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Identification, Characterization, and Natural Selection of Mutations Driving Airborne Transmission of A/H5N1 virus

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SUMMARY

Recently, A/H5N1 influenza viruses were shown to acquire airborne transmissibility between ferrets upon targeted mutagenesis and virus passage. The critical genetic changes in airborne A/Indonesia/5/05 were not yet identified. Here, five substitutions proved to be sufficient to determine this airborne transmission phenotype. Substitutions in PB1 and PB2 collectively caused enhanced transcription and virus replication. One substitution increased HA thermostability and lowered the pH of membrane fusion. Two substitutions independently changed HA binding preference from α2,3 linked to α2,6 linked sialic acid receptors. The loss of a glycosylation site in HA enhanced overall binding to receptors. The acquired substitutions emerged early during ferret passage as minor variants and became dominant rapidly. Identification of substitutions that are essential for airborne transmission of avian influenza viruses between ferrets and their associated phenotypes advances our fundamental understanding of virus transmission and will increase the value of future surveillance programs and public health risk assessments.

INTRODUCTION

Since the first detection in the late 1990s (Claas et al., 1998), highly pathogenic avian influenza (HPAI) A/H5N1 viruses continue to circulate in poultry in Asia and the Middle East. Hundreds of millions of domestic birds have died as a result of infection and during culling activities to control the spread of the A/H5N1 virus. Occasional cross-species

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transmission events have been reported for several species of wild birds, pigs, felids, dogs, mustelids. To date, 650 laboratory-confirmed cases of A/H5N1 virus infection in humans have been reported to the World Health Organization from 16 countries, of which approximately 60% with fatal outcome. Sustained human-to-human transmission has not yet been described. However, the enzootic nature of A/H5N1 virus, its broad host range, the large number of infected hosts, and the observed accumulation of mammalian adaptive substitutions in the virus, could potentially increase the risk of a future A/H5N1 virus pandemic.

For 15 years, one of the key questions for pandemic preparedness has been whether the A/H5N1 virus might acquire the ability to transmit via aerosols or respiratory droplets ("airborne transmission") among humans, a trait necessary for the virus to become pandemic. It was recently shown that a fully avian A/H5N1 virus can become airborne transmissible between ferrets (Herfst et al., 2012). Three other groups demonstrated that reassortant viruses between A/H5N1 and 2009 pandemic A/H1N1 viruses that contain the H5 hemagglutinin (HA) were also transmitted between ferrets or guinea pigs via the airborne route (Chen et al., 2012; Imai et al., 2012; Zhang et al., 2013b).

Herfst et al. introduced the well-known glutamic acid to lysine substitution at position 627 (E627K) of the basic polymerase 2 protein (PB2) that is associated with increased replication in mammalian cells at relatively low temperatures (Aggarwal et al., 2011; Subbarao et al., 1993; Taubenberger et al., 2005) and was shown to be important for airborne transmission of 1918 A/H1N1 and 1999 A/H3N2 viruses between ferrets and guinea pigs (Steel et al., 2009; Van Hoeven et al., 2009). In addition, two substitutions were introduced in the receptor binding site (RBS) of HA that are known to switch receptor specificity from "avian" α2,3-linked sialic acids (α2,3-SA) to "human" α2,6-SA (Matrosovich et al., 2000); glutamine to leucine at position 222 and glycine to serine at position 224 (Q222L, G224S in H5 HA numbering). These three substitutions or other polymerase and RBS mutations with similar phenotypes were found in all pandemic influenza viruses of the last century, and were therefore postulated to represent minimal requirements for adaptation of animal influenza viruses to humans to yield pandemic strains (Chutinimitkul et al., 2010; Sorrell et al., 2011). This 'triple mutant' virus was passaged ten times in the upper respiratory tract of ferrets to yield mutant A/H5N1 viruses that were able to transmit via the airborne route between ferrets. In addition to the three substitutions introduced by reverse genetics, two substitutions in HA (H103Y and T156A) were consistently found in all transmitted A/H5N1 viruses. However, all airborne-transmitted viruses had accumulated additional substitutions. The transmissible virus with the lowest number of amino acid substitutions compared to the A/H5N1 wildtype virus had a total of nine substitutions.

We here describe the identification of a minimal set of substitutions required for airborne transmission of influenza virus A/Indonesia/5/05 between ferrets and provide a detailed characterization of the phenotypic changes caused by each of these substitutions. We show that the substitutions acquired upon ferret passage of the 'triple mutant' A/Indonesia/5/05 virus emerged rapidly, suggestive for strong natural selection. The identification of previously unrecognized substitutions and phenotypic traits responsible for influenza virus

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transmission are key to increase our fundamental understanding of airborne spread of influenza virus and may ultimately increase prognostic capabilities and diagnostic value of surveillance studies necessary for pandemic preparedness.

RESULTS

Airborne transmission of A/H5N1 virus between ferrets is determined by a minimum of 5 amino acid substitutions

To define a minimal number of substitutions in A/Indonesia/5/05 that confer airborne transmission between ferrets, transmission experiments were performed as described previously (Munster et al., 2009). First, a recombinant virus was produced based on the consensus sequence of a previously identified virus that was airborne-transmissible and that contained the lowest number of substitutions (N=9) compared to the wildtype virus (PB2-E627K, PB1-H99Y and PB1-I368V, HA-H103Y, HA-T156A, HA-Q222L and HA-G224S, and NP-R99K and NP-S345N; (Herfst et al., 2012)). This virus was transmitted to two out of two recipient ferrets, thus reproducing with a recombinant clonal virus our earlier results with a virus isolate (Figure 1A, V1). Next, we omitted either the two substitutions in NP (V2) or the two substitutions in PB1 (V3). While the recombinant virus missing two substitutions in NP was transmitted to one out of two animals, the virus missing two substitutions in PB1 was not transmitted (Figure 1A, V2 and V3). To investigate this further, the two substitutions in PB1 (H99Y and I368V) were tested individually. The virus harbouring I368V in addition to the set of 5 substitutions consistently found in the airborne transmitted viruses (PB2-E627K and HA-H103Y, HA-T156A, HA-Q222L, HA-G224S) was not transmitted to recipient ferrets (Figure 1A, V4), whereas the addition of PB1-H99Y yielded a recombinant virus that was detected in three out of four exposed ferrets (Figure 1A, V5).

Starting with virus V5 that had six substitutions compared to wildtype A/Indonesia/5/05 (HA-Q222L, HA-G224S, HA-H103Y, HA-T156A, PB2-E627K, and PB1-H99Y), all individual substitutions were omitted one by one (V7-V12). Viruses lacking either the receptor-binding substitution HA-G224S or HA-Q222L were detected in 1 and 2 out of 4 naïve ferrets respectively upon exposure to inoculated ferrets (Figure 1B: V7, V8). In contrast, when both HA-Q222L and HA-G224S were omitted, the virus was not transmitted between ferrets (Figure 1B: V6). Viruses lacking HA-T156A, HA-H103Y, PB1-H99Y, or PB2-E627K were not detected in recipient ferrets upon exposure either (Figure 1B: V9-V12). From this set of experiments, we conclude that PB2-E627K, PB1-H99Y, HA-H103Y, HA-T156A, and either HA-Q222L or HA-G224S in HA represent minimal sets of substitutions required for airborne transmission of A/Indonesia/5/05 between ferrets. Individual virus titers of nose and throat samples obtained from all donor-recipient pairs are shown in Figure S1.

