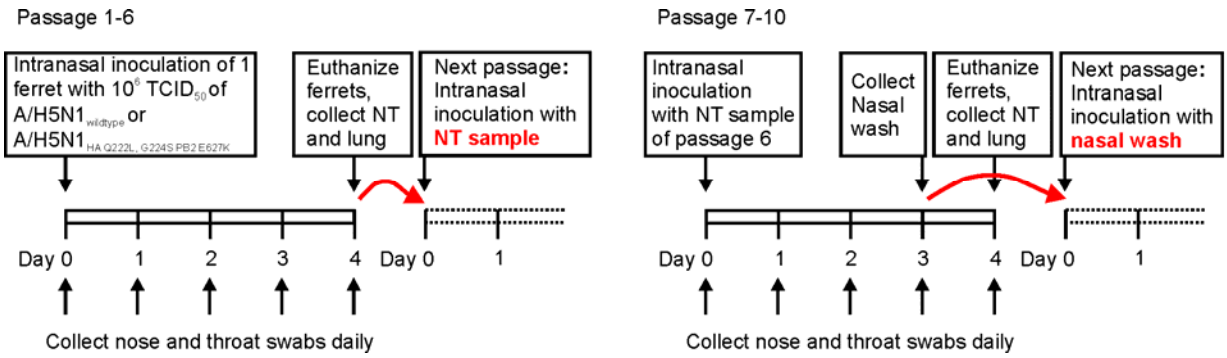

Fig. S6. Schematic overview of experiment 3 to allow adaptation for efficient replication in the upper respiratory tract by serial passaging in ferrets. NT; nasal turbinates.

