nature

https://doi.org/10.1038/s41586-024-07766-6

Accelerated Article Preview

Pathogenicity and transmissibility of bovine H5N1 influenza virus

Received: 1 June 2024

Accepted: 28 June 2024

Accelerated Article Preview

Cite this article as: Eisfeld, A. J. et al. Pathogenicity and transmissibility of bovine H5N1 influenza virus. *Nature* https://doi.org/10.1038/s41586-024-07766-6 (2024)

Amie J. Eisfeld, Asim Biswas, Lizheng Guan, Chunyang Gu, Tadashi Maemura, Sanja Trifkovic, Tong Wang, Lavanya Babujee, Randall Dahn, Peter J. Halfmann, Tera Barnhardt, Gabriele Neumann, Yasuo Suzuki, Alexis Thompson, Amy K. Swinford, Kiril M. Dimitrov, Keith Poulsen & Yoshihiro Kawaoka

Pathogenicity and transmissibility of bovine H5N1 influenza virus

Authors

Amie J. Eisfeld[1,*], Asim Biswas[1,*], Lizheng Guan[1,*], Chunyang Gu[1,*], Tadashi Maemura[1,*], Sanja Trifkovic[1], Tong Wang[1], Lavanya Babujee[1], Randall Dahn[1], Peter J. Halfmann[1], Tera Barnhardt[2], Gabriele Neumann[1], Yasuo Suzuki[3], Alexis Thompson[4], Amy K. Swinford[5], Kiril M. Dimitrov[5], Keith Poulsen[6], Yoshihiro Kawaoka[1,7,8,9,†]

Affiliations

[1]Influenza Research Institute, Dept. of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI 53711, USA

[2]Heritage Vet Partners, Johnson, KS 67855, USA

[3]Department of Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

[4]Texas A&M Veterinary Medical Diagnostic Laboratory, Canyon, TX 79016, USA

[5]Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX 77843, USA

[6]Wisconsin Veterinary Diagnostic Laboratory, University of Wisconsin-Madison, Madison, WI 53706, USA

[7]Department of Virology, Institute of Medical Science, University of Tokyo, Tokyo, 108-8639, Japan

[8]The University of Tokyo Pandemic Preparedness, Infection and Advanced research center (UTOPIA), University of Tokyo, Tokyo 108-8639, Japan

[9] The Research Center for Global Viral Diseases, National Center for Global Health and Medicine Research Institute, Tokyo, 162-8655, Japan

Other footnotes

[*]These authors contributed equally.

[†]Corresponding author; please address correspondence to Yoshihiro Kawaoka (yoshihiro.kawaoka@wisc.edu)

Abstract

Highly pathogenic H5N1 avian influenza (HPAI H5N1) viruses occasionally infect, but typically do not transmit, in mammals. In the Spring of 2024, an unprecedented outbreak of HPAI H5N1 in bovine herds occurred in the US, with virus spread within and between herds, infections in poultry and cats, and spillover into humans, collectively indicating an increased public health risk[1-4]. Here, we characterized an HPAI H5N1 virus isolated from infected cow milk in mice and ferrets. Like other HPAI H5N1 viruses, the bovine H5N1 virus spread systemically, including to the mammary glands of both species; however, this tropism was also observed for an older HPAI H5N1 virus isolate. Importantly, bovine HPAI H5N1 virus bound to sialic acids expressed in human upper airways and inefficiently transmitted to exposed ferrets (one of four exposed ferrets seroconverted without virus detection). Bovine HPAI H5N1 virus thus possesses features that may facilitate infection and transmission in mammals.

Introduction

After reports of unexplained symptoms including reduced milk production in lactating dairy cattle in Texas, USA, highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype was reported in milk and nasal wash samples of an infected cow on March 25, 2024, marking the first documented outbreak of HPAI H5N1 viruses in cattle. By May 30, 2024, the USDA had confirmed 69 infected bovine herds in nine states[1], with spread being attributed to cattle movement between states. Virus transmission among lactating dairy cattle may occur through contaminated milking equipment with virus infection through the udder, but this has not been confirmed. HPAI H5N1 viruses rarely infect mammals and typically do not transmit among them. The bovine H5N1 virus outbreak, along with reports of three HPAI H5N1 virus-infected dairy farm workers (presenting with conjunctivitis[4] or respiratory symptoms[3]), fatal HPAI H5N1 virus infections of cats on affected farms, and spillover to poultry highlight the public health risk of the current HPAI H5N1 virus outbreak in cattle.

The bovine H5N1 viruses isolated from cattle are closely related to HPAI H5N1 viruses circulating in North American wild birds[5-8]. These viruses belong to HA clade 2.3.4.4b and were introduced into North America in late 2021 through the Transatlantic flyway from Europe. Frequent reassortment with North American low pathogenic avian influenza viruses has resulted in multiple genotypes which have spread throughout the American continent, causing sizeable outbreaks in wild birds and sea mammals, some with high mortality rates and suspected virus transmission among sea mammals[9,10].

The basic characteristics of the bovine H5N1 viruses are unknown. Accordingly, here, we tested a bovine H5N1 virus isolated from the milk of an infected dairy cow in New Mexico, USA, for replication and pathogenicity in mice and ferrets, two mammalian animal models routinely used for influenza A virus studies, and for respiratory droplet transmission in ferrets. We also tested the vertical transmission of bovine HPAI H5N1 virus from lactating mice to their pups. Finally, we compared receptor specificity, an important factor for host range restriction, of bovine and avian H5N1 viruses and a seasonal human H1N1 influenza virus.

Results

Pathogenicity after oral ingestion. To evaluate the public health risk of H5N1 virus-containing milk, we previously demonstrated that oral consumption of milk from an HPAI H5N1-infected cow led to rapid induction of disease symptoms (by day 1 post-infection) and virus dissemination to respiratory and non-respiratory organs (by day 4 post-infection) in BALB/cJ mice[5]. To assess disease caused by oral inoculation in more detail, we repeated this experiment with smaller inoculation volumes of milk from infected cattle (25, 10, 5, and 1 µl per mouse; corresponding dosages: 3.25×10^3 plaque-forming units [PFU] per 25 µl; 1.3×10^3 PFU per 10 µl; 6.5×10^2 PFU per 5 µl; and 1.3×10^2 PFU per 1 µl; 10 mice per inoculation group). For five mice, we monitored body weight loss and survival daily over 14 days, and in the other five, we determined virus titres in the lung, nasal turbinate, and brain (the latter served as a proxy for virus dissemination to non-respiratory sites) on day 6 post-infection. Some mice inoculated with 25 µl or 10 µl of milk exhibited substantial weight loss (**Fig. 1a and Extended Data Fig. 1**) and a subset succumbed to the infection (**Fig. 1b**). Additionally, in mice euthanised on day 6 post-infection, high virus titres were observed in nasal turbinate, lung, and brain tissues (**Fig. 1c**; no statistically significant differences in nasal turbinate, brain, or lung titres were observed between the 25 µl and 10 µl inoculation groups). In contrast, mice inoculated with 25 µl of milk from a healthy cow (mock) showed no symptoms of disease (**Fig. 1a and 1b and Extended Data Fig. 1**). In mice inoculated with 5 µl of milk, disease was less apparent and virus replication in respiratory tissues and brain was sporadic. No disease or virus replication was observed in animals inoculated with 1 µl of milk. A hemagglutination inhibition (HI) assay of serum collected from all mice that survived inoculation with any volume of infected cow's milk revealed no seroconversion in any of the animals.

Pathogenicity after intranasal infection. Influenza A viruses typically infect humans by the respiratory route. To assess pathogenicity in mice after intranasal (*i.e.*, respiratory) exposure, we determined the mouse lethal dose 50 (MLD_{50}) and tissue tropism of A/dairy cattle/New Mexico/A240920343-93/2024 ('Cow-H5N1'). Female BALB/cJ mice were inoculated with 10-fold serial dilutions (10^0 to 10^6 PFU, 5 animals per dose) of Cow-H5N1, and body weight (**Fig. 2a**) and survival (**Fig. 2b**) were monitored daily for 15 days. All mice infected with ≥ 10^3 PFU of virus succumbed to the infection, whereas some mice infected with 10^2 or 10^1 PFU survived (**Fig. 2b**). No body weight loss or death was observed among mice infected with 10^0 PFU of virus (**Fig. 2a**). The resulting MLD_{50} of 31.6 PFU is comparable to that of two different clade 2.3.4.4b HPAI H5N1 mink viruses isolated during an outbreak in Spain in 2022 (A/mink/Spain/22VIR12774-13_3869-

2/2022, MLD_{50}: 48.1 PFU; A/mink/Spain/22VIR12774-14_3869-3/2022, MLD_{50}: 30 PFU), but slightly higher than that of A/Vietnam/1203/2004 ('VN1203-H5N1', MLD_{50}: 2.2 PFU)[11], that is, a typical avian H5N1 virus isolated from a human.

To examine tissue tropism after intranasal infection, we inoculated female BALB/cJ mice with 10^3 PFU of Cow-H5N1, VN1203-H5N1, or a pandemic H1N1 influenza virus (A/Isumi/UT-KK001-01/2018, 'Isumi-H1N1')[12] for comparison (10 mice per group). Three and six days later, five mice in each group were euthanised, tissues (blood, eye, teat, mammary gland, brain, intestine, liver, spleen, kidney, heart, nasal turbinate, trachea, lung, hamstring, and latissimus dorsi) were collected, and virus titres were determined by performing plaque assays in MDCK cells (**Fig. 2c**). For Cow-H5N1 and VN1203-H5N1, virus titres on day 6 were generally higher than those on day 3. Both viruses caused systemic infections with high titres in respiratory and non-respiratory organs, including the mammary glands, teats, and muscle tissues of the leg (hamstring) and back (latissimus dorsi). Virus was also found in the eye of a single mouse infected with VN1203-H5N1 (**Fig. 2c**), and in a similar experiment (performed under the same conditions, but without Isumi-H1N1 infections or collection of blood or muscle tissue), we found both Cow-H5N1 and VN1203-H5N1 in eyes (**Extended Data Fig. 2**). The consistent detection of HPAI H5N1 virus in the mammary glands and muscle tissues, and its sporadic detection in the eyes of mice is consistent with reports of HPAI H5N1 virus in the mammary glands[2,13] and muscle tissues of cows[14] and with reports of conjunctivitis and respiratory symptoms in humans infected with an HPAI H5N1 virus related to the outbreak in cattle[3,4]. In contrast to Cow-H5N1 and VN1203-H5N1, the Isumi-H1N1 virus was detected only in the respiratory tissues of mice (**Fig. 2c**). Since Cow-H5N1 and VN1203-H5N1 (but not Isumi-H1N1) were also found in the blood, it is possible that viral spread to non-respiratory tissues occurred through viremia.