Rapid emergence of substitutions required for airborne transmission of A/H5N1 in ferrets

We next determined at which passage substitutions H99Y in PB1 and H103Y and T156A in HA emerged during the ten repeated passages of the 'triple mutant' A/Indonesia/5/05. To this end, primers were designed to amplify virus genome fragments covering these amino

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acid positions and RT-PCR was performed on RNA isolated from nasal turbinates, lungs, and nasal swabs or washes collected from the ferrets after each passage. Amplicons were sequenced using the Roche 454 GS Junior platform.

PB1-99Y was already detected as a minor variant in the nasal turbinates in passage 1, increased during subsequent passages, to become the dominant variant in all tissues from passage 7 onwards (Figure 2). HA-103Y was first detected in the nasal swabs of passage 2, was detected again in passages 5 and 6, and became the major variant from passage 7 onwards. Of note, nasal washes instead of nasal turbinates were used to inoculate the ferrets from passage 6 onwards which may have contributed to rapid increase in the proportion of mutants containing HA-103Y after passage 7 (and perhaps also PB1-99Y). HA-156A was detected as a minor variant in passage 1, to rapidly become the dominant variant from passage 3 onwards. Thus, the three substitutions that emerged during ferret passage and that contributed to aerosol transmissibility of A/H5N1 virus arose as early as after one or two passages and became dominant by passage 7.

Nucleotide variations in the full genomes of A/H5N1$_{wildtype}$ and A/H5N1$_{HA\ Q222L,\ G224S\ PB2\ E627K}$ during ferret passaging and transmission

We next compared the full genome sequences of viruses described in Herfst et al. present in passage 4, 8, and 10 nasal turbinates of ferrets inoculated with A/H5N1$_{wildtype}$ or A/H5N1$_{HA\ Q222L,\ G224S\ PB2\ E627K}$ (Table S1). These samples were selected because they contained high copy numbers of viral RNA as determined by real-time PCR. Substitutions PB2-T23P, PA-T363P, PA-P211T, PA-L498F, NP-G462E, and NA-E239G emerged upon passage with both A/H5N1$_{wildtype}$ and A/H5N1$_{HA\ Q222L,\ G224S\ PB2\ E627K}$ but did not become dominant variants. In contrast, T156A became a major variant upon passage of both viruses. Thus, while substitutions H99Y in PB1 and H103Y in HA emerged only upon passage of A/H5N1$_{HA\ Q222L,\ G224S\ PB2\ E627K}$, HA-T156A emergence was independent of PB2-E627K, HA-Q222L and HA-G224S that were introduced by reverse genetics. The latter three substitutions remained dominant (>96.2%) throughout all passages tested, and were not detected throughout passage of A/H5N1$_{wildtype}$ (Table S1).

To study the effect of airborne-transmission on the viral intrahost nucleotide sequence variation, we compared the full virus genome sequences present in nasal wash samples of ferrets inoculated with A/H5N1$_{HA\ Q222L,\ G224S\ PB2\ E627K}$ after 10 passages in ferrets to nose swab samples of ferrets after two consecutive airborne transmission events (ferrets F5 and F6, Table S2). The number of nucleotide variations present in specimens from F5 (N=12) and F6 (N=16), were substantially less as compared to those in the passage 10 nasal wash sample (N=28). Moreover, substitution PB1-99Y increased in frequency from 44.9% in passage 10 nasal wash to 100% and 73.3% in F5 and F6 after the two consecutive airborne transmission events. Similarly, HA-103Y increased from 87.6% to 100% and 100%, and HA-156A increased from 89.9% to 99% and 98.8%. These collective data are indicative for a strong selection bottleneck occurring on intrahost nucleotide sequence variation.

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HA substitutions Q222L, G224S, and T156A affect receptor binding

To study the impact of the HA substitutions associated with airborne transmission on receptor preference, the attachment patterns of A/Puerto Rico/8/1934 (PR8) viruses harboring wildtype or mutant A/H5N1 HA proteins were first characterized using formalin-fixed paraffin-embedded tissue sections of ferret and human nasal turbinates, known to express "human-like" α2,6-SA receptors abundantly. PR8 virus with the wildtype H5 HA did not attach to nasal turbinate sections, while the same virus with a control human H3 HA showed abundant attachment, as expected (Figure 3A). Introduction of Q222L and G224S in wildtype H5 HA resulted in abundant virus attachment to the nasal turbinate sections, comparable to the H3 HA control, as shown previously (Chutinimitkul et al., 2010). Introduction of H103Y and T156A in either the wildtype H5 HA or HA $_{Q222L,\ G224S}$ did not result in obvious changes in these patterns of virus attachment. The attachment patterns of HAs to the ferret and human nasal turbinate tissue sections were indistinguishable.

We used two solid-phase enzyme-linked receptor-binding assays to determine HA binding of virus immobilized on a 96-well plate to receptor analogs in solution: a direct binding assay and a binding inhibition assay (Matrosovich and Gambaryan, 2012). PR8 viruses that harbor wildtype or mutant H5 HAs were tested in direct binding assays with receptor analogs 3′-fetuin and 6′-fetuin (Figure 3B). Introduction of the Q222L and G224S in HA$_{wildtype}$ resulted in a switch in receptor binding specificity from α2,3-SA to α2,6-SA as expected, with no residual α2,3-SA binding. Introduction of H103Y and T156A in HA$_{Q222L,\ G224S}$ resulted in increased binding to α2,6-SA receptors, but also low binding to α2,3-SA. Introduction of H103Y and T156A in HA$_{wildtype}$ resulted in increased binding to α2,3-SA and low binding to α2,6-SA. Thus in both the context of HA$_{wildtype}$ and HA$_{Q222L,\ G224S}$, substitutions H103Y and T156A resulted in increased binding avidity and limited dual receptor specificity. A direct binding assay performed with 3′-sialyl-N-acetyllactosamine- and 6′-sialyl-N-acetyllactosamine-containing synthetic sialylglycopolymers rather than 3′-fetuin and 6′-fetuin yielded similar results (Figure S2A). In a fetuin binding inhibition assay, we compared binding of the viruses to a panel of sialylglycopolymers containing several different sialyloligosaccharide moieties (Figure S2B). In this assay, the wildtype and mutant H5 viruses in general bound less avidly as compared to the control avian H1N1 virus A/Duck/Bavaria/1/1977 and human H3N2 virus A/HongKong/1/1968. HA$_{wildtype}$ and HA$_{H103Y,\ T156A}$ bound stronger to various α2,3-SA analogs than to α2,6-SA, whereas HA$_{Q222L,\ G224S}$ and HA$_{Q222L,\ G224S,\ H103Y,\ T156A}$ showed the opposite binding preference (Figure S2B), thus yielding similar results as the direct binding assays. In a third approach, we assessed the binding of PR8 viruses expressing wildtype or mutant H5 HA in a hemagglutination assay using normal turkey red blood cells (TRBC) that contain both α2,3-SA and α2,6-SA, or modified TRBC that contain either α2,3-SA or α2,6-SA alone, or no SA (Figure 3C). As shown previously, introduction of Q222L and G224S in H5 HA resulted in a switch in receptor binding preference from α2,3-SA to α2,6-SA (Chutinimitkul et al., 2010). Additional introduction of the H103Y and T156A substitutions required for airborne transmission increased the hemagglutination titers to both α2,3-SA and α2,6-SA containing TRBC. Each of the individual receptor-binding site substitutions Q222L and G224S in the context of changes H103Y and T156A displayed