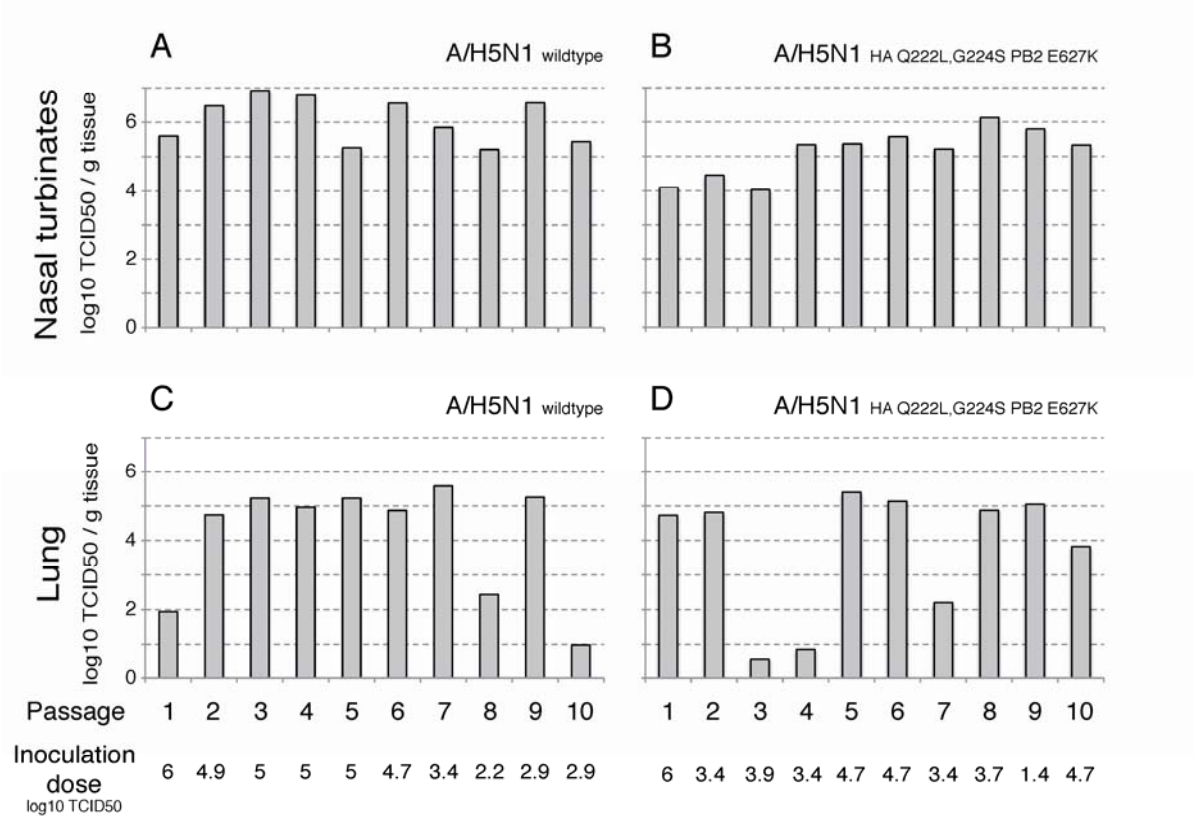

Fig. S7. Virus titers in the nasal turbinates (A, B) and lungs (C, D) collected on day 4 from ferrets inoculated with A/H5N1$_{wildtype}$ (A, C) and A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ (B, D) throughout the ten serial passages as numbered on the x axes. Virus titers were determined by end-point titration in MDCK cells. The total virus dose for each inoculation is shown below panels C and D; these numbers also correspond to parts A and B, respectively. This virus dose was given in a 0.5 ml volume (0.25 ml/nostril). After inoculation with A/H5N1$_{wildtype}$, virus titers in the nasal turbinates were variable but high, ranging from 1.6 x 10^5 to 7.9 x 10^6 $TCID_{50}$/gram tissue (panel A), with no further increase observed with repeated passage. After inoculation with A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$, virus titers in nasal turbinates averaged 1.6 x 10^4 in the first three passages, 2.5 x 10^5 in passage four to seven and 6.3 x 10^5 $TCID_{50}$/gram tissue in the last three passages, suggestive of improved replication and virus adaptation. In the lungs, no apparent adaptation was observed for animals inoculated with either virus. Virus titers in lungs were highly variable; presumably it was a matter of chance whether the virus reached the lower airways.

Sequencing of virus quasispecies using a 454 sequencing platform

Viral RNA was extracted from nasal washes of ferrets after 10 passages with influenza viruses A/H5N1$_{wildtype}$ and A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ using the High Pure RNA Isolation Kit (Roche). RNA was subjected to reverse transcriptase polymerase chain reaction (RT-PCR), using 32 primer sets that cover the full viral genome (*66*). These fragments, approximately 400-600 nucleotides in length, were sequenced using the 454/Roche GS-FLX 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 runs were performed according to instructions of the manufacturer (Roche). Sequence reads from the GS-FLX sequencing data were sorted by bar code and aligned to reference sequence A/Indonesia/5/2005 using CLC Genomics software 4.6.1. The sequence reads were trimmed at 30 nucleotides from the 3' and 5' ends to remove all primer sequences. The threshold for the detection of single nucleotide polymorphisms was manually set at 10% (Table S1, Table S2).

Table S1. Sequence analysis of virus quasispecies present in nasal wash sample of a ferret after 10 passages with A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ virus, using the 454/Roche GS-FLX sequencing platform (ferret P10 in figure 4 of the main text). The viral gene segment is indicated, along with the nucleotide substitutions, the frequency (percent) of sequence reads with the detected mutations, the coverage (total number of sequence reads covering the position), and the nucleotide (nt) and amino acid (aa) positions. All changes from A/H5N1$_{wildtype}$ are shown that have a frequency > 10; the introduced mutations are underlined.

Segment	Reference	Mutation	Frequency	Coverage	nt position	aa position	aa	aa subst
PB2	G	A	26.4	416	252	75	Gln	S[1]
<u>PB2</u>	G	A	99.6	260	1906	627	Glu	Lys
PB1	C	T	45.0	464	319	99	His	Tyr
PB1	G	T	34.3	542	885	287	Arg	Ser
PB1	G	A	39.2	102	982	320	Ala	Thr
PB1	A	G	50.8	177	1126	368	Ile	Val
PB1	C	T	18.2	390	1500	492	Phe	S
PA	G	A	10.6	416	579	185	Arg	S
PA	C	T	15.0	360	1569	515	Thr	S
PA	T	C	54.6	646	1653	543	Leu	S
PA	A	G	27.7	913	1864	614	Asn	Asp
HA	C	T	85.3	225	383	103	His	Tyr
HA	C	A	11.1	814	538	154	Asn	Lys
HA	A	G	89.9	975	542	156	Thr	Ala
<u>HA</u>	A	T	99.9	932	741	222	Gln	Leu
<u>HA</u>	G	A	99.7	998	746	224	Gly	Ser
<u>HA</u>	A	C	99.9	1033	748	224	Gly	Ser
HA	G	A	15.1	436	977	301	Glu	Lys
HA	C	T	12.9	708	1020	315	Thr	Ile
HA	G	A	17.4	619	1156	360	Gln	S
HA	G	A	11.8	977	1611	512	Gly	Glu
NP	C	T	11.1	36	29	NCR[2]		
NP	G	A	18.1	1189	341	99	Arg	Lys
NP	G	A	22.2	1083	1430	462	Gly	Glu
NP	A	G	12.0	1296	1431	462	Gly	S
NA	A	G	21.7	650	981	321	Asn	Asp
NS	G	A	41.4	596	219	65	Val	Met