We next intranasally infected female ferrets with 10^6 PFU of Cow-H5N1 or VN1203-H5N1 (4 animals per virus) and examined tissue tropism at days 3 and 6 post-infection. Ferrets infected with either virus exhibited elevated body temperatures and body weight loss after infection (**Extended Data Fig. 3**), consistent with clinical disease. As in mice, both viruses replicated to high titres in the upper and lower respiratory tracts, and spread to non-respiratory organs (including eyes, brain, colon, liver, spleen, kidney, and/or heart) in some of the infected ferrets (**Fig. 3**). Virus was also detected in the mammary glands and teats but only in a few animals in each group. No virus was detected in the blood or muscle tissues of ferrets infected with Cow-H5N1, VN1203-H5N1, or Isumi-H1N1 in a separate experiment (**Extended Data Fig. 4**). Currently, it is unclear whether the lack of virus in the blood and muscle tissues of ferrets is due

to differences in the animals or due to the inability of HPAI H5N1 viruses to spread to blood and/or muscle tissues in the ferret model. Nonetheless, these findings are consistent with other reports of the systemic spread of related HPAI H5N1 viruses in ferrets, including limited spread to the ocular tissues[15,16]; and further support the possibility that mammary gland and/or teat tropism are features of mammalian infection with HPAI H5N1 viruses, and not a specific characteristic of HPAI H5N1 isolated from lactating dairy cattle.

Together, our pathogenicity studies in mice and ferrets revealed that (1) HPAI H5N1 derived from lactating dairy cattle may induce severe disease after oral ingestion or respiratory infection; and (2) infection by either the oral or respiratory route can lead to systemic spread of virus to non-respiratory tissues including the eye, mammary gland, teat, and/or muscle.

Transmission from lactating mice to pups. HPAI H5N1 viruses have been detected in the milk of lactating dairy cattle and oral ingestion of milk can lead to severe disease in the mouse model (see [5] and **Fig. 1**). In our next set of experiments, we tested whether bovine H5N1 virus could be transferred from infected, lactating mice to uninfected, suckling offspring (*i.e.*, pups) or adult contact animals. Five-to-seven days after giving birth, lactating females were intranasally inoculated with 100 PFU of Cow-H5N1, and then either reunited with their pups or placed into cages with non-lactating female adults. At days 4, 7, and 9 post-infection, mice were euthanised and organs were collected for virus titration.

At day 4 post-infection, 5 of 6 lactating females showed virus replication in respiratory tissues, but no virus was detected in the brain or mammary glands (**Fig. 4a**). None of the 25 pups (distributed across 5 litters) exhibited any detectable virus in the brain, lung, or intestines; and none of the 3 adult contacts (co-housed with a single lactating female) exhibited any detectable virus in nasal turbinate, lung, or brain (**Extended Data Fig. 5**). At day 7 post-infection, lactating females (9 in total) exhibited higher virus loads in lung and nasal turbinate, and three lactating females (one co-housed with pups and two with adult contacts) also had virus in the brain and mammary gland (**Fig. 4b**; note, two lactating females co-housed with adult contacts also had virus in their milk). Of the 24 pups (distributed across 5 litters), 4 pups from 2 litters became infected (3 of the 4 infected pups were from a litter of a lactating female that had virus spread to the brain and mammary gland), but again, no virus was detected in any of the adult contact animals (**Extended Data Fig. 5**). At day 9 post-infection, virus was detected in the respiratory tissues and brain of all lactating females, as well as in the mammary glands or milk of 3 of the 6 lactating females (**Fig. 4c**). At this timepoint, 11 pups (of 30 pups distributed across 6 litters) became infected (4 of 6 litters had at least 1 infected pup); and in 3 of the litters with infected pups, we

also detected virus in the mammary gland and/or milk of their lactating mothers. As observed on day 4 and day 7, none of the adult contact animals on day 9 had detectable virus in the examined tissues (**Extended Data Fig. 5**). Therefore, Cow-H5N1 can be transmitted from lactating females to their pups, but not to adult animals with which they have direct contact. Since virus was detected in the mammary glands and milk of most of the lactating mice and the pups had direct exposure to the infected milk, it is conceivable that mother-to-pup vertical transmission occurred via the milk. Of note, vertical transmission was observed in the absence of virus detection in the mammary glands or milk of the lactating mother in two instances (one animal each at day 7 and day 9 post-infection, **Fig. 4b and 4c**). We hypothesize that this may be due to non-uniform dissemination of Cow-H5N1 to the mammary glands.

Inefficient transmission in ferrets. Currently, it is unknown whether bovine H5N1 viruses transmit among mammals via respiratory droplets. To test this possibility, we carried out a respiratory droplet transmission experiment in ferrets as described previously[17]. Groups of ferrets were infected with 10^6 PFU of either Cow-H5N1 or Isumi-H1N1 (4 ferrets per virus), which is known to transmit efficiently via respiratory droplets[11]. One day later, naïve animals were housed in cages next to the infected animals (1 contact ferret per infected donor), separated by about 5 cm to prevent transmission by direct contact. Nasal swab samples were collected from infected and exposed animals every other day starting on day 1 post-infection or post-exposure, respectively, and virus titres were assessed. Ferrets infected with either Cow-H5N1 or Isumi-H1N1 showed clinical signs of disease (**Extended Data Fig. 6**) and high virus titres in nasal swabs collected over multiple days, with a delay in the peak virus titre of animals infected with Cow-H5N1 (**Fig. 5a**). In contrast, only the exposed animals in the Isumi-H1N1 group exhibited signs of clinical disease (**Extended Data Fig. 6**) and virus in the nasal swabs (**Fig. 5b**). These data indicate that the Isumi-H1N1 virus, but not the Cow-H5N1 virus, transmits efficiently via respiratory droplets in ferrets. A hemagglutination inhibition (HI) assay carried out with serum collected from all ferrets that survived until day 21 post-infection or post-exposure revealed high neutralization titres for all infected and exposed animals in the Isumi-H1N1 group (**Fig. 5c**), consistent with their demonstrated infection. In addition, while no virus was detected in any of the animals exposed to the Cow-H5N1-infected ferrets (**Fig. 5a**), 1 of 4 exposed animals had a positive, albeit low, HI titre (**Fig. 5c**). No viral genomic sequences were detected in, and no virus was amplified from, any of the nasal swabs of the seroconverted ferret. Therefore, bovine H5N1 virus may transmit inefficiently by the respiratory droplet route in ferrets.

Receptor binding preference. Influenza A virus binds to sialic acid receptors on the surface of susceptible cells to initiate infection. Human influenza A viruses preferentially bind to sialic acids linked to galactose by an α2,6-linkage, whereas avian influenza A viruses preferentially bind to α2,3-linked sialic acid. Because α2,6-linked sialic acids are abundantly distributed in the upper respiratory tract of humans, influenza A viruses that can bind to α2,6-linked sialic acids may have a greater capacity to transmit among humans. To test the receptor specificity of Cow-H5N1, we employed an established assay utilizing α2,3- or α2,6-linked sialylglycopolymers to measure virion binding to α2,3- or α2,6-linked sialic acid[17,18]. As expected, the human Isumi-H1N1 virus exhibited a clear preference for α2,6-linked sialic acids, whereas the avian VN1203-H5N1 virus exhibited a clear preference for α2,3-linked sialic acids (**Fig. 6**). In contrast, the Cow-H5N1 virus bound to both α2,3- and α2,6-linked sialic acids, indicating that the Cow-H5N1 virus may have the ability to bind to cells in the upper respiratory tract of humans. The dual receptor binding specificity of Cow-H5N1 was confirmed by two independent replicate experiments (**Extended Data Fig. 7 and Extended Data Fig. 8**). Since dual receptor binding specificity was not observed for the older, distantly related VN1203-H5N1 isolate (**Fig. 6, Extended Data Fig. 7, and Extended Data Fig. 8**), it may be a feature unique to the HPAI H5N1 virus that recently emerged in dairy cattle.

Discussion

HPAI H5N1 influenza viruses do not transmit efficiently among mammals. Moreover, influenza A viruses have rarely been detected in cattle. Thus, the current outbreak of HPAI H5N1 influenza viruses in dairy cows and the spill-over into other mammalian species may have profound consequences for public health and the dairy industry.

Although >850 people and increasing numbers of mammals have been infected with HPAI H5N1 viruses, sustained transmission among mammals has not been reported, although we[12] and others[19,20] have suggested that it may be possible. Recently, mammal-to-mammal transmission may have occurred during outbreaks of HPAI H5N1 viruses in mink in Spain[21] and sea mammals South America[10]. Sutton and colleagues[22] reported respiratory droplet transmission of mink HPAI H5N1 virus from experimentally infected to exposed ferrets, but we did not detect respiratory droplet transmission of mink HPAI H5N1 viruses in ferrets[11]; these differences may result from the different virus isolates used and/or differences in experimental settings. Here, we found that a bovine HPAI H5N1 virus may have transmitted to exposed ferrets at low efficiency,

resulting in seroconversion in the absence of detectable virus in nasal swabs. Importantly, while this work was under review, the US CDC reported limited (33%) respiratory droplet transmission in ferrets of an HPAI H5N1 virus isolated from an infected farm worker in Texas (A/Texas/37/2024) during the current outbreak in dairy cattle[23], which supports our findings. The discovery that HPAI H5N1 viruses may acquire the ability to transmit among mammals is a paradigm shift and increases the pandemic potential of these viruses. The isolate we tested does not encode PB2-E627K, an amino acid substitution that facilitates the efficient replication of avian influenza viruses in mammals[24,25]. However, this substitution was detected in the HPAI H5N1 virus isolated from the infected farm worker in Texas. Additional studies with human HPAI H5N1 isolates are urgently needed to fully assess the risks they pose to the greater human population.

The host range of influenza viruses is determined, in part, by their receptor-binding specificity because avian influenza viruses prefer α2,3-linked sialic acids (expressed in the gastrointestinal tract of avian species), whereas human influenza viruses prefer α2,6-linked sialic acids (the predominant sialic acid species in the upper respiratory tract of humans). The 1957 and 1968 pandemic influenza viruses possess human-type receptor-binding specificity, even though their HAs originated from avian influenza viruses. The HPAI H5N1 viruses tested to date displayed avian-type receptor-binding specificity (for example, see [26-29]); however, here we detected human- and avian-type receptor-binding specific for a bovine HPAI H5N1 virus, consistent with the finding of both sialic acid species in udders of cattle[30]. Currently, we do not know whether this dual receptor-binding specificity reflects adaptive changes in cattle or is also a trait of other North American HPAI H5N1 viruses. Collectively, our study demonstrates that bovine H5N1 viruses may differ from previously circulating HPAI H5N1 viruses by possessing dual human/avian-type receptor-binding specificity with limited respiratory droplet transmission in ferrets.