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binding to α2,6-SA containing TRBC, in agreement with the fact that single RBS substitutions were sufficient for airborne transmission (Figure 1B).

Introduction of H103Y in HA$_{\text{wildtype}}$, HA$_{\text{T156A}}$, HA$_{\text{Q222L, G224S}}$, and HA$_{\text{T156A, Q222L, G224S}}$ did not result in consistent changes in hemagglutination titers. In contrast, introduction of T156A in HA$_{\text{wildtype}}$, HA$_{\text{H103Y}}$, HA$_{\text{Q222L, G224S}}$, and HA$_{\text{H103Y, Q222L, G224S}}$ resulted in dual receptor specificity as indicated by a consistent 2-fold increase in hemagglutination titers, irrespective of the HA used.

Collectively, we conclude from these studies that H103Y had no discernable effect on receptor binding preference, while T156A increased overall virus binding to both α2,3-SA and α2,6-SA thus resulting in dual receptor specificity.

HA substitution H103Y affects acid and temperature stability

Upon virus attachment to SA receptors on the cell surface and internalization into endosomes, a low-pH-triggered conformational change of HA mediates fusion of the viral and endosomal membranes to release the virus genome in the cytoplasm (Shaw and Palese, 2013). We measured the pH threshold required for fusion of wildtype and mutant HAs. Vero cells were transfected with HA-expression plasmids, exposed to trypsin to cleave and activate the HA, followed by acidification of the cell culture at a pH range of 5.2 to 6.0. Visual inspection of the cell cultures for the presence of syncytia (multinucleated cells) was used to determine the pH threshold required for fusion (Figure 4A). Fusion of HA$_{\text{wildtype}}$ was triggered at pH 5.6, similar to the threshold pH of the control H5 HA of A/Hongkong/156/97 (pH 5.8). HA of the control human H3N2 virus A/Netherlands/213/03 required a lower pH (5.2) for fusion to occur. This data is in agreement with the observation that avian influenza virus HAs generally trigger fusion at a higher pH than human virus HAs (Galloway et al., 2013). The three substitutions that affect receptor binding (T156A, Q222L, G224S; see above) did not result in a reduction of the threshold pH for fusion as compared to HA$_{\text{wildtype}}$. In contrast, upon introduction of H103Y in HA$_{\text{wildtype}}$ and HA$_{\text{T156A, Q222L, G224S}}$, fusion was triggered only at pH 5.2 and lower. Moreover, HA$_{\text{H103Y, T156A, Q222L}}$ and HA$_{\text{H103Y, T156A, G224S}}$ also triggered fusion at relatively low pH (pH 5.2 and pH 5.4 respectively).

As a second read-out for fusion, we used a “cell content mixing assay”, in which two populations of Vero cells were transfected with HA and either a chloramphenicol- acetyl-transferase construct under control of the HIV-1 promoter (LTR-CAT) or an HIV-1 transactivator construct (pTat). Upon mixing of the two cell populations and after acidification of the cell culture at a pH range of 5.2 to 6.0, fusion was quantified by measuring CAT expression that is dependent on the expression of Tat. Also in this assay, introduction of H103Y in HA$_{\text{wildtype}}$ and HA$_{\text{T156A, Q222L, G224S}}$ resulted in a lower threshold pH for fusion, as indicated by the dotted lines in Figures 4B and 4C respectively. The difference in pH required for fusion between HA with and without H103Y was similar to that observed for the reference A/H3N2 and A/H5N1 HAs (Figure 4D).

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

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temperature. This conformational change of HA is biochemically indistinguishable from the change triggered by low pH (Carr et al., 1997) and results in a loss in the ability to bind receptor. To further investigate HA stability, PR8 viruses harboring wild type or mutant HAs were incubated at increasing temperatures, after which the ability of the viruses to agglutinate TRBCs was quantified. The PR8 virus with the H3 HA of A/Netherlands/213/03 retained hemagglutination activity even upon treatment for 30 minutes at 60°C. In contrast, PR8 with H5 HA of A/Indonesia/5/05 lost hemagglutination activity upon treatment at 56°C for 30 minutes. Irrespective of the presence or absence of substitutions affecting receptor binding ($HA_{wildtype}$, HA_{T156A}, $HA_{Q222L, G224S}$, and $HA_{T156A, Q222L, G224S}$ were tested), H103Y resulted in increased temperature stability as measured in the hemagglutination assay (Figure 4E).

Collectively, these data indicate that H103Y has a stabilizing effect on the HA of A/Indonesia/5/05 – with respect to both low pH and high temperature treatment – irrespective of the presence or absence of substitutions that affect receptor binding.

PB2-E627K and PB1-H99Y affect polymerase activity

The influenza virus polymerase complex - consisting of the polymerase proteins PA, PB1 and PB2 - transcribes the negative sense viral RNA ((−)vRNA) in mRNA and positive sense copy RNA ((+)cRNA), the latter of which is used as the template to yield newly synthesized (−)vRNA. To read out polymerase complex function, we used a (−)vRNA reporter construct consisting of the firefly luciferase open reading frame flanked by the non-coding regions of segment 8 of influenza A virus (de Wit et al., 2010). Upon co-transfection of the reporter with expression plasmids encoding PB1, PB2, PA, and NP, the (−)vRNA reporter is transcribed and the firefly luciferase protein is expressed. A plasmid that constitutively expresses *Renilla* luciferase was co-transfected as an internal control to standardize transfection efficiency and sample processing. Substitution E627K in PB2 resulted in a 12-fold increase in firefly luciferase expression as compared to the wildtype PB2. Substitution H99Y in PB1 resulted in a 3-fold increase. The polymerase complex with both PB2-E627K and PB1-H99Y yielded a 25-fold increase in firefly luciferase expression as compared to the polymerase complex of the wildtype virus (Figure 5A).