1. S; silent substitution, 2. NCR; non-coding region.

Table S2. Sequence analysis of virus quasispecies present in nasal wash sample of a ferret after 10 passages with A/H5N1$_{wildtype}$ virus, using the 454/Roche GS-FLX sequencing platform. The viral gene segment is indicated, along with the nucleotide substitutions, the frequency (percent) of sequence reads with the detected mutations, the coverage (total number of sequence reads covering the position), and the nucleotide (nt) and amino acid (aa) positions. All changes from A/H5N1$_{wildtype}$ are shown that have a frequency > 10.

Segment	Reference	Mutation	Frequency	Coverage	nt position	aa position	aa	aa subst
PB2	A	G	100.0	415	1296	423	Arg	S[1]
PB2	G	A	100.0	37	2008	661	Ala	Thr
PB1	T	C	99.8	567	303	93	Ala	S
PB1	A	G	12.1	174	1398	458	Gly	S
PA	G	A	100.0	522	816	264	Thr	S
PA	G	A	99.8	515	1821	599	Glu	S
HA	A	G	99.6	926	542	156	Thr	Ala
HA	T	C	99.5	605	1123	349	Asp	S
NP	G	A	17.9	1497	1430	462	Gly	Glu
NA	C	T	100.0	561	1337	439	Asp	S

1. S; silent substitution

Experiment 4

Nasal wash samples collected at 3 dpi from ferrets at passage ten (experiment 3) were used in transmission experiments, to test if airborne-transmissible virus was present in the virus quasispecies. Transmission experiments were done as described above for experiment 2 (Fig. S4). Four and two donor ferrets were inoculated intranasally with nasal wash samples collected from passage 10 (P10) of the A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ and A/H5N1$_{wildtype}$ virus, respectively. As shown in figure 5 of the main text, three out of four recipients ferrets became infected upon airborne transmission of the P10 A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$ virus (F1, F2 and F3). As shown in Figure 6A and 6B of the main text, virus isolated from a throat swab collected at 3 dpi from ferret F2 was subsequently used to inoculate two additional donor ferrets and both of these animals again transmitted the virus to two other recipient ferrets (F5, F6). As shown in figure 6C and 6D of the main text, a virus isolate from F5 that was passaged once in MDCK cells, was next inoculated intranasally into two more donor ferrets. When these ferrets were paired with two recipient ferrets (F7, F8) the next day, one of them (F7) became infected. See Figure 4 of the main text for overall summary.

Sanger sequencing to determine the consensus genome sequences of airborne-transmissible viruses

To determine the consensus sequence of airborne-transmissible viruses in throat and nose swabs obtained from recipient ferrets in experiment 4, viral RNA was extracted using the High Pure RNA Isolation Kit (Roche). All eight influenza virus gene segments were amplified by RT-PCR (*67*) using 32 primer sets that cover the full viral genome (*66*) and sequenced using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) and a 3130XL genetic analyzer (Applied Biosystems), according to the instructions of the manufacturer (Table S3, Fig. 4).

Table S3. Sanger sequence analysis of full viral genomes of airborne transmitted influenza viruses. Data are shown for individual ferrets (F1-F3, F5-F7) upon three subsequent transmission experiments, as described in figure 4 of the main text. The consensus sequence was determined for viruses isolated from the following samples: F1: throat swab 7 dpi, F2: throat swab 3 dpi, F3: throat swab 5 dpi, F5: throat swab 5 dpi, F6: nose swab 5 dpi and F7: throat swab 5 dpi. Substitutions detected in the virus samples that differ from the wildtype A/Indonesia/5/2005 virus are marked with "x". The introduced mutations are underlined.

