The soft palate is an important site of adaptation for transmissible influenza viruses

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Influenza A viruses pose a major public health threat by causing seasonal epidemics and sporadic pandemics. Their epidemiological success relies on airborne transmission from person to person; however, the viral properties governing airborne transmission of influenza A viruses are complex. Influenza A virus infection is mediated via binding of the viral haemagglutinin (HA) to terminally attached α2,3 or α2,6 sialic acids on cell surface glycoproteins. Human influenza A viruses preferentially bind 2,6-linked sialic acids whereas avian influenza A viruses bind 2,3-linked sialic acids on complex glycans on airway epithelial cells1–4. Historically, influenza A viruses with preferential association with 2,3-linked sialic acids have not been transmitted efficiently by the airborne route in ferrets5,6. Here we observe efficient airborne transmission of a 2009 pandemic H1N1 (H1N1pdm) virus (A/California/07/2009) engineered to preferentially bind α2,3-linked sialic acids. Airborne transmission was associated with rapid selection of virus with a change at a single HA site that conferred binding to long-chain α2,6-linked sialic acids, without loss of α2,3-linked sialic acid binding. The transmissible virus emerged in experimentally infected ferrets within 24 hours after infection and was remarkably enriched in the soft palate, where long-chain α2,6-linked sialic acids predominate on the nasopharyngeal surface. Notably, presence of long-chain α2,6-linked sialic acids is conserved in ferret, pig and human soft palate. Using a loss-of-function approach with this one virus, we demonstrate that the ferret soft palate, a tissue not normally sampled in animal models of influenza, rapidly selects for transmissible influenza A viruses with human receptor (α2,6-linked sialic acids) preference.

Receptor-binding specificity is an important determinant of host-range restriction and transmission of influenza A viruses (refs 4, 5 and reviewed in ref. 6). The ability of zoonotic influenza A viruses to transmit via the airborne route increases their pandemic potential7. Recently, several investigators have attempted to identify viral determinants of airborne transmission by generating transmissible H5 and H7 avian influenza A viruses8–10. We approached the question differently and used an epidemiologically successful influenza A virus in which we altered receptor preference from the human (α2,6-linked sialic acids) to the avian receptor (α2,3-linked sialic acids).

We previously generated H1N1pdm virus variants with highly specific binding to either α2,6-linked or α2,3-linked sialic acids (referred to as α2,6 or α2,3 H1N1pdm virus, respectively)11. The α2,3 H1N1pdm virus was generated by introducing four amino acid mutations in the receptor binding site of HA (D187E, I216A, D222G and E224A)11. Theoretical structural analysis suggests that the G222D reversion makes the receptor binding site better suited to bind α2,6-linked sialic acids while retaining contacts with α2,3-linked sialic acids via glutamic acid at amino acid 187 (Extended Data Fig. 1). Glycan binding data corroborated this structural prediction because the G222D mutation makes the receptor binding site better suited to bind α2,6-linked sialic acids whereas retaining contacts with α2,3-linked sialic acids via glutamic acid at amino acid 187 (Extended Data Fig. 1). Glycan binding data corroborated this structural prediction because the G222D mutation caused no change in α2,3-linked sialic acid binding but substantially increased binding to long-chain α2,6-linked sialic acids (Fig. 2b).

A delay in peak viral shedding was noted in the airborne-contact animals (see Fig. 1 legend for details) in the α2,3 virus group (red arrows, Fig. 1), suggesting that the virus evolves before transmission. Deep sequence analysis of viral RNA (vRNA) extracted from nasal washes of α2,3 H1N1pdm virus-infected ferrets revealed a mixed population at amino acid position 222 (H1 numbering) with the engineered glycine (G) and wild-type aspartic acid (D), while the other three engineered changes in the HA were retained (Fig. 2a and Supplementary Table 2). Interestingly, the vRNA from the nasal washes of airborne-contact ferrets contained only the G222D HA mutation (Fig. 2a and Supplementary Table 2), suggesting that this sequence at amino acid 222 in the α2,3 H1N1pdm virus was associated with airborne transmission. The virus inoculum did not contain a mixture at this residue (Fig. 2a), and associated changes were not observed in the neuraminidase gene (Supplementary Table 3).