References

1 USDA Animal and Plant Health Inspection Service. *Highly Pathogenic Avian Influenza (HPAI) Detections in Livestock*, <https://www.aphis.usda.gov/livestock-poultry-disease/avian/avian-influenza/hpai-detections/livestock> (2024).

2 Caserta, L. C. *et al.* From birds to mammals: spillover of highly pathogenic avian influenza H5N1 virus to dairy cattle led to efficient intra- and interspecies transmission. *Preprint at* https://www.biorxiv.org/content/10.1101/2024.05.22.595317v1, doi:https://doi.org/10.1101/2024.05.22.595317 (2024).

3 CDC Newsroom. *CDC Confirms Second Human H5 Bird Flu Case in Michigan; Third Case Tied to Dairy Outbreak*, <https://www.cdc.gov/media/releases/2024/p0530-h5-human-case-michigan.html> (2024).

4 Uyeki, T. M. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Virus Infection in a Dairy Farm Worker. *N Engl J Med*, doi:10.1056/NEJMc2405371 (2024).

5 Guan, L. *et al.* Cow's Milk Containing Avian Influenza A(H5N1) Virus - Heat Inactivation and Infectivity in Mice. *N Engl J Med*, doi:10.1056/NEJMc2405495 (2024).

6 Worobey, M. *et al. Preliminary report on genomic epidemiology of the 2024 H5N1 influenza A virus outbreak in U.S. cattle (Part 1 of 2)*, <https://virological.org/t/preliminary-report-on-genomic-epidemiology-of-the-2024-h5n1-influenza-a-virus-outbreak-in-u-s-cattle-part-1-of-2/970> (2024).

7 Worobey, M. *et al. Preliminary report on genomic epidemiology of the 2024 H5N1 influenza A virus outbreak in U.S. cattle (Part 2 of 2)*, <https://virological.org/t/preliminary-report-on-genomic-epidemiology-of-the-2024-h5n1-influenza-a-virus-outbreak-in-u-s-cattle-part-2-of-2/971> (2024).

8 Meade, P. S. *et al.* Detection of clade 2.3.4.4b highly pathogenic H5N1 influenza virus in New York City. *J Virol*, e0062624, doi:10.1128/jvi.00626-24 (2024).

9 Puryear, W. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Virus Outbreak in New England Seals, United States. *Emerg Infect Dis* **29**, 786-791, doi:10.3201/eid2904.221538 (2023).

10 Tomas, G. *et al.* Highly pathogenic avian influenza H5N1 virus infections in pinnipeds and seabirds in Uruguay: Implications for bird-mammal transmission in South America. *Virus Evol* **10**, veae031, doi:10.1093/ve/veae031 (2024).

11 Maemura, T. *et al.* Characterization of highly pathogenic clade 2.3.4.4b H5N1 mink influenza viruses. *EBioMedicine* **97**, 104827, doi:10.1016/j.ebiom.2023.104827 (2023).

12 Imai, M. *et al.* Influenza A variants with reduced susceptibility to baloxavir isolated from Japanese patients are fit and transmit through respiratory droplets. *Nat Microbiol* **5**, 27-33, doi:10.1038/s41564-019-0609-0 (2020).

13 Burrough, E. R. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus Infection in Domestic Dairy Cattle and Cats, United States, 2024. *Emerg Infect Dis* **30**, doi:10.3201/eid3007.240508 (2024).

14 USDA Animal and Plant Health Inspection Service. *Updates on H5N1 Beef Safety Studies*, <https://www.aphis.usda.gov/livestock-poultry-disease/avian/avian-influenza/hpai-detections/livestock/h5n1-beef-safety-studies> (2024).

15 Belser, J. A., Sun, X., Pulit-Penaloza, J. A. & Maines, T. R. Fatal infection in ferrets after ocular inoculation with highly pathogenic avian influenza A(H5N1) virus. *Emerg Infect Dis* **30**, doi:10.3201/eid3007.240520 (2024).

16 Pulit-Penaloza, J. A. *et al.* Highly pathogenic avian influenza A(H5N1) virus of clade 2.3.4.4b isolated from a human case in Chile causes fatal disease and transmits between co-housed ferrets. *Emerg Microbes Infect* **13**, 2332667, doi:10.1080/22221751.2024.2332667 (2024).

17 Imai, M. *et al.* Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* **486**, 420-428, doi:10.1038/nature10831 (2012).

18 Gambaryan, A. S. & Matrosovich, M. N. A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. *J Virol Methods* **39**, 111-123, doi:10.1016/0166-0934(92)90130-6 (1992).

19 Herfst, S. *et al.* Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* **336**, 1534-1541, doi:10.1126/science.1213362 (2012).

20 Chen, L. M. *et al.* In vitro evolution of H5N1 avian influenza virus toward human-type receptor specificity. *Virology* **422**, 105-113, doi:10.1016/j.virol.2011.10.006 (2012).

21 Aguero, M. *et al.* Highly pathogenic avian influenza A(H5N1) virus infection in farmed minks, Spain, October 2022. *Euro Surveill* **28**, doi:10.2807/1560-7917.ES.2023.28.3.2300001 (2023).
22 Restori, K. H. *et al.* Risk assessment of a highly pathogenic H5N1 influenza virus from mink. *Nat Commun* **15**, 4112, doi:10.1038/s41467-024-48475-y (2024).
23 CDC Avian Influenza (Bird Flu). *CDC Reports A(H5N1) Ferret Study Results*, <https://www.cdc.gov/bird-flu/spotlights/ferret-study-results.html?CDC_AAref_Val=https://www.cdc.gov/flu/avianflu/spotlights/2023-2024/ferret-study-results.htm> (2024).
24 Hatta, M., Gao, P., Halfmann, P. & Kawaoka, Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* **293**, 1840-1842, doi:10.1126/science.1062882 (2001).
25 Hatta, M. *et al.* Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathog* **3**, 1374-1379, doi:07-PLPA-RA-0307 [pii] 10.1371/journal.ppat.0030133 (2007).
26 Chang, P. *et al.* Characterization of the haemagglutinin properties of the H5N1 avian influenza virus that caused human infections in Cambodia. *Emerg Microbes Infect* **12**, 2244091, doi:10.1080/22221751.2023.2244091 (2023).
27 Xing, X. *et al.* Evolution and biological characterization of H5N1 influenza viruses bearing the clade 2.3.2.1 hemagglutinin gene. *Emerg Microbes Infect* **13**, 2284294, doi:10.1080/22221751.2023.2284294 (2024).
28 Bi, Y. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Virus Struck Migratory Birds in China in 2015. *Sci Rep* **5**, 12986, doi:10.1038/srep12986 (2015).
29 Hui, K. P. *et al.* Tropism and innate host responses of influenza A/H5N6 virus: an analysis of ex vivo and in vitro cultures of the human respiratory tract. *Eur Respir J* **49**, doi:10.1183/13993003.01710-2016 (2017).
30 Kristensen, C., Jensen, H. E., Trebbien, R., Webby, R. J. & Larsen, L. E. The avian and human influenza A virus receptors sialic acid (SA)-α2,3 and SA-α2,6 are widely expressed in the bovine mammary gland. *Preprint at* https://www.biorxiv.org/content/10.1101/2024.05.03.592326v1, doi:https://doi.org/10.1101/2024.05.03.592326 (2024).

Figure Legends

Figure 1. Pathogenicity in mice orally inoculated with milk from an HPAI H5N1 virus-infected cow. Female BALB/cJ mice (8 weeks old) were lightly anaesthetized and orally inoculated with 25 µl of milk from a healthy cow ('mock'; n=5 biologically independent animals per inoculation volume) or different volumes (25, 10, 5, or 1 µl containing 3.25 x 10^3 PFU per 25 µl, 1.3 x 10^3 PFU per 10 µl, 6.5 x 10^2 PFU per 5 µl, and 1.3 x 10^2 PFU per 1 µl; n=10 biologically independent animals per inoculation volume) of milk from a dairy cow infected with HPAI H5N1 virus. For five mice per inoculation volume, body weights (**A**) and survival (**B**) were monitored daily for 14 days. In panel A, datapoints represent mean values for each inoculation volume at each time point and error is represented by standard deviation. The other 5 mice in each inoculation group were euthanised at 6 days post-infection and nasal turbinate (NT), lung, or brain

tissues were collected for virus titration in MDCK cells (**C**). In panel C, the floating bars show the median titre for each tissue of each inoculation group and variability is represented by the range. When virus was not detected in a tissue, an arbitrary value below the limit of detection was assigned to enable visualization of the datapoint on the graph. Non-parametric, two-tailed Mann-Whitney tests were used to compare titres of the 25 µl and 10 µl inoculation groups and no significant differences were found (NT, p = 0.4603; lung, p = 0.5397; brain, p = 0.3016). PFU/g, plaque-forming units per gram of tissue.

Figure 2. Pathogenicity and tissue tropism in mice intranasally inoculated with bovine H5N1 virus. (**A**) and (**B**) BALB/cJ mice (7 weeks old, n=5 biologically independent animals per dosage) were deeply anaesthetized and intranasally inoculated with 10-fold-serial dilutions of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1) in 50 µl of PBS. (**A**) Body weight and (**B**) survival were monitored daily for 15 days. In panel A, the error bars represent the standard deviation. (**C**) BALB/cJ mice (10 weeks old, n=10 biologically independent animals per virus) were deeply anaesthetized and intranasally inoculated with 10^3 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1; 'Cow-H5N1'), A/Vietnam/1203/2004 (H5N1; 'VN1203-H5N1'), or A/Isumi/UT-KK001-01/2018 (H1N1; 'Isumi-H1N1') in 50 µl of PBS. At 3 and 6 days post-infection, five mice infected with Cow-H5N1 or Isumi-H1N1 were euthanised and tissues were collected for plaque assays in MDCK cells. For VN1203-H5N1 infections, four mice were euthanised at the day 3 timepoint since one mouse succumbed at day 1 post-infection, and five mice were euthanized at the day 6 timepoint. In panel C, the floating bars show the median titre for each tissue of each inoculation group and variability is represented by the range. When virus was not detected in a tissue, an arbitrary value below the limit of detection was assigned to enable visualization of the datapoint on the graph. PFU/g, plaque-forming units per gram of tissue; PFU/ml, plaque-forming units per millilitre.