Segment	nt pos	nt wildtype	nt mutant	aa pos	aa wildtype	aa mutant	Transmission 1			Transm 2		Transm 3
							F1	F2	F3	F5	F6	F7
PB2	1298	C	T	424	Ala	Val					x	
PB2	1884	A	G	619	Leu	S[1]			x			
<u>PB2</u>	1906	G	A	627	Glu	Lys	x	x	x	x	x	x
PB1	319	C	T	99	His	Tyr		x		x		x
PB1	591	G	A	189	Arg	S		x		x		x
PB1	885	G	T	287	Arg	Ser	x		x			
PB1	982	G	A	320	Ala	Thr					x	
PB1	1113	G	A	363	Lys	S					x	
PB1	1126	A	G	368	Ile	Val	x	x		x		x
PB1	1500	C	T	492	Phe	S					x	
PB1	1672	C	T	550	Leu	S		x		x	x	x
PA	1719	G	A	565	Val	S		x				
PA	1864	A	G	614	Asn	Asp			x			
HA	383	C	T	103	His	Tyr	x	x	x	x	x	x
HA	538	C	A	154	Asn	Lys	x					
HA	542	A	G	156	Thr	Ala	x	x	x	x	x	x
<u>HA</u>	741	A	T	222	Gln	Leu	x	x	x	x	x	x
<u>HA</u>	746	G	A	224	Gly	Ser	x	x	x	x	x	x
<u>HA</u>	748	A	C	224	Gly	Ser	x	x	x	x	x	x
HA	1020	C	T	315	Thr	Ile	x					
NP	29	C	T	NCR[2]	C	T			x			
NP	341	G	A	99	Arg	Lys	x	x	x	x		x
NP	1079	G	A	345	Ser	Asn				x		x
NA	324	A	G	102	Ile	Val					x	
NA	447	G	A	143	Val	Met					x	
NA	995	G	A	325	Gly	S					x	
NS1	219	G	A	65	Val	Met			x		x	