To study the levels of transcription of (−)vRNA, (+)cRNA and mRNA during the course of a viral infection, we performed primer-extension assays using total cellular RNA isolated upon virus inoculation of MDCK cells. Three hours after inoculation with $A/H5N1_{wildtype}$, all three RNA species were detected (Figure 5B). Introduction of E627K in PB2 resulted in elevated levels of the viral RNAs, predominantly increasing the amount of (−)vRNA and (+)cRNA, while marginally changing mRNA. Substitution H99Y in PB1 resulted in slightly reduced levels of all detected RNA species as compared with $A/H5N1_{wildtype}$. Upon inoculation with a virus containing both PB2-E627K and PB1-H99Y, the ratio between (−)vRNA, (+)cRNA, and mRNA was similar as observed for $A/H5N1_{wildtype}$, but with higher overall levels of all three RNA species. As a third test of polymerase function, we studied *in vitro* replication of the recombinant A/H5N1 viruses with and without polymerase substitutions by measuring plaque sizes upon inoculation of MDCK cells. At 48 hours after inoculation, cells were fixed, stained with an anti-NP antibody, and the number of pixels

representing ~100 to 500 plaques on digital images were quantified. As compared to A/ $H5N1_{wildtype}$, A/$H5N1_{PB2\ E627K}$ and A/$H5N1_{PB1\ H99Y}$ displayed a slight reduction in plaque size. However combination of these two substitutions (A/$H5N1_{PB2\ E627K,\ PB1\ H99Y}$) yielded plaques that were significantly larger than those observed for A/$H5N1_{wildtype}$ (Figure 5C).

From these assays we conclude that PB2-E627K and PB1-H99Y collectively resulted in increased levels of (−)vRNA, (+)cRNA, and mRNA transcription, and increased virus replication in MDCK cells.

DISCUSSION

Here, we show that of the 9 substitutions observed in an airborne transmissible A/H5N1 virus (Herfst et al., 2012), two alternative sets of 5 mutations are identified (E627K in PB2; H99Y in PB1; H103Y, T156A, and either Q222L or G224S in HA), either of which is sufficient to confer ferret transmissibility on A/Indonesia/5/05. Keeping in mind that the design of this ferret transmission model is qualitative rather than quantitative, and that such studies need to take the principles of Replacement, Reduction, and Refinement in animal experiments into account (Belser et al., 2013; Russell and Burch, 1959), it should be noted that the present study is limited by the number animals that were used and was purposely designed to define a minimal set of substitutions rather than the definitive minimal set of substitutions required for airborne transmission in ferrets. Starting with the recombinant A/ H5N1 virus harboring 9 substitutions, omission of 2 substitutions in NP still yielded virus transmission to 4 out of 6 airborne-exposed ferrets (V2 and V5). Subsequently, a virus lacking PB1-H99Y (V4) was not detected by virus isolation and serology in 2 out of 2 airborne-exposed ferrets, while a virus lacking PB1-I386V was detected by virus isolation in 3 out of 4 airborne exposed ferrets, thus providing evidence that H99Y and not I386V was required for airborne transmission. In the subsequent experiment, all individual substitutions from the remaining set of six (PB2-E627K, PB1-H99Y, HA-H103Y, HA-T156A, HA-Q222L, HA-G224S; Fig. 1B) were eliminated one by one. Viruses lacking either HA-Q222L or HA-G224S still resulted in airborne transmission in 2 and 1 of 4 ferrets tested, indicating that a single receptor-binding site substitution was sufficient for transmission. In contrast, in transmission experiments using viruses from which each of the other single substitutions were eliminated, virus was not detected in exposed ferrets. However, some of these exposed animals seroconverted despite a lack of virus detection. Apparently, although transmission may occur for some viruses with 5 substitutions as measured by serology or single time-points of virus detection (Figure S1), the viruses were insufficiently replication-competent to cause robust infection and seroconversion consistently in the exposed ferrets. As a consequence, refining the minimal set of substitutions required for airborne transmission would require substantial numbers of ferret pairs, given the statistical considerations for this type of experiment (Belser et al., 2013; Nishiura et al., 2013).

The substitutions required for airborne transmission in ferrets were determined in *in vitro* assays to either affect HA binding to receptors, HA stability, or activity of the polymerase complex. Two substitutions introduced by reverse genetics (Q222L and G224S) are known to change the receptor binding preference of the H5 HA from avian-like α2,3-SA to human-like α2,6-SA (Chutinimitkul et al., 2010). This change in receptor binding preference, either

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through single or double substitutions, was required for airborne-transmissibility, in agreement with loss-of-function transmission studies using 1918 A/H1N1 and 1957 A/H2N2 viruses (Pappas et al., 2010; Tumpey et al., 2007). Although HAs with single Q222L or G224S substitutions were less efficient in binding α2,6-SA-containing TRBC as compared to the double mutant, viruses with the single RBS substitutions were still transmissible. HA substitution T156A increased virus binding to both α2,6-SA and α2,3-SA in quantitative binding assays. Imai et al. showed that substitution N154D in HA also affected transmission of a reassortant H5 virus. T156A and N154D result in the loss of the same glycosylation site in HA, suggesting that loss of this glycosylation site rather than the specific amino acid substitutions were important for the change in phenotype. Loss of glycosylation in H5 HA in combination with substitutions Q222L and G224S was previously shown to enhance virus replication in ferrets (Wang et al., 2010). The airborne transmissible H5 virus of Imai et al. also contained Q222L in HA, but had N220K as a second substitution in the RBS. In a third study, Q222L/G224S along with Q192R in the context of A/Vietnam/1203/04 HA also resulted in slightly increased transmission in ferrets (Chen et al., 2012). These studies thus indicate that changes in receptor specificity critically contribute to airborne transmission of H5 viruses in ferrets. Affinity measurements using A/Indonesia/5/05, A/Vietnam/1203/04, and A/Vietnam/1194/04 HAs with substitutions associated with airborne transmission revealed a binding preference for human receptors (Figure 3) (de Vries et al., 2014; Lu et al., 2013; Xiong et al., 2013; Zhang et al., 2013a). Structural studies of HA further showed that human and avian receptor analogs were bound in the RBS in the same "folded-back" conformation as seen for HA from H1, H2, and H3 pandemic viruses, which is distinct from typical avian virus HAs, including H5 (Lu et al., 2013; Xiong et al., 2013; Zhang et al., 2013a). In these studies, the affinity of the mutant H5 HA was relatively low as compared to HA of human H2 and H3 viruses (Liu et al., 2009; Skehel and Wiley, 2000; Xiong et al., 2013; Zhang et al., 2013a). Although it was speculated that an N-linked glycan at the tip of HA might sterically hinder SA binding (Xiong et al., 2013), direct evidence from structural studies on HA of the airborne transmissible viruses is still lacking.