A D222G change in the 2009 H1N1pdm virus HA has occurred in natural isolates, and reports suggest an association with increased virulence in humans and no effect on airborne transmission16–18. Theoretical structural analysis suggests that the G222D reversion makes the receptor binding site better suited to bind α2,6-linked sialic acids while retaining contacts with α2,3-linked sialic acids via glutamic acid at amino acid 187 (Extended Data Fig. 1). Glycan binding data corroborated this structural prediction because the G222D mutation caused no change in α2,3-linked sialic acid binding but substantially increased binding to long-chain α2,6-linked sialic acids (Fig. 2b).

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Figure 1 | Airborne transmission of receptor-specific H1N1pdm viruses. Transmission studies were performed with 4 pairs of animals (8 animals total) in double secure cages with perforated dividers12. One ferret in each pair was infected with 105 50% tissue culture infectious dose (TCID50) of the indicated virus; a naïve ferret (referred to as airborne-contact) was introduced into the adjacent compartment 24 h later. Nasal secretions were collected every other day for 14 days. Viral titres from the nasal washes of airborne-contact ferrets were measured every 2 days (Fig. 1 and Supplementary Table 1) and with a similar efficiency as observed previously for wild-type H1N1pdm virus12–15.

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To determine whether the rapid enrichment of G222D revertant virus in the soft palate was responsible for infection of the airborne-contact animal, we performed an airborne transmission study where naive ferrets were exposed to experimentally infected donor ferrets for only 2 days. Surprisingly, even within this shortened exposure time, two airborne-contact animals shed virus and 3 out of 4 airborne-contact animals seroconverted (Extended Data Fig. 4 and Supplementary Table 1). Sequence analysis of vRNA from the two airborne-contact animals with detectable virus in the nasal washes revealed presence of the G222D revertant. These data suggest that the selection of the α2,3 H1N1pdm virus with the D222 sequence occurs within 3 dpi in the donor ferret and that the airborne-contact ferrets were possibly infected with virus originating in the soft palate because there was nearly complete selection of the G222D mutant by 3 dpi in this tissue.

The soft palate, with mucosal surfaces facing the oral cavity and nasopharynx, is not usually examined in animal models of influenza. To understand what drives the enrichment of the long-chain α2,6-linked-sialic-acid-binding G222D revertant virus at this site, we stained the soft palate with lectins specific for α2,6 or α2,3 sialic acids (Extended Data Fig. 5). The ciliated respiratory epithelium and mucus secreting goblet cells in the respiratory epithelium and submucosal glands (SMG) contained α2,6-linked sialic acids (SNA staining) (Extended Data Fig. 5). Expression of α2,3-linked sialic acid (MAL II staining) was present in the connective tissue underlying the respiratory epithelium and in the serous cells of the SMG. Using a purified HA protein (SC18) that selectively binds long-chain α2,6-linked sialic acids, we found high expression of long-chain α2,6-linked sialic acids in the soft palate compared to the trachea and lungs of ferrets (Fig. 4 and Extended Data Fig. 6). A recent report detailing the glycan profile of the ferret respiratory tract confirms that the soft palate abundantly expresses α2,6 sialylated LacNAc structures, similar to the long-chain α2,6-linked sialic acids recognized by SC18 HA. Interestingly, both the respiratory epithelium and olfactory epithelium from the nasal turbinates of ferrets expressed high levels of long-chain α2,6-linked sialic acid, but the respiratory epithelium of the nasal turbinates was not enriched for the G222D mutant (Fig. 3b and Extended Data Fig. 6). These data suggest that the soft palate is unusual in driving selection for the G222D virus.

To determine the relevance for humans, we evaluated the expression of long-chain α2,6-linked sialic acids in the soft palate of humans and pigs. Interestingly, expression of long-chain α2,6-linked sialic acids was conserved on the respiratory epithelium and goblet cells of the soft palate of both species (Fig. 4). In addition, staining with plant lectins specific for α2,6-linked or α2,3-linked sialic acids (Extended Data Fig. 7) revealed

Figure 3 | Emergence of the α2,3 G222D H1N1pdm virus in the ferret respiratory tract.