1. S; silent substitution, 2. NCR; Non-coding region

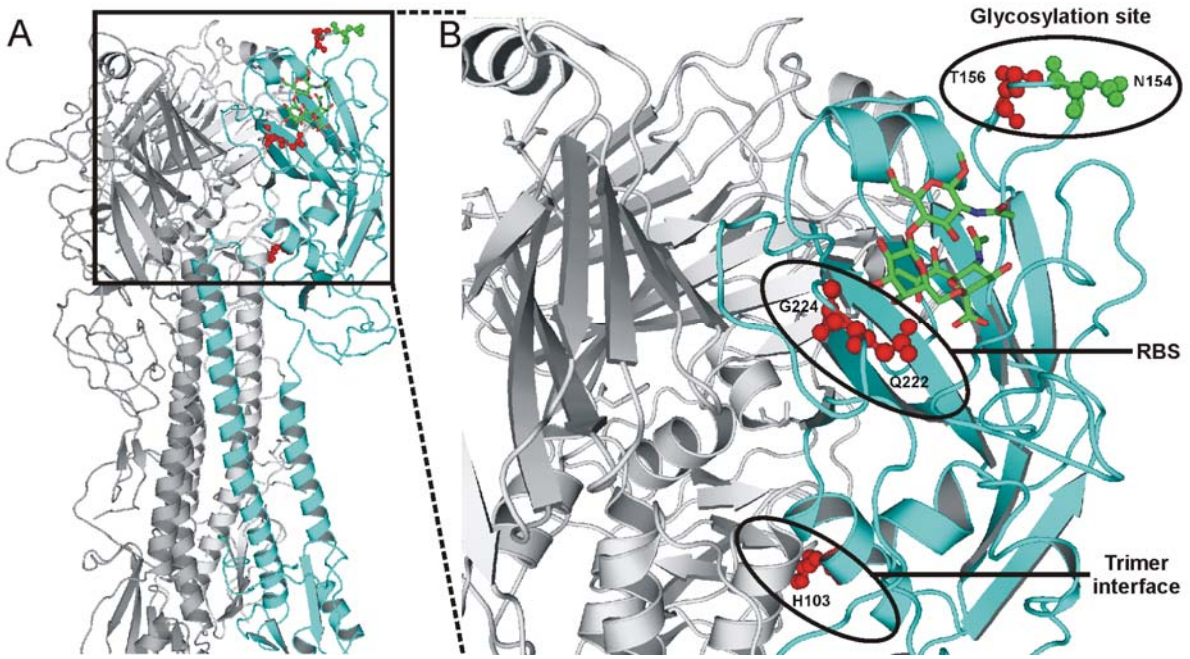

Fig. S8. Cartoon representation of a model of the trimer structure HA of A/Indonesia/5/2005 (*68*) (PDB ID: 1JSM). One monomer is colored cyan for clarity. The structure of the three-sugar glycan NeuAcα2,6Galβ1-4GlcNAc is docked into the receptor binding site (RBS). The substitutions discussed in the text are shown as sticks and balls and colored red (H103, T156, Q222 and G224). N154 of the 154-156 potential N-glycosylation site at the tip of HA is also shown as sticks and balls and colored green.

Amino acid T156 is proximal but not immediately adjacent to the RBS and does not interact directly with the three-sugar glycan that is depicted in the figure. Thus, T156A is unlikely to affect binding to three sugar glycans. Longer glycans may interact with positions as distal from the RBS as amino acid position 156, but little is currently known for such binding. Residue T156 is part of a N-glycosylation sequon (Asn-X-Thr/Ser) and mutation at T156A (as well as N154K) would delete this potential glycosylation site. Wang *et al.* have shown that this N-glycosylation site is used in H5N1 viruses (*69*). Loss of N-glycosylation sites at the tip of HA has been shown to enhance virulence of strains of H1 (*48-49*) and H5 (*50*). Mir-Shekari *et al.* (*49*) postulated that large, complex N-linked glycans at a site so close to the RBS may occlude binding to the cells, and loss of such a site may improve binding to sialic acids. The T156A substitution was detected in 99.6% of the A/H5N1$_{wildtype}$ sequences after

ten passages. While T156A was detected in only 89% of the A/H5N1$_{\text{HA Q222L,G224S PB2 E627K}}$ sequences after ten passages, the other 11% of sequences had N154K, which removes the same potential N-linked glycosylation site in HA.

Substitutions Q222L and G224S have previously been shown to be sufficient to switch receptor-binding specificity of avian influenza strains to that of human strains (*20, 35, 46-47*) G224S can form an additional polar interaction with atom O9 of the NeuAc sugar and Q222L forms favorable van der Waals interactions with the C8 atom of NeuAc as well as the C6 atom of the α2,6 linkage (*46*). Amino acid position 103 is distal from the RBS and thus H103Y is unlikely to affect receptor specificity directly. This amino acid forms part of the trimer interface, making both hydrophobic interactions and charge interactions with neighboring amino acids. H103Y maintains the aromatic characteristic at this position but with a slight increase in size. Thus, we expect H103Y would largely maintain its ability to form polar interactions.

Experiment 5

To test whether the airborne-transmissible H5N1 virus could cause pneumonia upon high-dose intra-tracheal challenge, six ferrets were inoculated with 1 x 10^6 $TCID_{50}$ of virus in a 3 ml volume (Fig. S9). This virus was obtained from a nose swab collected from ferret F5 at 7 dpi (experiment 4) and passaged once in MDCK cells. After inoculation, animals were monitored daily for clinical signs. We initially planned to euthanize 3 ferrets each at 3 and 7 dpi, however two animals were found dead at 3 dpi and all other animals were moribund, requiring euthanasia at 3 dpi. Necropsies were performed and nasal turbinates, trachea and lungs were collected for virological examination (Fig S9).

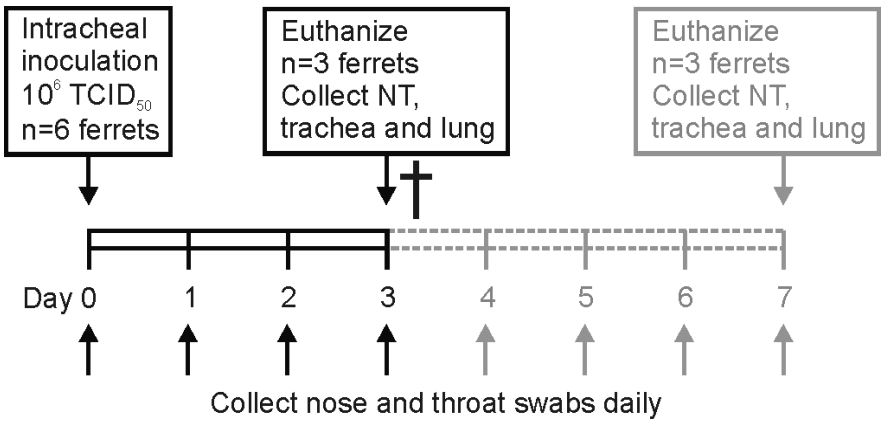

Fig. S9. Schematic overview of experiment 5, to test whether airborne-transmissible H5N1 virus caused pneumonia upon high dose intra-tracheal inoculation. The experiment was halted at day 3.