Arguably, one of the more intriguing findings of the Imai et al. and Herfst et al. studies is the requirement of HA mutations that affect stability in terms of temperature and pH. H103Y in A/Indonesia/5/05 HA resulted in increased temperature stability and requirement of lower pH treatment to trigger membrane fusion, similar as described for T315I by Imai et al. H103Y was recently shown to increase the thermostability of HA (de Vries et al., 2014) and temperature-dependent circular dichroism spectroscopic experiments revealed hydrogen bond formation between 103Y and 413N in adjacent monomers that stabilized the trimeric protein (Zhang et al., 2013a). In contrast, T315I stabilized the position of the fusion peptide within the HA monomer (Xiong et al., 2013), indicating that multiple mechanisms can lead to increased HA stability. Galloway et al. suggested that an altered pH of fusion may be associated with virus adaptation to new hosts (Galloway et al., 2013). Furthermore, substitutions that decrease the pH of fusion increased virus replication in the upper respiratory tract of ferrets and in mice (Krenn et al., 2011; Shelton et al., 2013; Zaraket et al., 2013). Although the contribution of H103Y and T315I to increase airborne transmission between ferrets may be related to the pH of fusion or thermostability, these properties may

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merely be a surrogate for another – as yet unknown – phenotype, such as stability of HA in aerosols, resistance to drought, stability in mucus, or altered pH in the host environment.

The requirement of increased polymerase activity to yield an airborne transmissible H5 virus was also expected (Sorrell et al., 2011). PB2 E627K has been identified as a major determinant of host adaptation of pandemic influenza viruses (Aggarwal et al., 2011; Steel et al., 2009; Subbarao et al., 1993; Taubenberger et al., 2005; Van Hoeven et al., 2009). Here, E627K also resulted in increased polymerase activity. More importantly however, we identified a previously unknown substitution acting in concert with E627K to increase polymerase activity. Like PB2 E627K, PB1 H99Y alone resulted in increased polymerase activity in minigenome assays, but decreased virus replication. When combined, these two substitutions had a synergistic effect in minigenome assays, and increased virus replication. In primer extension assays, PB2 E627K predominantly caused an increase in vRNA and cRNA, changing the ratio between RNA replication and mRNA transcription. Addition of PB1 H99Y lowered vRNA and cRNA while further increasing mRNA levels, yielding a similar ratio as observed for A/H5N1$_{wildtype}$, but at overall increased levels. We here thus describe a new "adaptive" substitution in PB1 that potentially improved the levels of, and balance between, vRNA, mRNA, and cRNA in concert with PB2-E627K in A/Indonesia/5/05. Beyond HA, only these two amino acid substitutions were required to generate airborne transmissible A/H5N1 virus. Such conclusion cannot be obtained from the A/H5 virus transmission experiments that use reassortant viruses. Zhang et al. showed that the PA or NS1 genes of a pH1N1 virus contributed to airborne transmission of reassortant A/H5N1 viruses in guinea pigs (Zhang et al., 2013b). At present, it is unclear how the guinea pig and ferret models compare with respect to A/H5 virus transmission studies.

Although some substitutions leading to an airborne phenotype have been observed in nature, the required combination of substitutions has not yet been detected (Herfst et al., 2012; Neumann et al., 2012; Russell et al., 2012). Keeping in mind that the ferret model may not be predictive for airborne A/H5N1 virus emergence in humans, it is of interest to note that upon acquisition of PB2 E627K and the receptor binding changes Q222L/G224S, the additional substitutions associated with the airborne phenotype of A/H5N1 emerged within only one or two passages in ferrets, became dominant after 6 passages, and appeared to be selected for during transmission events. Other sets of mutations than those identified here that similarly result in altered receptor specificity and stability of HA and polymerase function in mammalian cells may also be sufficient to increase airborne transmission. The specific mutations to increase transmission may be dependent on virus strain or subtype (Tharakaraman et al., 2013). Given that some of the required substitutions (PB2 E627K and HA T156A) are commonly found in field isolates and that functionally equivalent substitutions exist for most of the identified substitutions, emergence of transmissible A/H5N1 influenza A viruses in nature cannot be excluded (Russell et al., 2012). In this light, the A/H7N9 virus outbreak in China is a cause of concern, as some A/H7N9 field strains contain E627K in PB2 and Q222L in HA and this H7 lineage lacks a glycosylation site at the tip of HA (Liu et al., 2013). These viruses were shown to have increased preference for α2,6-SA and decreased binding to α2,3-SA (Kageyama et al., 2013; Richard et al., 2013; van Riel et al., 2013). Although airborne A/H7N9 virus transmission in ferrets was shown to be limited (Lam et al., 2013; Richard et al., 2013; Shi et al., 2013; Zhu et al., 2013), it

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cannot be excluded that fully avian viruses adapt upon repeated passage in mammals to gain transmissibility. In analogy to H5, it can be speculated that the A/H7N9 virus needs to acquire increased binding preference for α2,6-SA over α2,3-SA, increased HA stability, and increased polymerase activity. For both A/H5N1 and A/H7N9, appropriate surveillance for emergence of mutations that affect HA receptor binding, HA stability, polymerase activity, and transmission, is thus warranted. It has been argued that different sets of substitutions may lead to similar virus phenotypes, and hence sequence-based virus surveillance may be misleading. However, such surveillance may be improved by including phenotyping assays – using relatively simple methods as described here – in the future. Such surveillance may further be improved by deep-sequencing, in addition to sequencing consensus virus genomes. While it is clear that studies in ferrets may not be predictive for influenza virus outbreaks in humans, the ferret transmission model is one of the best models for influenza available today (Maher and DeStefano, 2004). Experiments like the ones presented here are crucial to increase our basic understanding of airborne virus transmission, as such knowledge is currently very limited. The results of this study do not imply that an H5 influenza pandemic is imminent, but warrant an intensified and broadened approach to detect emerging influenza viruses early and take immediate action once viruses naturally gain functions that might enable them to become a pandemic threat.

EXPERIMENTAL PROCEDURES

See Supplemental Information for additional details and references.

Viruses and cells

Madin-Darby Canine kidney (MDCK) cells, 293T (human kidney epithelial cells) and subclone 118 of Vero-WHO cells (African green monkey kidney epithelial cells) were used for virus propagation, plasmid transfections, and fusion assays, respectively. Influenza virus A/Indonesia/5/05 (A/H5N1) was isolated from a human case of HPAI virus infection. All viruses used in this study were generated upon transfection of 293T cells with reverse genetics plasmids. Recombinant viruses were propagated in MDCK cells and viral titers were determined by end-point titration. For binding and stability assays, recombinant viruses with seven gene segments of A/PR/8/34 and the wildtype or mutant HA segment of A/Indonesia/5/05 were used.