Figure 3. Tissue tropism in ferrets intranasally inoculated with bovine H5N1 virus. Ferrets (4—6 months old, n=8 biologically independent animals per virus) were deeply anaesthetized and intranasally inoculated with 10^6 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1; 'Cow-H5N1') or A/Vietnam/1203/2004 (H5N1; 'VN1203-H5N1') in 500 µl of PBS. At 3 and 6 days post-infection, four ferrets infected with Cow-H5N1 were euthanised and tissues were collected for plaque assays in MDCK cells. For VN1203-H5N1 infections, four ferrets were euthanised at the day 3 timepoint, one ferret succumbed to its infection on day 4, one succumbed on day 5, and two others were euthanised at the day 6 timepoint. Tissues from animals that succumbed on day 4 or day 5 post-infection are represented by triangles and squares, respectively. In the figure

panels, the floating bars show the median titre for each tissue of each inoculation group and variability is represented by the range. For VN1203-H5N1-infected animals, medians and ranges are shown only for the day 3 timepoint since some animals in the day 6 timepoint group succumbed earlier. When virus was not detected in a tissue, an arbitrary value below the limit of detection was assigned to enable visualization of the datapoint on the graph. PFU/g, plaque-forming units per gram of tissue.

Figure 4. Transmission of bovine H5N1 virus from lactating female mice to offspring. Lactating female BALB/c mice (10—12 weeks old) were deeply anaesthetized, intranasally inoculated with 10^2 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1; 'Cow-H5N1'), and then reunited with their suckling offspring ('pups'). At day 4 (n=5 biologically independent animals) (**A**), day 7 (n=5 biologically independent animals) (**B**), or day 9 (n=6 biologically independent animals) (**C**) post-infection, lactating females and their pups were euthanised and tissues were collected for plaque assays in MDCK cells. Milk was collected from 5 of 6 lactating females on day 9 post-infection only, as indicated, and tested by plaque assays in MDCK cells. In the figure, each box represents one cage with a lactating female and her pups. Animals for which Cow-H5N1 virus was detected in at least one tissue are coloured blue. At the lower left corner of each box, the status of each tissue or milk sample collected from the lactating females is indicated. Gray text indicates that no virus was detected, while red text indicates that virus was detected. Tissue abbreviations are given at the lower left of the figure. For the day 9 timepoint group, some of the lactating females succumbed to their infections prior to the designated endpoints, but within 12 h of tissue collection (indicated by asterisks). Tissues were collected from these mice and analysed along with the others.

Figure 5. Bovine H5N1 virus transmits inefficiently by respiratory droplets in ferrets. Ferrets (4-6 months old, n=4 biologically independent animals per virus) were deeply anaesthetized and intranasally inoculated with 10^6 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1; 'Cow-H5N1') (**A**) or A/Isumi/UT-KK001-01/2018 (H1N1; 'Isumi-H1N1') (**B**) in 500 µl of PBS. One day later, naïve ferrets (n=1 biologically independent animal per infected animal) were placed in adjacent cages allowing for air flow but no direct contact with the infected animals. Nasal swab samples were collected at the indicated timepoints and tested by plaque assays in MDCK cells. In panels A and B, the dotted lines represent the limit of detection. (**C**) Sera collected from recovered ferrets were subjected to hemagglutination inhibition (HI) assays with Cow-H5N1 or Isumi-H1N1, and HI titres are shown. The floating bars represent the mean HI titre for each group and error bars represent standard deviation. Ferrets exhibiting no seroconversion were assigned

arbitrary values below the limit of detection so they could be represented on the graph. PFU/ml, plaque-forming units per millilitre.

Figure 6. Bovine H5N1 virus binds to both α2,3 and α2,6 sialic acid residues. Four-fold serial dilutions of α2,3 and α2,6 sialylglycopolymers adhered to microtitre plates were incubated with 32 hemagglutination (HA) units of the indicated viruses or PBS (negative control). After washing, virus binding was detected by an anti-HA human monoclonal antibody (CR9114) and an HRP-conjugated secondary antibody. The absorbance values for each condition with each virus or PBS are shown. Each dot represents a single biologically independent replicate value.

Methods

Ethics Statement. All animal experiments and procedures were approved by the Institutional Care and Use Committees of the University of Wisconsin-Madison School of Veterinary Medicine (protocol # V006426-A04). The ambient conditions of the animal facilities were 25-28°C and 35-45% humidity. Animals were acclimated to the facilities before the start of the experiments, maintained on a 12 h on/off light cycle, given access to food and water *ad libitum*, and provided with enrichment. Humane endpoint criteria for both ferrets and mice after infection comprised the following: ≥ 35% body weight loss or inability to remain upright.

Biosafety. In the US, highly pathogenic avian influenza viruses are 'Select Agents' as described in title 9, Code of Federal Regulations Parts 121 and 122. After the identification of HPAI H5 viruses, they were reported immediately to the Federal Select Agent Program. All experiments were carried out in Biosafety Level 3 (BSL-3) containment laboratories (ferret experiments were performed under BSL-3-Ag containment) at the Influenza Research Institute at the University of Wisconsin-Madison, which is approved by the Federal Select Agent Program for studies with these viruses. Funding for this study came in part from the NIAID Centers of Excellence for Influenza Research and Response (CEIRR, Contract Number 75N93021C00014). All experiments were approved by the University of Wisconsin-Madison Institutional Biosafety Committee (IBC) and all animal experiments were approved by the University of Wisconsin-Madison Animal Care and Use Committee. The NIAID grant for the studies conducted was reviewed by the University of Wisconsin-Madison Dual Use Research of Concern (DURC) Subcommittee in accordance with the United States Government September 2014 DURC Policy and determined to not meet the criteria of DURC. The University of Wisconsin-Madison Institutional Contact for Dual Use Research reviewed this manuscript and confirmed that the studies described herein do not meet the criteria of DURC.

Cells and viruses. MDCK cells (obtained from the ATCC; no authentication was performed) were grown in Eagle's minimal essential medium (MEM) containing 5% newborn calf serum and were routinely monitored for mycoplasma contamination. A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1) was isolated in MDCK cells from a milk sample provided by the Texas A&M Veterinary Medical Diagnostic Laboratory[5]. The isolated virus was fully sequenced (GISAID EPI_ISL_19091702), amplified in MDCK cells, and sequenced again. No mutations emerged during passage in MDCK cells. This virus isolate does not encode the mammalian-adapting mutations PB2-E627K[24,31] or PB2-D701N[32,33], but possesses the PB2-M631L substitution, the effect of which is like that of the PB2-E627K substitution[34,35]. In addition, in our previous publication[5], we showed that this virus isolate is part of the same clade as other publicly available cow H5N1 virus sequences. The amplified virus stock was used for all studies described, except when otherwise stated. As indicated, control viruses included a highly pathogenic H5N1 avian influenza virus (A/Vietnam/1203/2004)[25], which was originally isolated from a human; and a human H1N1 influenza virus (A/Isumi/UT-KK001-01/2018)[11]. Oral inoculation of mice was conducted with milk from an HPAI H5N1 virus-infected cow. An HPAI H5N1 virus was isolated from this milk sample, which was designated A/dairy cattle/Kansas/SM-3/2024. The consensus sequences of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1) and A/dairy cattle/Kansas/SM-3/2024 differ by nine amino acids: PB2-E249G, PB1-P384S, PA-K497R, PA-K613E, HA-N319S, NA-N71S, NS1-R21Q, NS1-R77L, and NS1-K229E.

Oral inoculation of mice. Eight-week-old female BALB/cJ mice (Jackson Laboratories, Bar Harbor, ME, USA) were lightly anaesthetized with isoflurane and inoculated with the milk sample containing A/dairy cattle/Kansas/SM-3/2024 (25, 10, 5, or 1 µl; 10 mice per inoculation volume) by applying the virus to the back of the throat with a micropipette. All mice swallowed the inoculum. Following inoculation, five animals per inoculation volume were monitored daily for signs of illness for 14 days; and the other five animals per inoculation volume were euthanised on day 6 post-inoculation, at which time organs (nasal turbinate, lung, and brain) were collected for virus titration. For all animals that survived beyond 14 days post-inoculation, blood was collected as follows: mice were deeply anaesthetized with isoflurane, cardiac puncture was performed to collect blood, and then the mice were euthanised. Blood was immediately transferred to serum separator tubes, centrifuged at 2,000 x *g* for 10 minutes, and the resultant serum was frozen at –80°C.

Mouse lethal dose 50 determination. To determine the mouse lethal dose 50 (MLD_{50}), seven-week-old female BALB/cJ mice were anaesthetized by i.p. injection of ketamine and

dexmedetomidine (45–75 mg/kg ketamine + 0.25–1 mg/kg dexmedetomidine) and intranasally inoculated with 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, or 10^6 plaque-forming units (PFU) in 50 µl of phosphate-buffered saline (PBS) of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1) (5 mice per dosage). To reverse the effects of dexmedetomidine, mice were injected i.p. with atipamezole (0.1–1 mg/kg). Body weight changes and survival were monitored daily for 15 days. Infected mice were euthanised if they lost more than 35% of their initial body weight. Lethal dose 50 values were calculated according to the method of Reed and Muench[36].

Tissue tropism in mice. Seven- to ten-week-old female BALB/cJ mice were anaesthetized by i.p. injection of ketamine and dexmedetomidine (45–75 mg/kg ketamine + 0.25–1 mg/kg dexmedetomidine) and intranasally inoculated with 10^3 PFU (in 50 µl of PBS) of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1), A/Vietnam/1203/2004 (H5N1), or A/Isumi/UT-KK001-01/2018 (H1N1). At days 3 and 6 post-infection, groups of 5 mice were euthanised and the following tissues were collected in the order listed and frozen at –80˚C: whole blood, eye, teat, mammary gland, brain, colon, liver, spleen, kidney, heart, nasal turbinate, trachea, lung, hamstring, and latissimus dorsi. Instruments used for tissue dissection were disinfected after each tissue was collected to prevent cross-contamination of virus between organs. Whole blood was snap-frozen on dry ice immediately after collection in the absence of anticoagulant. Later, frozen tissue samples were thawed, mixed with 1 ml of MEM medium containing 0.3% bovine serum albumin (BSA) and homogenised by using a TissueLyser II (Qiagen) at 30-Hz oscillation frequency for 3 min. Homogenates were clarified by centrifugation (14,000 rpm for 10 minutes) and used for plaque assays in MDCK cells. Whole blood was thawed and used directly for plaque assays.