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that 2,6-linked sialic acids were present on the nasopharyngeal surface and SMG of both pigs and humans. Expression of 2,3-linked sialic acids was detected in the basal cells of the oral surface and on the nasopharyngeal surface of the human soft palate; these findings are consistent with reports describing the sialic acid distribution in the human nasopharynx. Other investigators have also reported replication of seasonal and pandemic influenza A viruses in tissue sections obtained from the human nasopharynx. Taken together, these data highlight the importance of the nasopharynx, of which the soft palate forms the floor, as a site for host adaptation of influenza A viruses.

Influenza A virus infection of the soft palate may contribute to airborne transmission by providing a mucin-rich microenvironment for generation of airborne virus during coughing, sneezing or breathing. Infection with 2,3 H1N1pdm virus resulted in severe inflammation and necrosis of the respiratory epithelial cells and SMG in the soft palate (Extended Data Fig. 8). Since the soft palate is innervated by the trigeminal nerve, inflammation of this tissue could stimulate sneezing. Alternatively, the soft palate may be the site where infection is initiated during airborne transmission; therefore binding to this tissue would provide a fitness advantage.

These results, albeit with one virus, enhance our understanding of the properties necessary for airborne transmission of influenza A viruses in the ferret model. Loss of 2,3-linked sialic acid specificity is not necessary but gain of long-chain 2,6-linked sialic acid binding is critical for efficient airborne transmission of influenza A viruses. H7N9 viruses from China show dual receptor binding but variable airborne transmission efficiency in ferrets. Interestingly, the 1918 H7N9 viruses from China show dual receptor binding but variable airborne transmission efficiency in ferrets. Interestingly, the 1918 H7N9 viruses from China show dual receptor binding but variable airborne transmission efficiency in ferrets.

Although we did not observe adaptive mutations in the HA stalk of the 2,3 H1N1pdm virus, perhaps because H1N1pdm HA is already adapted to humans, a mixed population was observed at four lysine residues around the receptor binding site (Extended Data Fig. 9 and Supplementary Table 2). Some are known to be egg adaptive mutations or are components of the proposed positively charged ‘lysine fence’ around the base of the receptor binding site, positioned to anchor the N-acetylneuraminic acid and galactose sugar of 2,3-linked and 2,6-linked sialic acid glycans. Interestingly, the lysine residues were restored in the rVNA isolated from nasal washes of airborne-contact ferrets and the soft palate of experimentally infected ferrets (Extended Data Figs 9 and 10).

Taken together with our previously published data, long-chain 2,6-linked sialic acid binding and a highly active neuraminidase contribute to the airborne transmission of the H1N1pdm virus. Importantly, we have identified the previously overlooked soft palate as an important site of isolation of transmissible virus and perhaps the initial site of infection. Analysis of the replicative fitness of influenza A viruses in this tissue may be warranted in assessment of their pandemic potential.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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

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Author Contributions S.S.L., A.J., R.S., D.E.W. and K.S. designed the study. S.S.L., A.J., E.W.L., A.R.S., X. L., A.S., C.T.H., L.V., M.P. and M.M. performed the experiments. S.S.L., R.A.H., T.B.S., I.M., M.O. and S.R.D. analysed the data. S.S.L., K.S., A.J. and R.S. wrote the paper.

Author Information The sequences detailed in this manuscript can be found in GenBank under accession numbers CY184674–CY185309. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.S. (rams@mit.edu) and K.S. (ksubbarao@niaid.nih.gov).
METHODS
Ethics statement and animal studies. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The National Institutes of Health Animal Care and Use Committee (ACUC) approved the animal experiments that were conducted. All studies were conducted under ABLSL2 conditions and all efforts were made to minimize suffering. No statistical methods were used to predetermine sample size. In our animal study protocol, we state that the number of animals in each experimental group varies, and is based on our prior experience. We use the minimum number of animals per group that will provide meaningful results. Randomization was used not to allocate animals to experimental groups and the animal studies were not blinded.