Ferret model for airborne transmission

Ethical, biosafety, and biosecurity considerations related to the experiments are described in detail in the Supplemental Information. One to two year old ferrets (*mustela putorius furo*), free of antibodies against H5 HA, were inoculated with virus. The next day, a second ferret was housed in the same cage, which was divided into two separate sections by a pair of metal grids, allowing the transfer of air between cage mates but preventing direct contact. Nose and throat swabs of inoculated and naïve animals were taken every other day to test for virus presence. Blood from naïve contact animals was collected 14 days after first exposure to inoculated animals, and tested for the presence of antibodies against H5 HA.

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Given that airborne transmission in ferrets has never been observed for A/Indonesia/5/05 or any other avian A/H5N1 virus (Table S3), we define every single event of virus detection in naive ferrets as “airborne transmission”. The use of small group sizes could result in an underestimation of airborne transmission. Thus, while “not transmissible” does not mean “will never transmit”, “transmissible” is a clearly defined virus phenotype.

Deep sequencing

Viral RNA was extracted from ferret samples upon virus passaging, converted to cDNA, and amplified by PCR using primers covering the full viral genome. PCR fragments for each virus were pooled in equal concentrations, and libraries were created for each virus. Emulsion PCR and GS Junior sequencing runs were performed according to instructions of the manufacturer (Roche). Sequence reads were sorted by bar code, trimmed at 30 nucleotides from the 3′ and 5′ ends to remove primer sequences, and the 3′ ends were further trimmed to improve quality using a Phred score of 20. Reads were aligned to reference sequence A/Indonesia/5/05 using CLC Genomics software 4.6.1. The threshold for mutation detection was manually set at 1%.

Virus binding assays

Formalin-fixed, paraffin-embedded tissues from ferrets and humans were used for virus histochemistry. Sucrose-purified viruses were labeled with fluorescein isothiocyanate (FITC) and incubated with the tissue sections overnight. The FITC label was detected with a peroxidase-labeled anti-FITC antibody and the signal was amplified using a Tyramide Signal Amplification System. Peroxidase was revealed with 3-amino-9-ethyl-cabazole and tissues were counterstained with hematoxylin and embedded in glycerol-gelatin.

For direct fetuin binding assays, 96 well plates were first coated with bovine fetuin, and subsequently incubated with BPL-inactivated viruses, washed, and blocked with desialylated bovine serum albumin. The virus-coated wells were incubated with twofold dilutions of receptor analogs (re-sialylated fetuin preparations containing either α2,3-linked SAs (3′Fetuin) or α2,6-linked SAs (6′Fetuin) labeled with horseradish peroxidase). Plates were washed and tetramethylbenzidine substrate was added, after which the absorbance at 450nm was determined. The association constants (K_{ass}) of virus complexes with analogs were determined from the slopes of Scatchard plots.

For modified red blood cell assays, all SAs were removed from the surface of TRBC by incubation with *Vibrio cholerae* neuraminidase. Complete removal of SAs was confirmed by loss of hemagglutination using control viruses. Resialylation was done using α2,3-(N)-sialyltraferase or α2,6-(N)-sialyltransferase to produce α2,3-TRBC and α2,6-TRBC respectively. Resialylation of either α2,3 or α2,6 was confirmed using viruses with known receptor specificity. Viruses were tested in standard HA assay using native and resialylated TRBCs.

Fusion assays

HA-mediated membrane fusion was tested by transfecting populations of Vero-118 cells with plasmids expressing HA and β-galactosidase (β-Gal) along with either pLTR-CAT (a

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chloramphenicol acetyltransferase (CAT) gene under the control of the human immunodeficiency virus type 1 long terminal repeat), or pTat (expressing the HIV-1 transactivator of transcription Tat). One day after transfection, both cell populations were harvested, pooled, and re-plated. The next morning, cells were exposed to PBS at different pH for 10 minutes. Cell lysates were harvested 24 hours after the pH pulse, and CAT and β-Gal expression were quantified by enzyme-linked immunosorbent assays. As an alternative to CAT quantification, cells were fixed, washed, and stained with Giemsa for microscopy.

Stability assay

Viruses were incubated for 30 minutes at different temperatures before performing an HA assay using TRBCs. Two-fold dilutions of virus in PBS containing 0.25% red blood cells were prepared in a U-shaped 96 well plate and were incubated for one hour at 4°C and agglutination was recorded.

Minigenome assay

A model vRNA, consisting of the firefly luciferase flanked by the noncoding regions of segment 8 of influenza A virus, under the control of a T7 RNA polymerase promoter was transfected into 293T cells, along with plasmids expressing T7 RNA polymerase, PB2, PB1, PA, and NP, and a constitutive Renilla luciferase expression plasmid. 24 hours after transfection, firefly and Renilla luminescence was measured. Relative light units were calculated as the ratio of firefly and Renilla luciferase.

Plaque assay

MDCK cells were inoculated with virus and grown with an Avicel (FMC biopolymers, Brussels, Belgium) overlay. Two days later, cells were washed, fixed, permeabilized, and stained with anti-NP monoclonal antibody and goat-anti-mouse-HRP. True blue reagent (KPL Inc., Maryland, USA) was added to the cells and digital images were taken to quantify plaque size using ImageQuant TL software (GE Healthcare Life Sciences).

Primer extension assay

MDCK cells were inoculated with virus and RNA was isolated 3 hours later using Trizol reagent (Invitrogen). Radioactive labelled primers specific for mRNA/cRNA, vRNA of PB1, and 5S rRNA were annealed to prime a reverse transcription reaction. The reaction was stopped and transcription products were separated on 6% polyacrylamide gels containing 7M urea in trisborate-EDTA buffer and detected using autoradiography films.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(PIEF-GA-2009-237505). All experiments involving A/H5N1 virus were performed before January 2012 in agreement with a moratorium on A/H5N1 gain of function research and restarted after February 2013, but without NIH/NIAID funding. Special arrangements are in place with the NIH and the contractor at Icahn School of Medicine at Mount Sinai, New York, for sharing the viruses (and plasmids) in the present paper.