Tissue tropism in ferrets. Four- to six-month-old female ferrets (Triple F Farms) (confirmed to be serologically negative to the following influenza viruses, A/Hong Kong/4/2022 (H3N2), A/Wisconsin/588/2019 (H1N1), B/Washington/02/2019 and A/Astrakhan/3212/2020 (H5N8)) were anaesthetized intramuscularly with ketamine and dexmedetomidine (4-5 mg/kg and 10-40 µg/kg of body weight, respectively) and infected intranasally with 10^6 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1), A/Vietnam/1203/2004 (H5N1), or A/Isumi/UT-KK001-01/2018 (H1N1) in 500 µl of PBS as indicated in the text and figure legends. Body weights and body temperatures were monitored daily. At day 3 or 6 post-infection, groups of four ferrets were euthanised, and the following tissues were collected and frozen at –80°C: eye, teat, mammary gland, hamstring, latissimus dorsi, brain, whole blood (collected from the jugular vein), colon, liver, spleen, kidney, heart, nasal turbinate, trachea, and lung. Tissues were collected in

the order listed to prevent cross-contamination of virus from respiratory organs. As done for mice, whole blood was immediately snap-frozen on dry ice and stored without anticoagulant. Ferret tissues were prepared for plaque assays in MDCK cells as follows: organs were mixed with 1 ml of MEM medium containing 0.3% BSA, homogenised at 1,850 rpm for six cycles (ON: 6 seconds; OFF: 4 seconds) in a multi-bead homogeniser (Yasui Kikai Corporation, Japan), centrifuged at 14,000 rpm for 10 min, and then used for plaque assays in MDCK cells.

Transmission in mice. Ten- to twelve-week-old lactating female BALB/c mice (Jackson Laboratories or Taconic Biosciences) at 5-7 days post-delivery were intranasally inoculated with 100 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1) in 50 µl of PBS under isoflurane anesthesia. Two hours after inoculation, the mice were returned to cages with their litters or co-housed with 3 adult BALB/cJ mice (8-12-weeks old). Co-housed adults were added to cages with infected, lactating females either 2 hours (day 7 time point, lactating females #1-6) or 24 hours (day 4, all lactating females; day 7, lactating females #7-9; and day 9, all lactating females) after infection. At days 4, 7, or 9 post-infection, lactating females, pups, and contacts were euthanised and tissues were collected and frozen at –80°C. From lactating females, mammary gland, brain, nasal turbinate, and lung tissues were collected. From pups of lactating females, brain, lung, and intestine tissues were collected. From adult contacts co-housed with lactating females, brain, nasal turbinate, and lung tissues were collected. Tissues were prepared for plaque assays in MDCK cells as described for other mouse tissues above. At the day 9 timepoint, milk was collected from infected lactating females under isoflurane anesthesia by squeezing the mammary gland after i.p. oxytocin injection (2 IU/mouse; Bimeda). For lactating females that succumbed prior to euthanasia, no oxytocin was given. A micropipette was used to collect the milk (up to 5 µl) directly from the teat, and milk was mixed with 100 µl of PBS prior to virus titration by plaque assay in MDCK cells.

Respiratory droplet transmissibility. Female ferrets were infected intranasally with 10^6 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1) or A/Isumi/UT-KK001-01/2018 (H1N1) in 500 µl of PBS (4 ferrets per virus). One day later, naïve ferrets (aerosol contacts; 1 contact per infected animal) were placed in cages adjacent to infected ferrets in an isolator rack. The cages housing the infected or exposed ferrets were separated by about 5 cm. The transmission study was carried out under controlled conditions of 20–25°C and relative humidity of 38.4% ± 8.8%. The airflow was from the front to the back of the isolator rack; thus, the airflow direction was perpendicular to the direction of virus transmission between the ferrets. Nasal swab samples were collected on day 1 after infection or exposure, respectively, and then every other

day. The swabs were pre-soaked in PBS, inserted into the ferret's nasal cavity, and then placed in a tube containing 1.0 ml of MEM with 50 U/ml penicillin and 50 µg/ml streptomycin and vortexed for 1 minute. The virus titre was determined by plaque assay in MDCK cells. At 21 days post-infection, blood was collected from the infected and contact ferrets in both groups, transferred to serum separator tubes, centrifuged at 2,000 x *g* for 10 minutes, and the resultant serum was frozen at –80°C.

Plaque assays. Plaque assays were performed by using standard methods. Briefly, confluent MDCK cells were washed with 1X MEM containing 0.3% BSA (MEM/BSA), followed by infection with serial dilutions of virus. Infected cells were incubated at 37°C for 1 h, washed with 1X PBS, and then covered with 1X MEM/BSA plus 1% low melting point agarose in the presence of 0.6 µg/ml TPCK-treated trypsin. Plates were incubated at 37 °C and 5% CO_2 for 2-3 days, and the monolayers were then fixed with 10% formalin. After removal of the agar overlay and air-drying, the virus plaques were counted under fluorescent light.

Hemagglutination inhibition assay. Ferret or mouse sera were treated with receptor destroying enzyme (Denka Seiken Co., Ltd., Tokyo, Japan) at 37°C for 18–20 h, followed by heat inactivation at 56 °C for 50 minutes and then adsorbed with turkey red blood cells for 1 h at room temperature with gentle shaking. Then, two-fold serial dilutions of treated sera were prepared in 96-well V-bottom plates and mixed with 4 hemagglutination (HA) units of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1) or A/Isumi/UT-KK001-01/2018 (H1N1). After 30 minutes at room temperature, 0.5% TRBC were added to each well, and the plate was incubated at room temperature for 1 hour. The HI titre was read as the reciprocal of the last dilution of serum that completely prevented hemagglutination.

Virus growth in embryonated chicken eggs. Ten-day-old embryonated chicken eggs were inoculated with nasal swab samples as described[37]. Two days later, eggs were killed by incubation at 4°C overnight. The next morning, allantoic fluids were collected and a small aliquot was assessed by use of the hemagglutination assay according to standard methods.

Quantitative PCR. RNA was extracted from ferret nasal swab samples or egg allantoic fluids by using the MagMAX™-96 Total RNA Isolation Kit (Invitrogen). qPCR reactions were carried out with the TaqMan™ Fast Virus 1-Step Master Mix for qPCR (Applied Biosystems) and the following primers: H5-forward, 5'-TACCAGATACTGTCAATTTATTCAAC-3'; H5-reverse, 5'-GTAACGACCCATTGGAGCACATCC-3'; H5 FAM probe, 5'-56-FAM/CTGGCAATCATGATGGCTGGTCT/3BHQ_1-3'; M-forward, 5'-CTTCTAACCGAGGTCGAAACGTA-3'; M-reverse, 5'-GGTGACAGGATTGGTCTTGTCTTTA -3'; and M VIC probe, 5'-5HEX/TCGGGCCCCCTCAAAGCCGAG/3BHQ_1-3'. qPCR reactions were

performed with the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) as follows: (1) 50°C for 20 minutes, (2) 95°C for 5 minutes, and (3) 40 cycles of 95° for 15 seconds and 60°C for 45 seconds; and then cycle threshold (Ct) values were determined.

Solid-phase binding assay. Microtitre plates (Nunc) were incubated with 4-fold serial dilutions (2.5, 0.625, 0.156, 0.039, 0.01, 0.002, and 0.001 µg/ml) of the sodium salts of sialylglycopolymers (Yamasa Corporation Co. Ltd)—Neu5Acα2,3Galβ1,4GlcNAcβ1-poly-Glu (α2,3SA) and Neu5Acα2,6Galβ1,4GlcNAcβ1-poly-Glu (α2,6SA)—in PBS at 4°C overnight. The next day, glycopolymer solutions were removed and non-specific binding was blocked by the addition of PBS containing 4% BSA at room temperature for 1 h. Plates were washed with cold PBS, and then solutions containing influenza viruses [16 hemagglutination (HA) units in PBS for the data shown in **Extended Data Fig. 8** and 32 HA units in PBS for the data shown in **Fig. 6 and Extended Data Fig. 7**] were added and plates were incubated at 4°C overnight. Plates were washed with cold PBS and then incubated with broadly reactive human monoclonal CR9114 antibody (HumImmu; 1:1000 dilution, catalog no. A90001) for 1 h at room temperature (for the data shown in **Extended Data Fig. 8**) or 1 h at 4°C (for the data shown in **Fig. 6 and Extended Data Fig. 7**). The plates are washed again as before and incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG (Abcam, catalog no. ab6858) for 1 h at room temperature. After being washed, the plates were incubated with o-phenylenediamine (Sigma) in PBS containing 0.03% H_2O_2 for 10 min at room temperature. Absorbance was measured at 450 nm using an optical plate reader (BioTek). The data shown in **Fig. 6** and **Extended Data Fig. 8** represent a single technical replicate per condition, whereas the data shown in **Extended Data Fig. 7** represent two technical replicates per condition.

Statistics and reproducibility. All animals were randomly allocated to experimental groups. No blinding was performed in any experiment. Sample sizes were based on our previous work. All graphs were generated with GraphPad Prism software, version 9.5.1. Basic summary statistics (*i.e.*, calculations of means, standard deviations, medians, and data ranges) were calculated and plotted by using GraphPad Prism. Virus titers of nasal turbinate, lung, and brain tissues from orally inoculated mice (25 µl and 10 µl groups) were $\log_{10}$-transformed and compared by using non-parametric, two-tailed Mann-Whitney tests in GraphPad Prism software, and *p*-values are reported in the **Fig. 1C** legend. No adjustment for multiple comparisons was performed. Except for experiments with lactating mice (**Fig. 4 and Extended Data Fig. 5**), all other figures represent data derived from a single experiment. Mouse experiments shown in **Fig. 2C** and **Extended Data Fig. 2** are similar, except that the experiment shown in **Fig. 2C** included mice infected with Isumi-H1N1 and collection of muscle tissues and blood. Ferret experiments

shown in **Fig. 3** and **Extended Data Fig. 4** are similar, except that the experiment shown in **Extended Data Fig. 4** included ferrets infected with Isumi-H1N1, a single timepoint for tissue collection (day 6), and collection of muscle tissues and blood. For the data shown in **Fig. 4**, lactating females at each timepoint were infected on the same days (*i.e.*, day 4 animals were infected on the same day, day 7 animals were infected on the same day, and day 9 animals were infected on the same day). For the data shown in **Extended Data Fig. 5**: the single lactating female on day 4 (**Extended Data Fig. 5A**) was infected on the same day as those from the same timepoint in **Fig. 4A**; one lactating female on day 7 (**Extended Data Fig. 5B, top panel**) was infected on the same day as those from the same timepoint in **Fig. 4B** and the other three were infected in another experiment; and all four lactating females on day 9 (**Extended Data Fig. 5C**) were infected on the same day as those from the same timepoint in **Fig. 4C**). Three independent receptor binding experiments were performed, and the data from all three are shown separately (**Fig. 6, Extended Data Fig. 7, Extended Data Fig. 8**).

Data availability. All source data underlying animal and receptor binding experiments described herein are available in the online version of the paper.