Virus rescue. The 2009 H1N1 pdm virus used in this study is influenza A/California/07/2009. Generation and characterization of the s2,3 H1N1 pdm and s2,6 H1N1 pdm viruses have been described previously\(^1\). Genomic sequencing and dose-dependent glycan binding assays confirmed the identity and receptor specificity of viruses generated by reverse genetics. All experiments were performed using viruses passaged no more than three times in MDCK cells (ATCC) or 10-day old embryonated chicken eggs.

Ferret transmission study. All ferrets (Mustela putorius furo) were obtained from Triple F Farms (Sayre, PA) and screened by haemagglutination inhibition (HAI) assay before infection to ensure that they were naive to seasonal influenza A and B viruses and the viruses used in this study. The transmission studies were conducted in adult ferrets as previously described\(^1\), male and female ferrets were used and the viruses used in this study. The transmission studies were conducted by investigators who were blinded to the experimental groups. For these samples, nested HA-specific small amplicons were generated using HA-specific PCR primers (outer primer pair H1-399F: 5’-AGCTCAGTGTCACTCA TTGAAAAG-3’ and H1-961R: 5’-TGAAATGGGGCTGTGTTGTT-3’; and inner primer pair H1-468F 5’-AACAAGGTGTTAACGGACG-3’ and H1-884R: 5’- AATGATATACAGTACCGCATG-3’). Illumina libraries were prepared from M-RTPCR products and from HA-specific RT–PCR products using the Nextera DNA Sample Preparation Kit (Illumina, Inc.) with half-reaction volumes.

After PCR amplification, 10 μl of each library derived from M-RTPCR products was pooled into a 1.5 ml tube; separately, 10 μl of each library derived from HA-specific amplicons was pooled into a 1.5 ml tube. Each pool was cleaned twice with Ampure XP Reagent (Beckman Coulter) to remove all leftover primers and small DNA fragments. The first and second cleanings used 1× and 0.6× volumes of Ampure XP Reagent, respectively. The cleaned pool derived from M-RTPCR products was sequenced on the Illumina HiSeq 2000 instrument (Illumina, Inc.) with 100-bp paired-end reads, while the cleaned pool derived from HA-specific amplicons was sequenced on the Illumina MiSeq v2 instrument with 300-bp paired-end reads. For additional sequencing coverage, and the HA-specific small amplicons, samples were re-sequenced using the Ion Torrent platform. M-RTPCR products were sheared for 7 min, and Ion-Torrent-compatible barcode adapters were ligated to the sheared DNA using the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA) to create 400-bp libraries. Libraries were pooled in equal volumes and cleaned with the Ampure XP Reagent. Quantitative PCR was performed on the pooled, barcode libraries to assess the quality of the pool and to determine the template dilution factor for emulsion PCR. The pool was diluted appropriately and amplified on ion sphere particles (ISPs) during emulsion PCR on the Ion One Touch 2 instrument (Thermo Fisher Scientific). The emulsion was broken, and the pool was cleaned and enriched for template-positive ISPs on the Ion One Touch ES instrument (Thermo Fisher Scientific). Sequencing was performed on the Ion Torrent PGM using a 318v2 chip (Thermo Fisher Scientific).

DNA sequencing analysis. Depth of sequencing data preparation, collection and analysis were conducted by investigators who were blinded to the experimental groups. For virus sequence assembly, all sequence reads were sorted by barcode, trimmed, and de novo assembled using CLC Bio’s clc_novo_assemble program (Qiagen, Hilden, Germany). The resulting contigs were searched against custom full-length influenza segment nucleotide databases to find the closest reference sequence for each segment. All sequence reads were then mapped to the selected reference influenza A virus segments using CLC Bio’s clc_ref_assemble_long program.