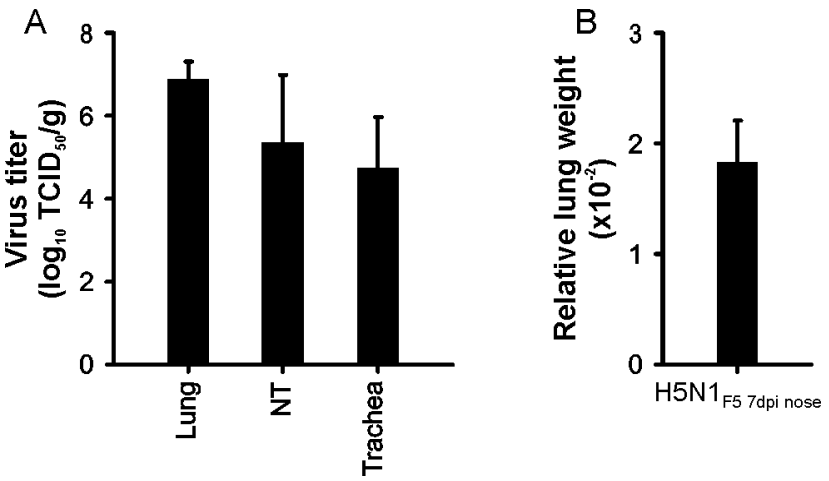

Fig. S10. (A) Virus titers and (B) relative lung weight in six ferrets inoculated intra-tracheally with 1 x 10^6 $TCID_{50}$ (in 3 ml) airborne-transmissible H5N1 virus obtained from the nose swab of transmission ferret F5 ($A/H5N1_{F5\ 7dpi\ nose}$), which was passaged once in MDCK cells and used as inoculum. At 3 dpi, lungs, nasal turbinates (NT) and trachea were collected and virus titers were determined by means of end-point titration in MDCK cells. Geometric mean titer ± SD is indicated. At 3 dpi, two out of six ferrets had succumbed to the infection. The four remaining animals were euthanized at this time point since they were moribund. Intra-tracheal inoculation with the wild-type A/H5N1 A/Indonesia/5/2005 virus had a similar effect (*45*). In this previous work two and four animals were euthanized when they were moribund at 2 and 3 dpi respectively. Mean virus titers in the lungs of animals inoculated with the airborne-transmissible A/H5N1 virus obtained in this passage experiment (6.9 ± 0.4 log_{10} $TCID_{50}$/gram) were slightly but not significantly higher than titers found in $A/H5N1_{wildtype}$-inoculated animals (6.2 ± 0.6 log_{10} $TCID_{50}$/gram) (p=0.093, Mann-Whitney). The relative lung weights of animals inoculated with $A/H5N1_{wildtype}$ were slightly higher compared to those of the $A/H5N1_{F5\ 7dpi\ nose}$-inoculated animals (0,024 ± 0,006 and 0,018 ± 0,004 respectively, p=0.180, Mann-Whitney) (*45*).

Experiment 6: Antiviral resistance

MDCK cells were seeded at $3x10^4$ cells per well in 96-well plates and cultured overnight. Virus stocks of interest were diluted to 1 x 10^3 $TCID_{50}$/ml, and added to cells in a volume of 100μl per well. After incubation for one hour at 37°C, 100μl of serially diluted oseltamivir carboxylate was added (range 170pM - 50μM) in four replicates. After three days of incubation at 37°C, the presence of virus was determined in the supernatant of cell cultures using the agglutinating activity of turkey erythrocytes as an indicator of virus replication in the cells to calculate the 50% inhibitory concentration (IC_{50}).