Methods References

31 Subbarao, E. K., London, W. & Murphy, B. R. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J Virol* **67**, 1761-1764, doi:10.1128/JVI.67.4.1761-1764.1993 (1993).

32 Gabriel, G. *et al.* The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proc Natl Acad Sci U S A* **102**, 18590-18595, doi:10.1073/pnas.0507415102 (2005).

33 Fan, S. *et al.* Novel residues in avian influenza virus PB2 protein affect virulence in mammalian hosts. *Nat Commun* **5**, 5021, doi:10.1038/ncomms6021 (2014).

34 Idoko-Akoh, A. *et al.* Creating resistance to avian influenza infection through genome editing of the ANP32 gene family. *Nat Commun* **14**, 6136, doi:10.1038/s41467-023-41476-3 (2023).

35 Staller, E. *et al.* Structures of H5N1 influenza polymerase with ANP32B reveal mechanisms of genome replication and host adaptation. *Nat Commun* **15**, 4123, doi:10.1038/s41467-024-48470-3 (2024).

36 Reed, L. J. & Muench, H. A simple method of estimating fifty percent endpoints. *American Journal of Hygiene* **27**, 493-497 (1938).

37 Eisfeld, A. J., Neumann, G. & Kawaoka, Y. Influenza A virus isolation, culture and identification. *Nat Protoc* **9**, 2663-2681, doi:10.1038/nprot.2014.180 (2014).

Acknowledgements

This work was supported by the National Institute of Allergy and Infectious Diseases Centers of Excellence for Influenza Research and Response (contract 75N93021C00014), non-sponsored discretionary funding, and by grants from the Japan Agency for Medical Research and Development (JP24wm0125002, JP243fa627001, and JP24fk0108626, to Y.K.), the USDA National Institute of Food and Agriculture (2023-37624-40714 to K.P.), Colorado State University (USDA NIFA: AP23VSD&B000C020 to K.P.), the USDA Animal and Plant Health Inspection Service (APHIS) (AP22VSSP0000C024 to K.P.), the Wisconsin Department of Agriculture, Trade, and Consumer Protection (WIAE 2307 to K.P.), and the APHIS National Animal Health Laboratory Network Enhancement Project (AP21VSD&B000C005 to K.D.). The authors would like to extend their gratitude to John A. Lucey (University of Wisconsin—Madison) for providing whole, unpasteurised milk from a healthy cow, which was used in the mouse oral inoculation experiment. In addition, the authors thank Hai Duong Nguyen, Robert Presler, Jr., Peter Jester, and Kirstyn Loyva for technical assistance and Sue Watson for editing the manuscript. Mouse illustrations used in Figure 4 and Extended Data Figure 5 were created with BioRender.com.

Author Contributions

Author contributions are provided according to Contributor Roles Taxonomy (CRediT):

Conceptualization: AE, PH, GN, YK

Data curation: AE, AB, LG, CG, TM, ST, LB, RD, GN

Formal analysis: AE, ST, LB

Funding acquisition: YK, KP

Investigation: AE, AB, LG, CG, TM, TW, LB, RD, GN

Methodology: AE, AB, LG, CG, TM, LB, RD, PH, GN, YK

Project administration: AE, PH, ST, GN

Resources: AT, AS, KD, KP, YS, YK

Software: LB

Supervision: YK

Validation: AE, AB, LG, CG, TM, TW, LB, RD

Visualization: AE, ST

Writing – original draft: AE, GN, YK

Writing – review and editing: AE, AB, LG, CG, TM, ST, TW, LB, RD, PH, TB, GN, YS, AT, AS, KD, KP, YK

Author contributions to specific experiments:

The mouse oral inoculation experiment was performed AB, AE, LG, and CG. Mouse intranasal inoculation experiments were performed by AB, AE, LG, CG, and TM. Ferret experiments were performed by AB, LG, CG, TM, and TW. Receptor binding experiments were performed by TM. Sequence analysis was performed by LB, RD, and GN.

Competing Interests

The authors do not have any competing interests to declare.

Additional Information

Supplementary Information is available for this paper.

Correspondence and requests for materials should be addressed to Yoshihiro Kawaoka (yoshihiro.kawaoka@wisc.edu).

Extended Data Figure Legends

Extended Data Figure 1. Individual body weight profiles of mice orally inoculated with milk from an infected dairy cow. Individual body weight profiles for the mice shown in **Fig. 1a** are shown in the four panels at the left (n=5 biologically independent animals per inoculation volume). Mock-infected body weights, shown in all four panels, are derived from the same mice. In the panel at the right, body weights are shown for mock-infected mice, and for mice that exhibited > 10% body weight loss after inoculation with milk from an infected dairy cow. For the mock-infected mice and mice inoculated with 10 µl of infected milk, the values are the means of 5 or 3 mice, respectively, while a single mouse body weight profile is shown for the 25 µl-infected milk inoculation group. Error bars represent one standard deviation.

Extended Data Figure 2. Tissue tropism in mice intranasally inoculated with bovine H5N1 virus, a replicate experiment. BALB/cJ mice (7 weeks old, n=10 biologically independent animals per virus) were deeply anaesthetized and intranasally inoculated with 10^3 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1; 'Cow-H5N1') or A/Vietnam/1203/2004 (H5N1; 'VN1203-H5N1') in 50 µl of PBS. At 3 and 6 days post-infection, five mice in each group were euthanised and tissues were collected for plaque assays in MDCK cells. In the figure panels, the floating bars show the median titre for each tissue of each inoculation group and variability is represented by the range. When virus was not detected in a tissue, an arbitrary value below the

limit of detection was assigned to enable visualization of the datapoint on the graph. PFU/g, plaque-forming units per gram of tissue.

Extended Data Figure 3. Clinical data associated with ferrets used to assess tissue tropism. For the same ferrets shown in Figure 3 (n=8 biologically independent animals per virus), daily body weights and body temperatures are given. For the VN1203-H5N1-infected group day 6 timepoint, two animals succumbed to their infections prior to the planned euthanasia date (ferret 7 at day 5 and ferret 8 at day 4 post-infection). The dotted lines indicate starting weights or body temperatures. F, ferret.

Extended Data Figure 4. Tissue tropism in ferrets intranasally inoculated with bovine H5N1, a replicate experiment. Ferrets (4—6 months old, n=4 biologically independent animals per virus) were deeply anaesthetized and intranasally inoculated with 10^6 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1; 'Cow-H5N1'), A/Vietnam/1203/2004 (H5N1; 'VN1203-H5N1'), or A/Isumi/UT-KK001-01/2018 (H1N1; 'Isumi-H1N1') in 500 µl of PBS. At 6 days post-infection, ferrets were euthanised and tissues were collected for plaque assays in MDCK cells. In the figure panels, the floating bars show the median titre for each tissue of each inoculation group and variability is represented by the range. When virus was not detected in a tissue, an arbitrary value below the limit of detection was assigned to enable visualization of the datapoint on the graph. PFU/g, plaque-forming units per gram of tissue; PFU/ml, plaque forming units per millilitre.

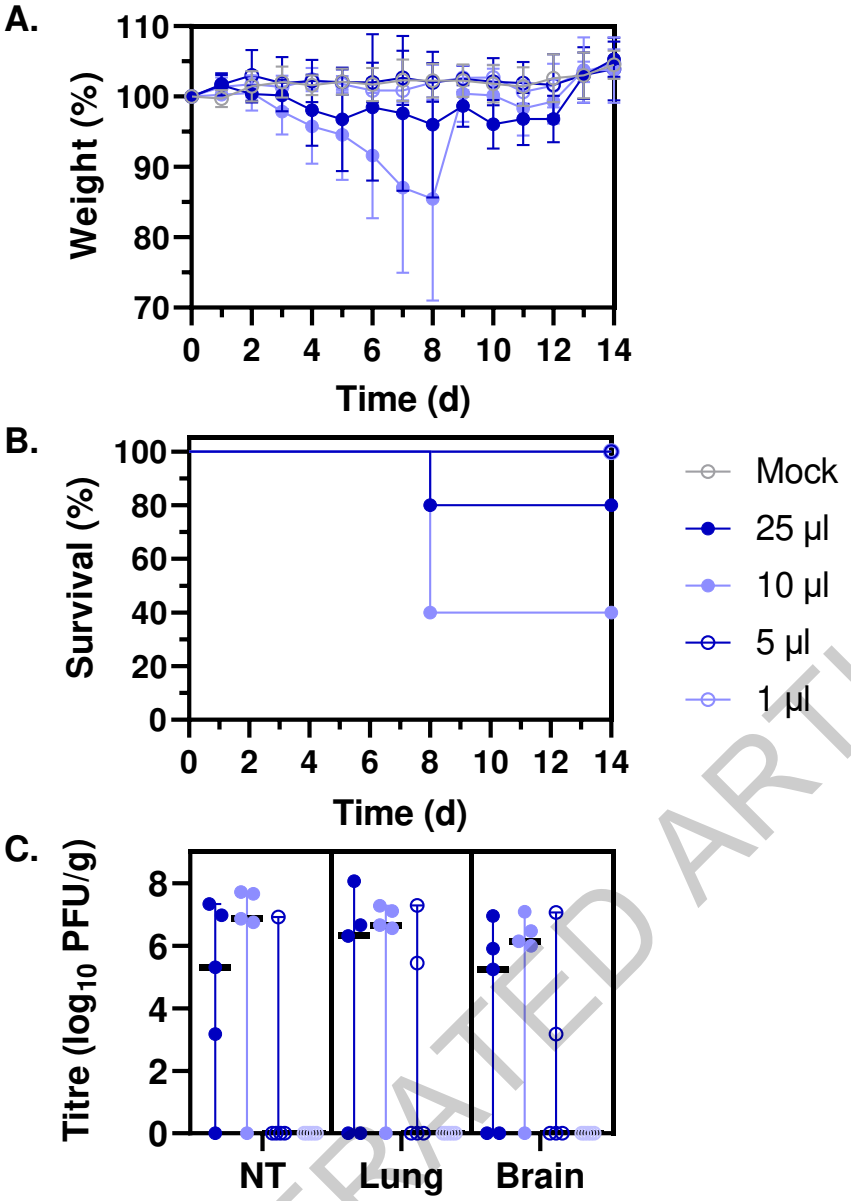
Extended Data Figure 5. Transmission of bovine H5N1 virus from lactating female mice to adult contacts. Lactating female BALB/cJ mice (10—12 weeks old) were deeply anaesthetized, intranasally inoculated with 10^2 PFU of A/dairy cattle/New Mexico/A240920343-93/2024 (H5N1; 'Cow-H5N1'), and then co-housed with adult female BALB/cJ mice (n=3biologically independent animals per lactating female). At day 4 (n=1 biologically independent lactating female) (**A**), day 7 (n=4 biologically independent lactating females) (**B**), or day 9 (n=4 biologically independent lactating females) (**C**) post-infection, lactating females and adult contacts were euthanised and tissues were collected for plaque assays in MDCK cells. Milk was collected from 3 of 4 lactating females on day 7 post-infection and all four lactating females on day 9 post-infection and tested by plaque assays in MDCK cells. In the figure, each box represents one cage with a lactating female and the associated adult contact animals. Animals for which Cow-H5N1 was detected in at least one tissue are coloured blue. At the lower left corner of each box, the status of each tissue or milk sample collected from the lactating females is indicated. Gray text indicates that no virus