Minor allele variants were identified using FindStatisticallySignificantVariants (FSSV) software (http://sourceforge.net/projects/elvira/). The FSSV software allows statistical tests to minimize false-positive SNP calls generated by Illumina sequence-specific errors (SSEs) described in ref. 33. SSEs usually result in false SNP calls if sequences are read in one sequencing direction. The FSSV analysis tool requires observing the same SNP at a statistically significant level in both sequencing directions. Once a minimum minor allele frequency threshold and significance level are established, the number of minor allele observations and major allele observations in each direction and the minimum minor allele frequency threshold are used to calculate P-values based on the binomial distribution cumulative probability. If the P-values calculated in both sequencing directions are less than the Bonferroni-corrected significance level, then the SNP calls are accepted. A significance level of 0.0025 (Bonferroni-corrected for tests in each direction to 0.025) and a minimum minor allele frequency threshold of 0.3% were applied for this analysis. Differences in the consensus sequence compared to the reference sequence were identified using CLC Bio’s find_variations software. The identified consensus and minor allele variations were analysed by assessing
the functional impact on coding sequences or other regions based on overlap with identified features of the genome. For each sample, the reference sequence was annotated using VIGOR software\textsuperscript{34}, and then the variant data and genome annotation were combined using VariantClassifier software\textsuperscript{35} to produce records describing the impacts of the identified variations.

**Lectin and immunohistochemistry.** Lectin histochemistry was performed as described previously for plant lectins\textsuperscript{36} and purified HA protein\textsuperscript{37}. For plant lectin staining, the soft palate was subjected to microwave-based antigen retrieval using a citrate buffer and was then incubated with FITC-conjugated *Sambucus nigra* agglutinin (SNA) and biotinylated *Maackia amurensis* agglutinin (MAL II) lectins (Vector Laboratories), followed by a streptavidin-Alexa-Fluor594 conjugate (Invitrogen). For SC18 staining, the tissue sections were incubated with pre-complexed purified His-tagged SC18 HA protein, mouse anti-His antibody (Abcam), and goat anti-mouse IgG secondary antibody conjugated to Alexa-Fluor 488 (Molecular Probes) at a 4:2:1 ratio. Nuclei were counter stained with DAPI (Vector Laboratories) or Fluoromount-G (Southern Biotech). Images were captured either on an Olympus BX51 microscope with an Olympus DP80 camera or a Leica SP5 confocal microscope.

Ferret nasal turbinate biopsy samples were obtained from an uninfected 8 month old ferret as follows: the head was dissected sagittally to expose two halves of the ferret nasal turbinates; biopsy of turbinates between the canine and second premolar represented respiratory epithelium and biopsy of turbinates at the molar tooth represented olfactory epithelium. A schematic depicting these two areas is shown in Extended Data Fig. 6h. Pig soft palate tissue sections were a gift from X. J. Meng and P. Pineyro. Pig soft palate tissues were collected from four 56-day-old mixed-breed commercial swine and fixed in 10% formalin. Soft palate tissues from four adult cadavers were obtained from the Maryland State Anatomy Board, Department of Health and Mental Hygiene in Baltimore, Maryland.

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Extended Data Figure 1 | Amino acids in the receptor binding site of the 2009 H1N1pdm HA that bind to α2,3-linked and α2,6-linked sialic acid glycan receptor. a–c, Ribbon diagrams of the 2009 H1N1pdm HA receptor binding pocket interacting with an α2,6-linked sialic acid glycan in the pocket (a), an α2,3 H1N1pdm HA with α2,3-linked sialic acid glycan (b), an α2,3 G222D revertant H1N1pdm HA with an α2,6-linked sialic acid glycan (c).
Extended Data Figure 2 | Replication of α2,3 H1N1pdm virus in ferret respiratory tract. a–f, We confirmed that the α2,3 H1N1pdm virus replicated to high titres on days 1, 3, 5 and 7 in different parts of the ferret respiratory tract. Each tissue homogenate is highlighted with a dashed circle; the grey circles represent washes. Each point represents a single animal. The horizontal black line indicates the mean viral titre on a given day.
Extended Data Figure 3 | Stability of engineered mutations in viruses replicating in the soft palate. Deep sequencing of the HA gene segment from virus populations in the soft palate from 1, 3, 5 and 7 dpi reveals a rapid change at position 222, but no change in the other engineered sites. The engineered sites are highlighted in blue, while the wild-type nucleotide is in orange. Each bar represents a single animal.
Extended Data Figure 4 | Airborne transmission of α2,3 H1N1pdm virus after 48 h exposure time. Transmission studies were performed with 4 pairs of animals (8 animals total) in double secure cages with perforated dividers. One ferret in each pair was infected with $10^6$ TCID$_{50}$ of the indicated virus; a naïve ferret (referred to as airborne-contact) was introduced into the adjacent compartment 24 h later. The airborne-contact animal was removed from the transmission cage on day 3 post-infection as indicated by the black arrow. Nasal secretions were collected every other day for 14 days. Viral titres from the nasal secretions are graphed for each infected or airborne-contact animal. The grey shading indicates the exposure time between the infected and airborne-contact animals.
Extended Data Figure 5 | Influenza receptor distribution on ferret soft palate. Haematoxylin and eosin (H&E) staining of the soft palate from an uninfected ferret highlights the nasopharyngeal and oral surfaces. Scale bar, 1.25 mm. a, Areas highlighted in parts b–g are marked with dashed polygons: square, nasopharyngeal surface (b and e); circle, submucosal gland (c and f); and triangle, oral surface (d and g). b–d, H&E staining of these regions, reproduced from Fig. 4a–c, are shown. e–g, Staining with plant lectins specific for α2,6 sialic acid (SNA) and α2,3 sialic acid (MAL II) are shown. Scale bars are 100 μm in images b–g.
Extended Data Figure 6 | SC18 staining of ferret respiratory tissues. 