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HIGHLIGHTS

- Five substitutions are sufficient for airborne transmission of A/H5N1 between ferrets
- Two substitutions in PB1 and PB2 increased RNA transcription and virus replication
- Three HA substitutions altered receptor binding preference and lowered pH of fusion
- Strong selective advantage of substitutions responsible for airborne transmission

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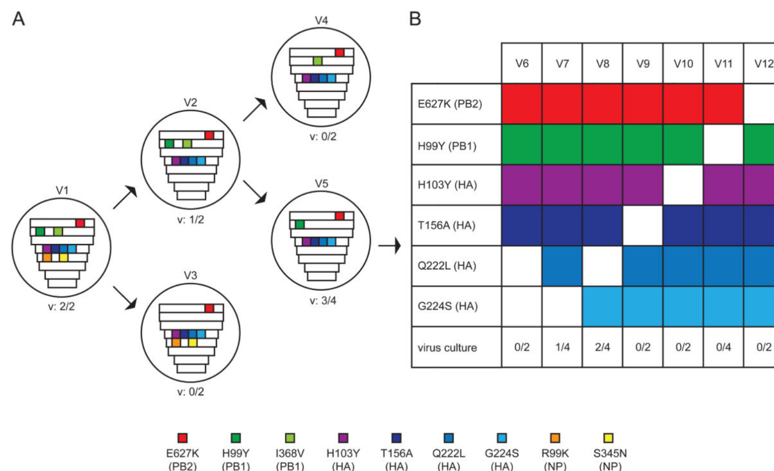

	V6	V7	V8	V9	V10	V11	V12
E627K (PB2)							
H99Y (PB1)							
H103Y (HA)							
T156A (HA)							
Q222L (HA)							
G224S (HA)							
virus culture	0/2	1/4	2/4	0/2	0/2	0/4	0/2

Figure 1. Summary of results to determine a minimal set of substitutions required for airborne A/H5N1 virus transmission between ferrets

(A) Starting with virus V1 that represents the airborne transmitted virus with the lowest number of amino acid substitutions (9) as compared to wildtype A/H5N1 (Herfst et al., 2012), the requirement of substitutions in the PB1 and NP segments was investigated. Recombinant viruses V1–V5 are shown with 8 gene segments, in which colored squares represent the presence or absence of indicated substitutions. The proportion of animals positive by virus isolation is indicated with 'v'. (B) All substitutions of virus V5 were omitted individually and transmission was again tested in ferrets for viruses V6–V12. Virus shedding patterns in donor and recipient ferrets for V1–V12 are provided in Figure S1.

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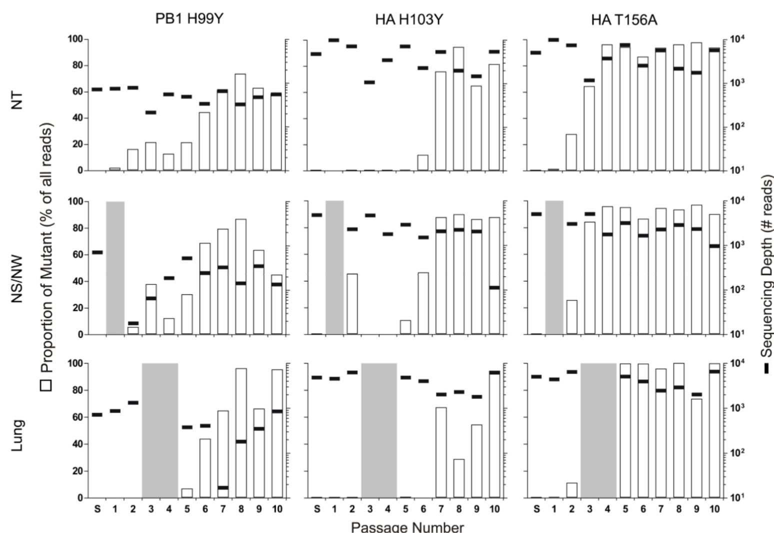

Figure 2. Single nucleotide variations that emerged upon repeated passaging of influenza virus A/H5N1$_{HA\ Q222L,\ G224S\ PB2\ E627K}$ in ferrets, as detected by deep sequencing

Passage number is indicated on the x-axis. Left and right y-axes indicated proportion of the mutant among all reads (white bars) and the sequencing depth in number of reads (black lines) respectively. The grey areas indicate that no virus sequences were amplified from these samples. S: virus stock used to inoculate the first ferret (P1); NT: nasal turbinate; NS: nasal swab; NW: nasal wash.

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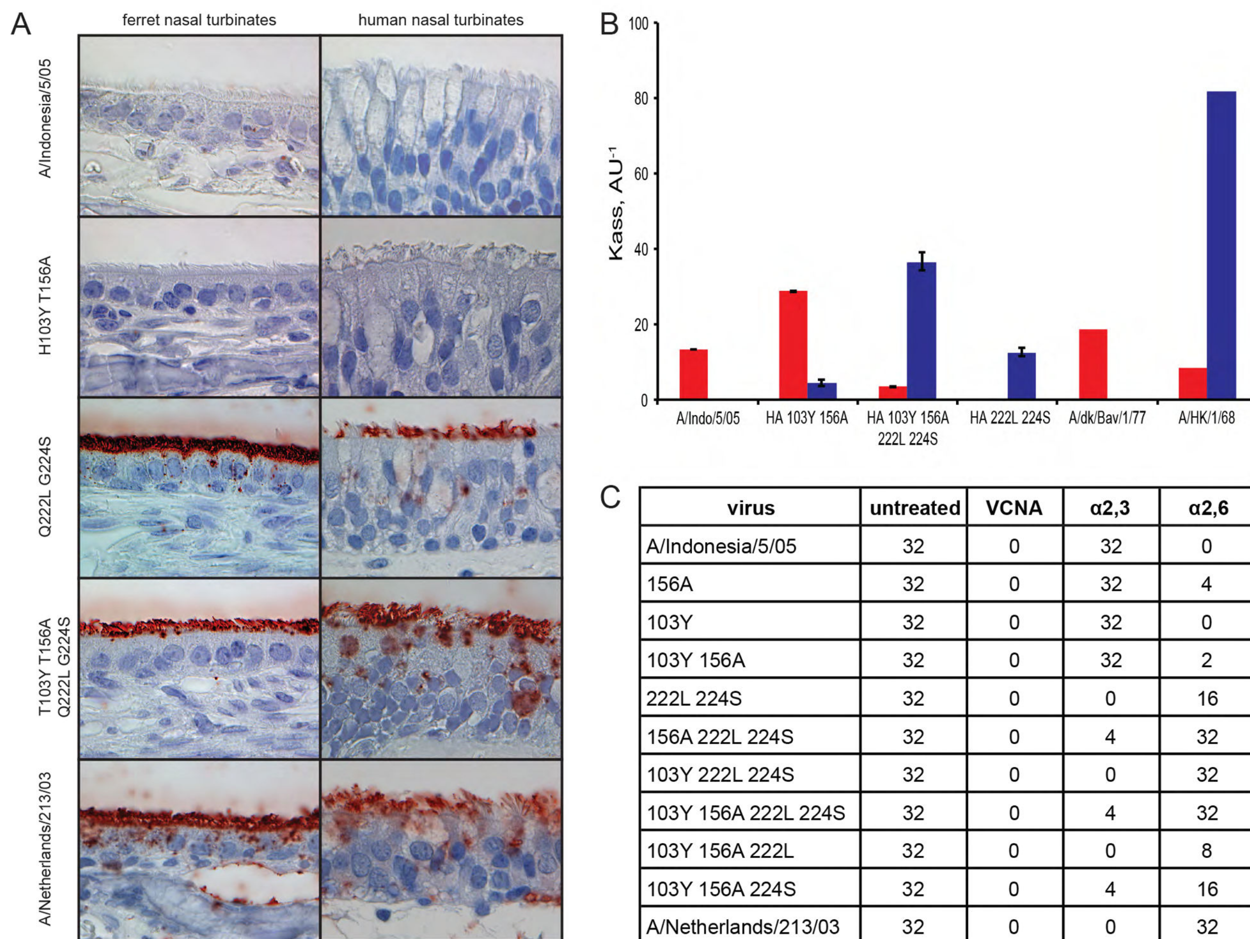

virus	untreated	VCNA	α2,3	α2,6
A/Indonesia/5/05	32	0	32	0
156A	32	0	32	4
103Y	32	0	32	0
103Y 156A	32	0	32	2
222L 224S	32	0	0	16
156A 222L 224S	32	0	4	32
103Y 222L 224S	32	0	0	32
103Y 156A 222L 224S	32	0	4	32
103Y 156A 222L	32	0	0	8
103Y 156A 224S	32	0	4	16
A/Netherlands/213/03	32	0	0	32