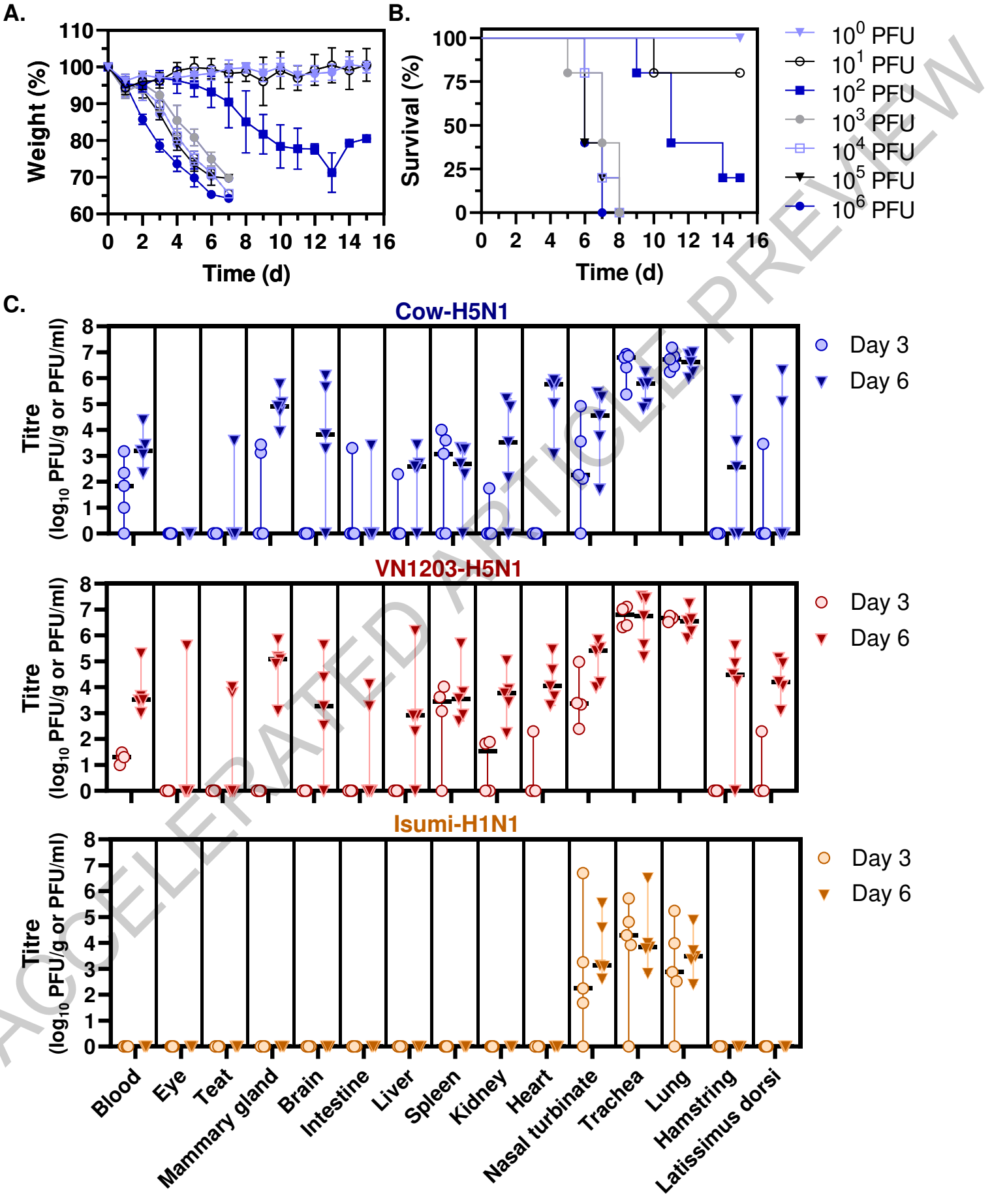
was detected, whereas red text indicates that virus was detected. Tissue abbreviations are given at the lower left of the figure.

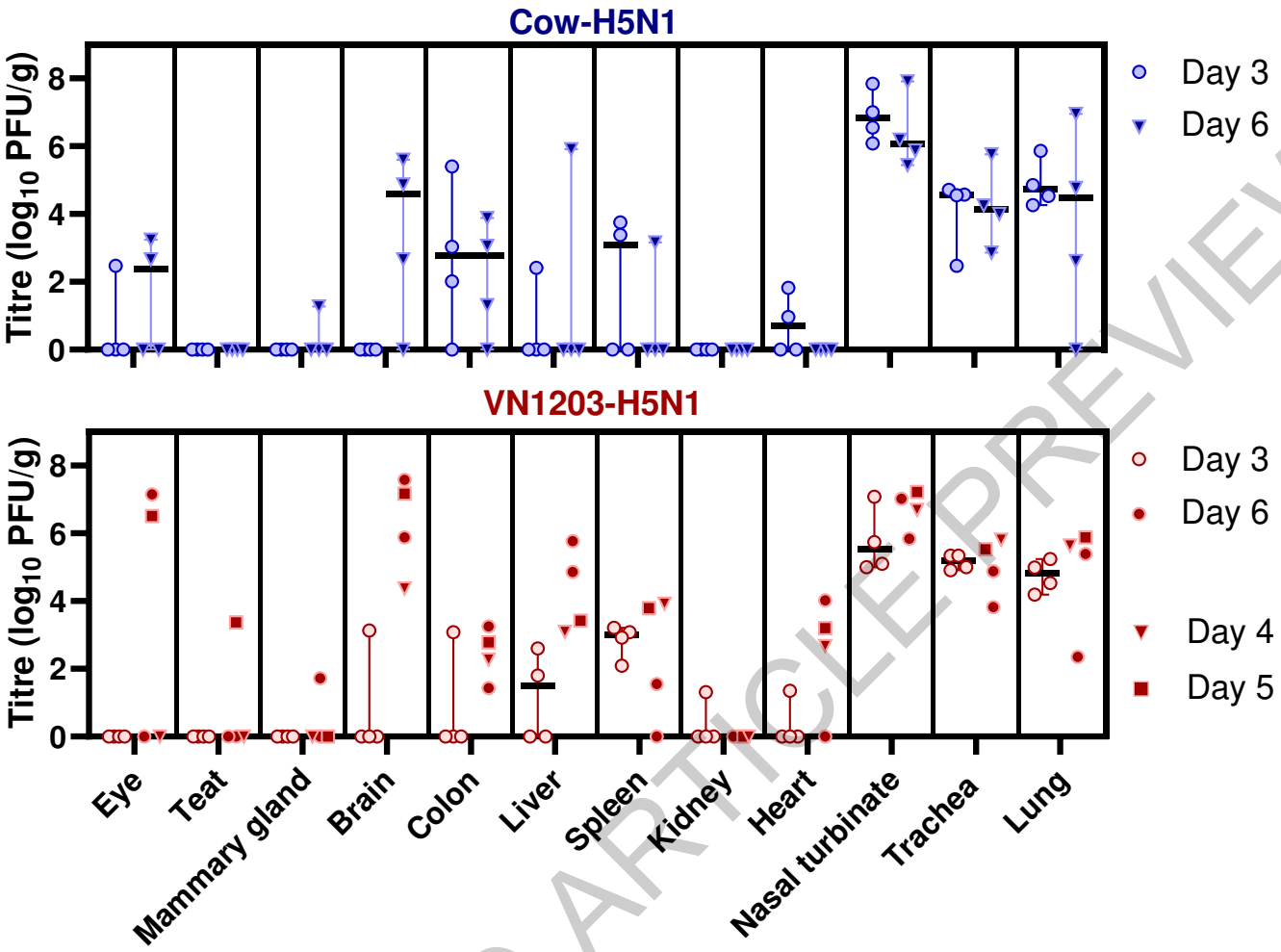
Extended Data Figure 6. Clinical data associated with ferrets used to assess respiratory droplet transmission. For the same ferrets shown in Figure 5 (n=4 biologically independent infected donor animals and n=4 biologically independent aerosol contact animals), daily body weights and body temperatures are shown. The dotted lines indicate starting weights or body temperatures. D, donor (infected) ferret; C, contact ferret.