a–d, f, g. Sections of ferret trachea (a), lung (b), soft palate (c, d), biopsy of nasal turbinate (NT) tissue with respiratory epithelium (RE) (f) and olfactory epithelium (OLF) (g) were stained with purified SC18 HA protein to identify areas expressing long-chain α2,6-linked sialic acids. h, Illustration of ferret head (sectioned along the midline) highlighting the anatomical locations of respiratory epithelium and OLF tissues. Goblet cells on the respiratory epithelium of the soft palate (nasopharyngeal surface) also stained positive for SC18 (d). e, Absence of SC18 staining after sialidase A treatment indicates the high specificity of SC18 for the respiratory epithelium of the soft palate. All scale bars are 100 μm unless indicated.
Extended Data Figure 7 | Influenza receptor distribution on pig and human soft palate. a–c, g–i, Pig (a–c) and human (g–i) soft palate tissues were stained with plant lectins SNA and MALII which are commonly used as markers for α2,6 and α2,3 sialic acids, respectively. d–f, j–l, Sialidase-A-treated control was run for each sample to ensure specificity of plant lectins and are displayed. Expression of α2,6 sialic acids (SNA staining) is found on the ciliated respiratory epithelium and goblet cells of the nasopharyngeal surface and in the submucosal glands of both the pig and human soft palate. Expression of α2,3-linked sialic acids is low in the pig soft palate and found primarily in goblet cells and submucosal glands. In the human soft palate, MALII (α2,3-linked sialic acids) staining sensitive to sialidase A treatment is found in the goblet cells and respiratory epithelium of the nasopharyngeal surface and in the basal cells of the oral surface. MALII staining in the submucosal glands was not sensitive to sialidase A treatment. Scale bars, 100 μm.
Extended Data Figure 8 | Pathology of the soft palate during infection with α2,3 H1N1pdm virus. The soft palate was removed from three ferrets infected with α2,3 H1N1pdm virus on 7 dpi. The tissue sections were stained with haematoxylin and eosin. Black arrows indicate the ciliated respiratory epithelium of the soft palate tissue (nasopharyngeal surface). Scale bars, 100 μm in all images.
Deep sequencing analysis of the α2,3 H1N1pdm inoculum revealed a mixed population at four lysine residues surrounding the receptor binding site of the HA protein. The lysine fence was restored in viruses from the nasal wash of airborne-contact animals from 6 days post-exposure (DPE). Each bar represents a single animal, and each amino acid (aa) that contained a quasi-species is indicated.
Extended Data Figure 10 | Quasi-species of lysine fence in various ferret respiratory tissue sections. Deep sequencing of viruses from respiratory tissues of ferrets infected with α2,3 H1N1 pdm virus. a–f, Virus populations from the soft palate (a), nasal wash (b), nasal turbinates (c), trachea (d), bronchoalveolar lavage (BAL) (e), or lung sections (f) were analysed and the proportion of lysine, glutamic acid, or asparagine are presented. Each bar represents a single animal. The lung section is an average of the right middle lung lobe and a portion of the left caudal lung tissue.