Table S4. Evaluation of the 50% inhibitory concentration (IC50) of oseltamivir for A/H5N1$_{wildtype}$, A/H5N1$_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$ and A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$. The 2009 pandemic A/H1N1 virus A/Netherlands/602/2009 was used as a control in this assay. Introduction of the well-known H275Y mutation or the I223R mutation in the A/Netherlands/602/09 NA resulted in a decreased susceptibility for oseltamivir as described previously (*70*). Introduction of mutations found in airborne-transmissible A/H5N1 viruses did not affect the sensitivity of A/H5N1 virus to oseltamivir.

Virus	IC50 (nM)
A/H5N1$_{wildtype}$	5000
A/H5N1$_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$	1100
A/H5N1$_{HA\ Q222L,G224S\ PB2\ E627K}$	1700
A/Netherlands/602/2009	5000
A/Netherlands/602/2009$_{NA\ H275Y}$	≥ 50000
A/Netherlands/602/2009$_{NA\ I223R}$	≥ 50000

Experiment 7: Hemagglutination inhibition assay with ferret sera

The hemagglutination inhibition (HI) assay is based on the ability of the influenza virus HA protein to cause agglutination of red blood cells (RBCs) and of specific antisera to block this reaction. HI assays were performed as described previously (*71*). Briefly, serum samples were first pre-treated with *Vibrio cholerae* neuraminidase to remove non-specific inhibitors of hemagglutination activity. Two-fold serial dilutions of the antisera (in 50μL) were incubated with four hemagglutinating units of virus (in 25μL) and incubated at 37°C for 30 minutes. Next, 25μL of turkey RBCs were added, followed by one hour incubation at 4°C. The highest serum dilution able to block the agglutination of RBCs was recorded as the HI-titer. For these experiments, ferret antisera raised against potential H5N1 vaccine strains were used to evaluate if pre-pandemic H5 vaccine candidates sufficiently matched the airborne-transmissible virus (Table S5). For all HI assays, we used recombinant viruses with six gene segments of influenza virus A/PR/8/34 and the HA and PB2 gene segments of A/H5N1 virus with or without substitutions Q222L, G224S, H103Y, T156A, and E627K. The multi basic cleavage site in HA was removed by changing the cleavage site PQRERRRKKR↓G in the H5 HA to PQIETR↓G by RT-PCR with specific primers as described (*35*). Due to this genetic modification, the HI assays could be performed under BSL2 conditions.

Table S5. Hemagglutination inhibition (HI) assay with ferret antisera raised against a panel of candidate H5N1 vaccine viruses for pandemic preparedness selected by the WHO network. Reactivity of ferret post-infection sera is shown for PR8/H5 recombinant influenza viruses with HA and PB2 of A/H5N1$_{wildtype}$, A/H5N1$_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ 627K}$ and A/H5N1$_{HA\ H103Y,T156A\ PB2\ E627K}$. Numbers in bold indicate HI-titers to wildtype A/Indonesia/5/2005 virus antiserum. The antiserum to A/Indonesia/5/2005 reacted with high titers with the HA of airborne-transmissible virus. HA of airborne-transmissible virus cross-reacted better with sera raised against other candidate vaccine viruses representing other clades of A/H5N1 virus, compared with A/H5N1$_{wildtype}$ (indicated in italic), presumably owing to changes in the receptor binding site and loss of the potential glycosylation site (69).

	Ferret antisera raised against:								
Viruses	A/Mallard/Netherlands/3/1999 (LPAI H5)	A/HongKong/156/1997 (clade 0)	A/Vietnam/1194/2004 (clade 1)	A/Indonesia/5/2005 (clade 2.1)	A/Turkey/Turkey/1/2005 (clade 2.2)	A/Anhui/1/2005 (clade 2.3)	IVR-148, A/Brisbane/059/2007 (sH1N1)	X175C, A/Uruguay/716/2007 (H3N2)	A/Netherlands/602/2009 (pH1N1)
A/H5N1$_{wildtype}$	<10	<20	10	**1280**	<10	10	<10	<10	<10
A/H5N1$_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$	*20*	*80*	*80*	***2560***	*480*	*120*	<10	<10	<10
A/H5N1$_{HA\ H103Y,T156A,\ PB2\ E627K}$	<10	<20	10	**1280**	160	10	<10	<10	<10
Serum control	<10	10	<10	**<10**	<10	<10	<10	<10	<10

Experiment 8: Hemagglutination inhibition assay with human sera

The effect of the introduced RBS mutations Q222L/G224S and co-mutations H103Y and T156A in HA on cross-reactivity with human sera was tested using HI assays as described for experiment 7. For this purpose, the reactivity of 24 human sera obtained from individuals over 70 years of age (chosen because these persons are more likely to have had a history of exposure to pandemic influenza viruses), to the HA of the airborne-transmissible A/H5N1 and to the currently circulating 2009 pandemic A/H1N1, seasonal A/H1N1 and seasonal A/H3N2 viruses was investigated (Table S6).