Figure 3. Receptor binding properties of wildtype and mutant A/H5N1 HA proteins

(A) Attachment patterns of viruses expressing wildtype or mutant H5 HA, to tissue sections of ferret and human nasal turbinates. Red color represents binding of influenza viruses. Images were chosen to reflect representative attachment patterns. (B) Direct binding of viruses expressing wildtype or mutant H5 HA to fetuin containing either α2,3-SA (red bars) or α2,6-SA (blue bars). A/dk/Bav/1/77 and A/HK/1/68 represent avian and human prototype strains A/duck/Bavaria/1/1977 (A/H1N1) and A/HongKong/1/1968 (A/H3N2) respectively. Error bars represent the standard deviation of the mean values (n=2). See also Figure S2. (C) Agglutination of TRBCs by viruses expressing wildtype or mutant H5 HA. TRBCs were left untreated, stripped from SA using *Vibrio cholerae* neuraminidase (VCNA), or modified to contain either α2,3-SA or α2,6-SA. Numbers show the hemagglutination titers determined with the indicated TRBCs using various mutant viruses. A/Netherlands/213/03 served as a typical human virus with α2,6 SA preference.

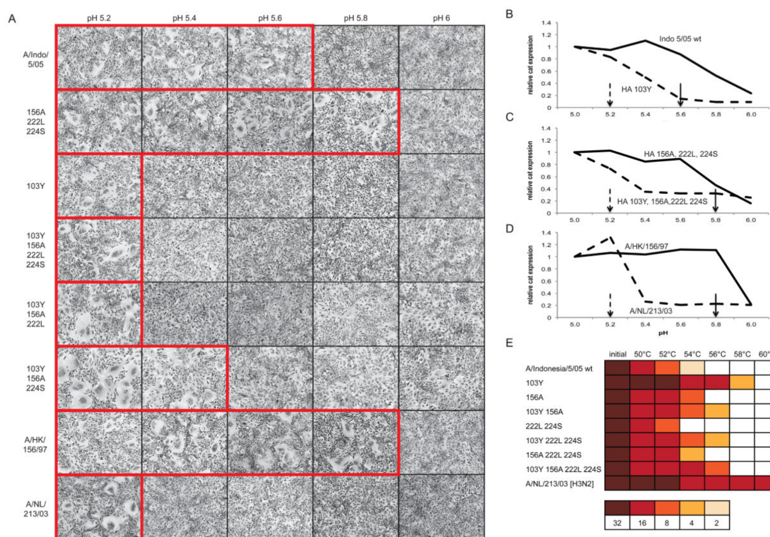

Figure 4. Analysis of pH threshold for fusion and thermostability of wildtype and mutant A/H5N1 HA proteins

(A) Syncytium formation in MDCK cells upon expression of wildtype or mutant HA proteins after exposure to different pH. The red line marks the range of pH values at which fusion was detected microscopically. HA of A/HongKong/156/97 (H5N1) and A/Netherlands/213/03 (H3N2) were included as typical avian and human control viruses. (B–D) Quantification of fusion as measured by the expression of a CAT reporter gene in a cell content mixing assay for: (B) influenza virus A/Indonesia/5/05 HA $_{\text{wildtype}}$ (solid line) and HA$_{\text{H103Y}}$ (dotted line), (C) HA $_{\text{T156A, Q222L, G224S}}$ (solid line) and HA$_{\text{H103Y, T156A, Q222L, G224S}}$ (dotted line), and (D) A/HongKong/156/97 (A/H5N1, solid line) and A/Netherlands/213/03 (A/H3N2, dotted line). Arrows indicate the pH threshold value at which syncytia were detected visually in (A). (E) HA protein stability as measured by the ability of viruses to agglutinate TRBCs after incubation at indicated temperatures for 30 minutes. Colors indicate the hemagglutination titers upon treatment at various temperatures for 30 minutes as shown in the legend.

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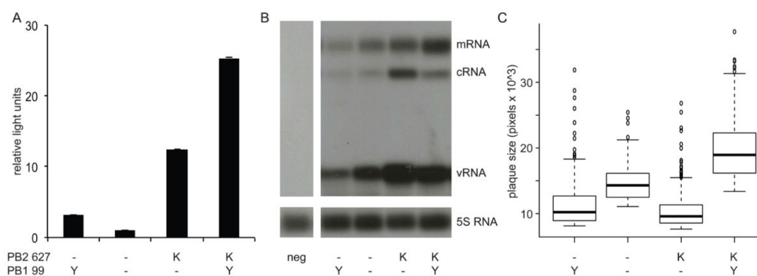

Figure 5. Effect of H99Y in PB1 and E627K in PB2 on polymerase activity and virus replication

(A) Minigenome reporter assay. Plasmids encoding PB2, PB1, PA, and NP were cotransfected with a vRNA reporter encoding firefly luciferase. Luminescence of firefly luciferase was standardized using a plasmid constitutively expressing *Renilla* luciferase. Results are calculated as relative light units (firefly luciferase/*Renilla* luciferase), and plotted as fold increase over wildtype. Error bars indicate the standard deviation from the average of two independent experiments. (B) Primer extension assay. MDCK cells were inoculated at an MOI of 1 with wildtype or mutant A/Indonesia/5/05 viruses. At 3 hours post inoculation, cells were lysed and viral RNA levels were determined by primer extension analysis using primers specific for mRNA, cRNA or vRNA of PB1. Determination of the 5S RNA levels served as an internal loading control. (C) Plaque assay. MDCK cells were inoculated with A/Indonesia/5/05 viruses containing the indicated substitutions. After 48 hours, plaque formation was visualized by influenza NP-specific staining. Digital images were analyzed using ImageQuant TL software to determine plaque size. The surface of individual plaques in pixels is plotted using boxplots indicating the median value and quartiles. Differences between individual groups were significant ($p<0.01$) in Student's t-test. In all panels, the mutations in PB1 and PB2 are indicated, with dashes representing the wildtype sequences.