Table S6. Hemagglutination inhibition assay using sera from human volunteers older than 70 years of age. The virus tested was a PR8/H5 recombinant influenza virus with HA and PB2 of A/H5N1$_{HA\ H103Y,T156A,Q222L,G224S\ PB2\ E627K}$. Titers of human sera against the vaccine strains NYMC-IVR-148 (seasonal A/H1N1, A/Brisbane/059/2007), NYMC X-181 (pandemic A/H1N1, A/California/7/2009) and NYMC X-187 (seasonal A/H3N2, A/Victoria/210/2009) were included as controls. Human sera did not react with the HA of airborne-transmissible H5N1 virus. In contrast, 22 of 24 human sera reacted with one or more A/H1N1 and A/H3N2 viruses (numbers in boldface).

Serum#	Serum control	H5N1	sH1N1	pH1N1	H3N2
1	<10	<10	**80**	**80**	**240**
2	<10	<10	<10	**20**	**320**
3	<10	<10	**30**	**20**	**10**
4	<10	<10	**30**	**40**	**60**
5	<10	<10	**10**	<10	<10
6	<10	<10	**30**	<10	**10**
7	20	<30	**80**	<30	<30
8	<10	<10	**80**	**40**	**160**
9	40	<60	**80**	<60	<60
10	30	<40	**40**	<40	<40
11	120	<160	<160	<160	<160
12	<10	<10	**40**	<10	**640**
13	<10	<10	<10	**30**	**1920**
14	20	<30	**30**	**240**	**640**
15	<10	<10	<10	**10**	**40**
16	<10	<10	**20**	**40**	**40**
17	<10	<10	<10	**20**	**240**
18	<10	<10	**640**	**40**	<10
19	<10	<10	**20**	**40**	**160**
20	80	<120	<120	<120	<120
21	<10	<10	**20**	<10	<10
22	<10	<10	**40**	**120**	**160**
23	<10	<10	**80**	**320**	**320**
24	<10	<10	**20**	**160**	**80**

Experiment 9: Modified TRBC hemagglutination assay

For the experiment shown in Table 2 of the main text, modified turkey red blood cells (TRBC) were prepared as described previously (*72*). Briefly, all α2,3-, α2,6-, α2,8-, and α2,9-linked sialic acids (SA) were removed from the surface of TRBC by incubating 62.5 μl of 1% TRBC in PBS with 50 mU *Vibrio cholerae* neuraminidase (VCNA; Roche, Almere, Netherlands) in 8 mM calcium chloride at 37°C for 1 hour. Removal of sialic acids was confirmed by observation of complete loss of hemagglutination of the TRBC by control influenza A viruses. Subsequently, resialylation was performed using 0.5 mU of α2,3-(N)-sialyltransferase (Calbiochem, San Diego, CA) or 2mU of α2,6-(N)-sialyltransferase (Japan Tobacco, Inc., Shizuoka, Japan) and 1.5 mM CMP-sialic acid (Sigma-Aldrich, Zwijndrecht, Netherlands) at 37°C in 75 μl for 2 h to produce α2,3-TRBC and α2,6-TRBC, respectively. After a washing step, the TRBC were resuspended in PBS containing 1% bovine serum albumin to a final concentration of 0.5% TRBC. Resialylation was confirmed by hemagglutination of viruses with known receptor specificity; chimeric PR8 viruses with the HA of A/Vietnam/1194/2004 (H5N1, no MBCS; affinity for α2,3-TRBC) or A/Netherlands/213/2003 (H3N2; affinity for α2,6-TRBC). The receptor specificity of recombinant viruses with six gene segments of PR8 and the HA and PB2 gene segments of A/H5N1 virus with or without substitutions Q222L, G224S, H103Y, T156A, and E627K, was tested by performing a standard hemagglutination assay with the modified TRBC. Serial two-fold dilutions of virus in PBS were made in a 50 μl volume; 50 μl of 0.5% TRBC was added, followed by incubation for 1 hour at 4°C before determining the hemagglutination titer.

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