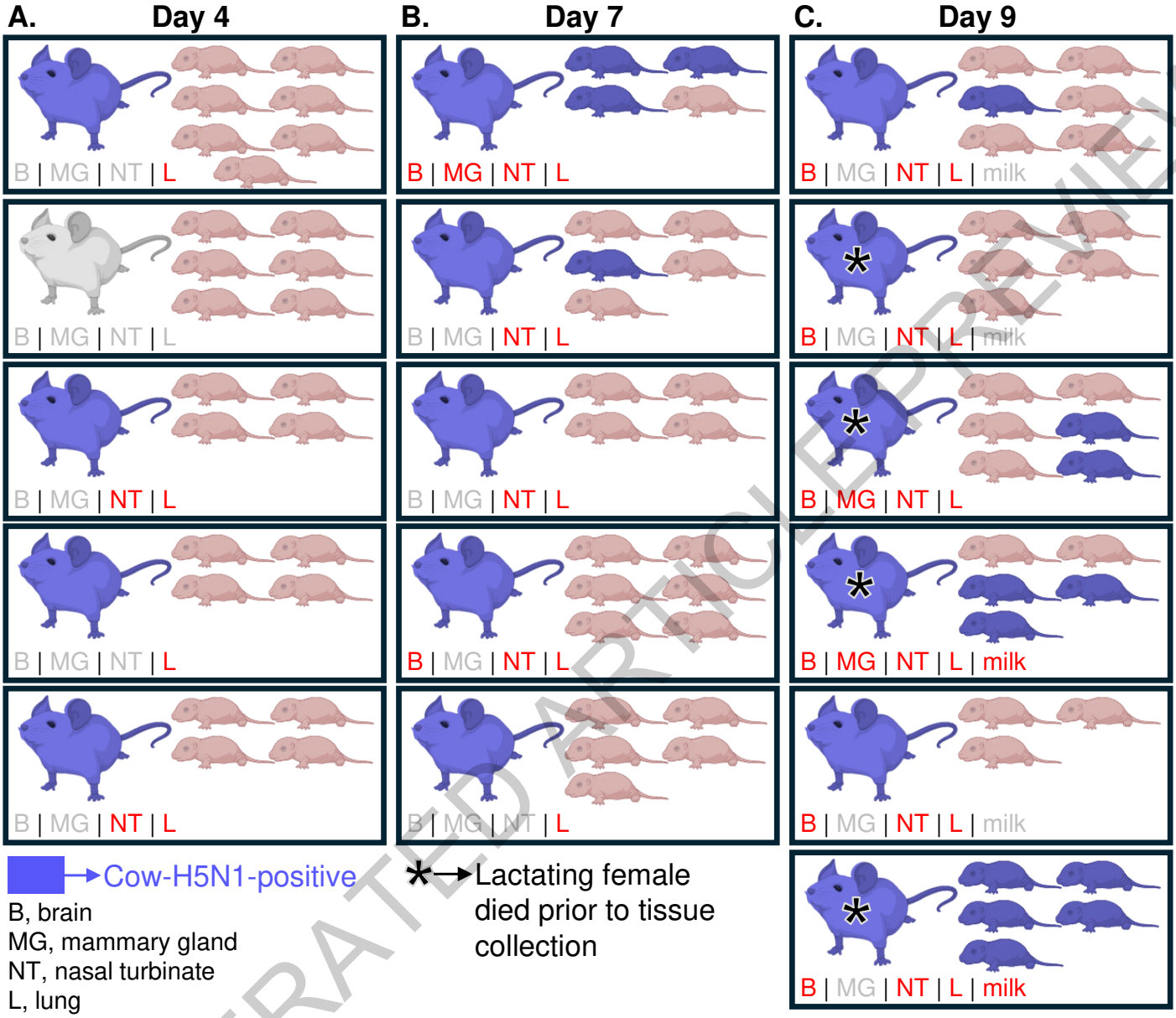
Extended Data Figure 7. Bovine H5N1 virus binds to both α2,3 and α2,6 sialic acid residues, replicate experiment 2. Four-fold serial dilutions of α2,3 and α2,6 sialylglycopolymers adhered to microtitre plates were incubated with 32 hemagglutination (HA) units of the indicated viruses or PBS (negative control). After washing, virus binding was detected by an anti-HA human monoclonal antibody (CR9114) and an HRP-conjugated secondary antibody. The absorbance values for each condition with each virus or PBS are shown. Each dot represents the mean of two biologically independent replicate values.

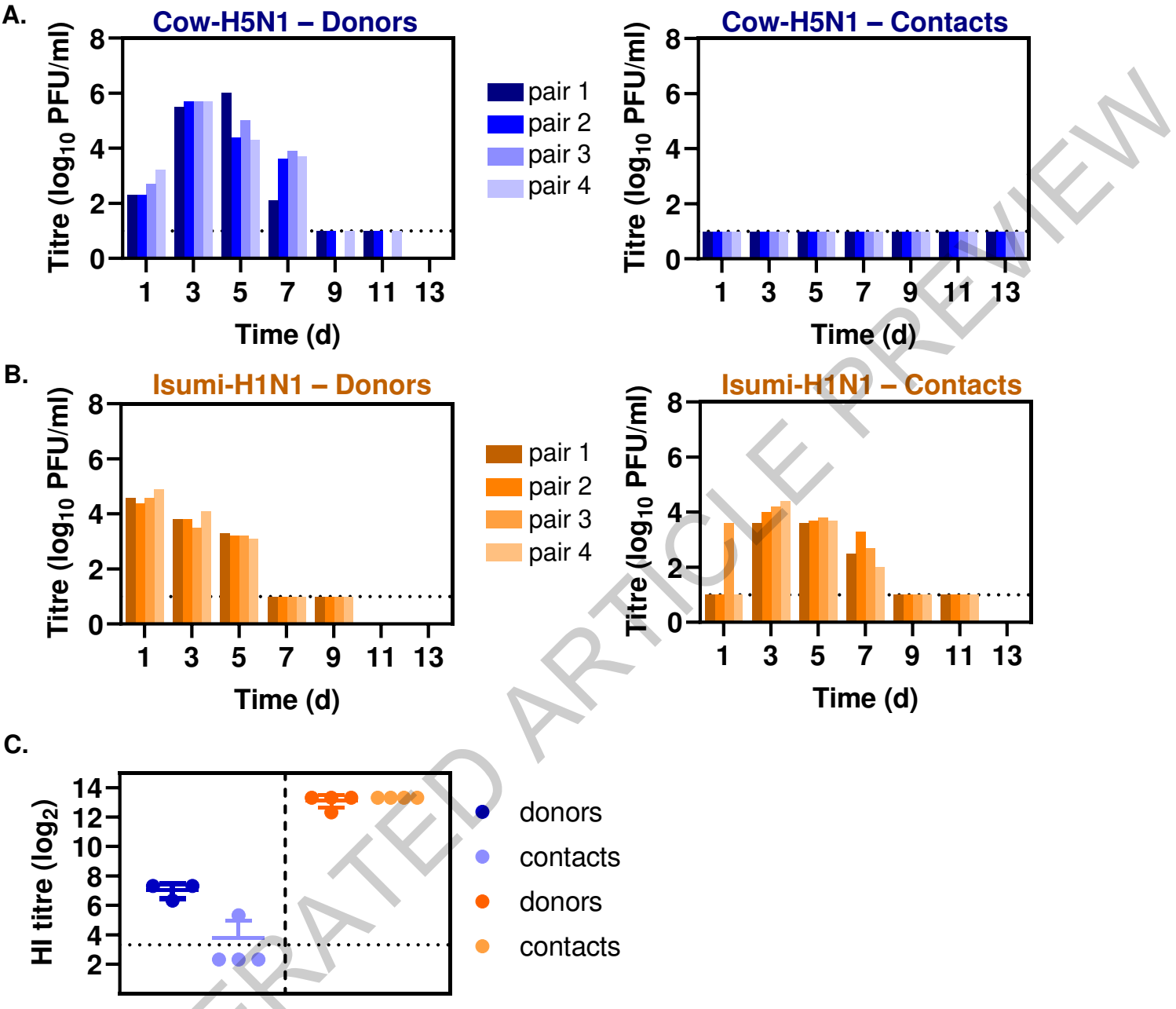
Extended Data Figure 8. Bovine H5N1 virus binds to both α2,3 and α2,6 sialic acid residues, replicate experiment 3. Four-fold serial dilutions of α2,3 and α2,6 sialylglycopolymers adhered to microtitre plates were incubated with 16 hemagglutination (HA) units of the indicated viruses or PBS (negative control). After washing, virus binding was detected by an anti-HA human monoclonal antibody (CR9114) and an HRP-conjugated secondary antibody. The absorbance values for each condition with each virus or PBS are shown. Each dot represents a single biologically independent replicate value. The dotted lines represent the average background signal.

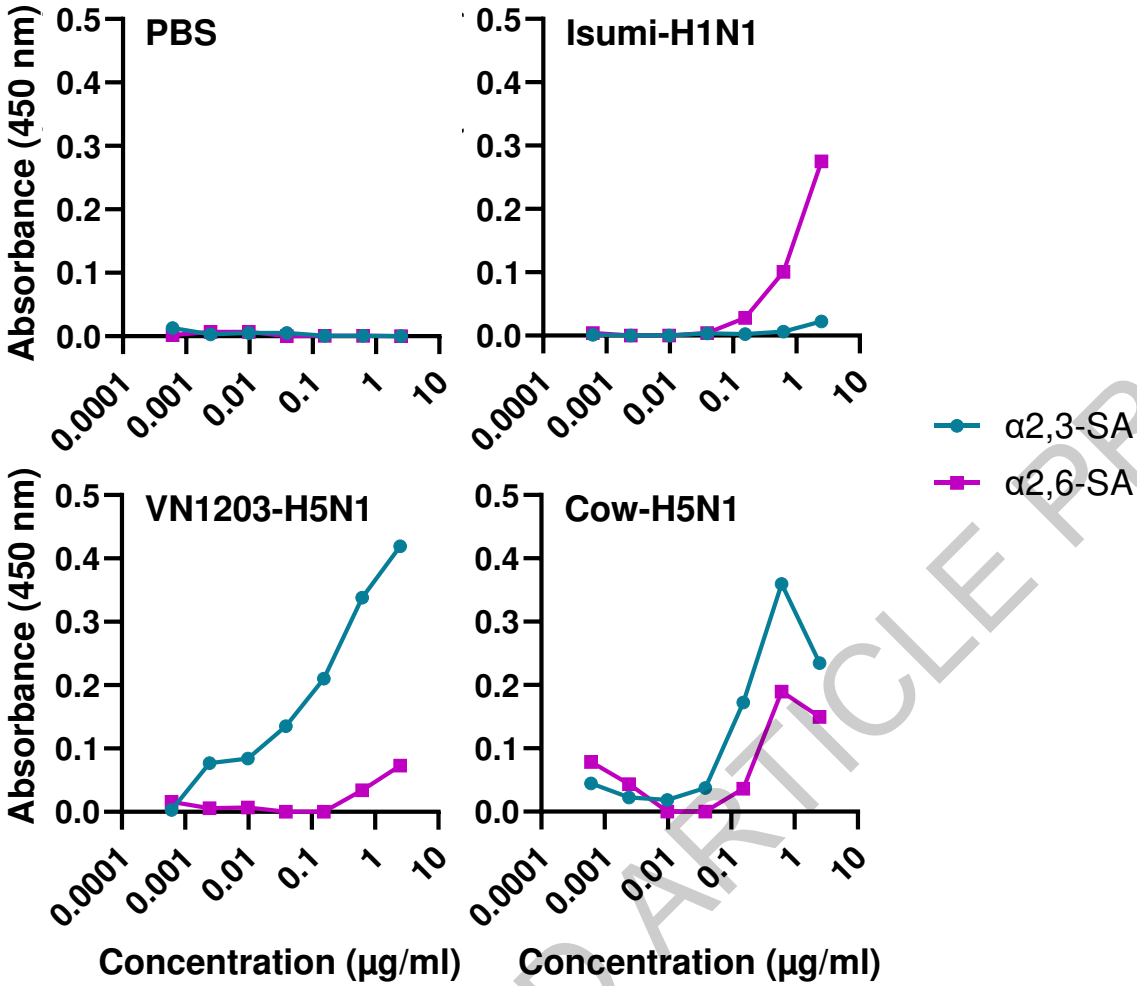




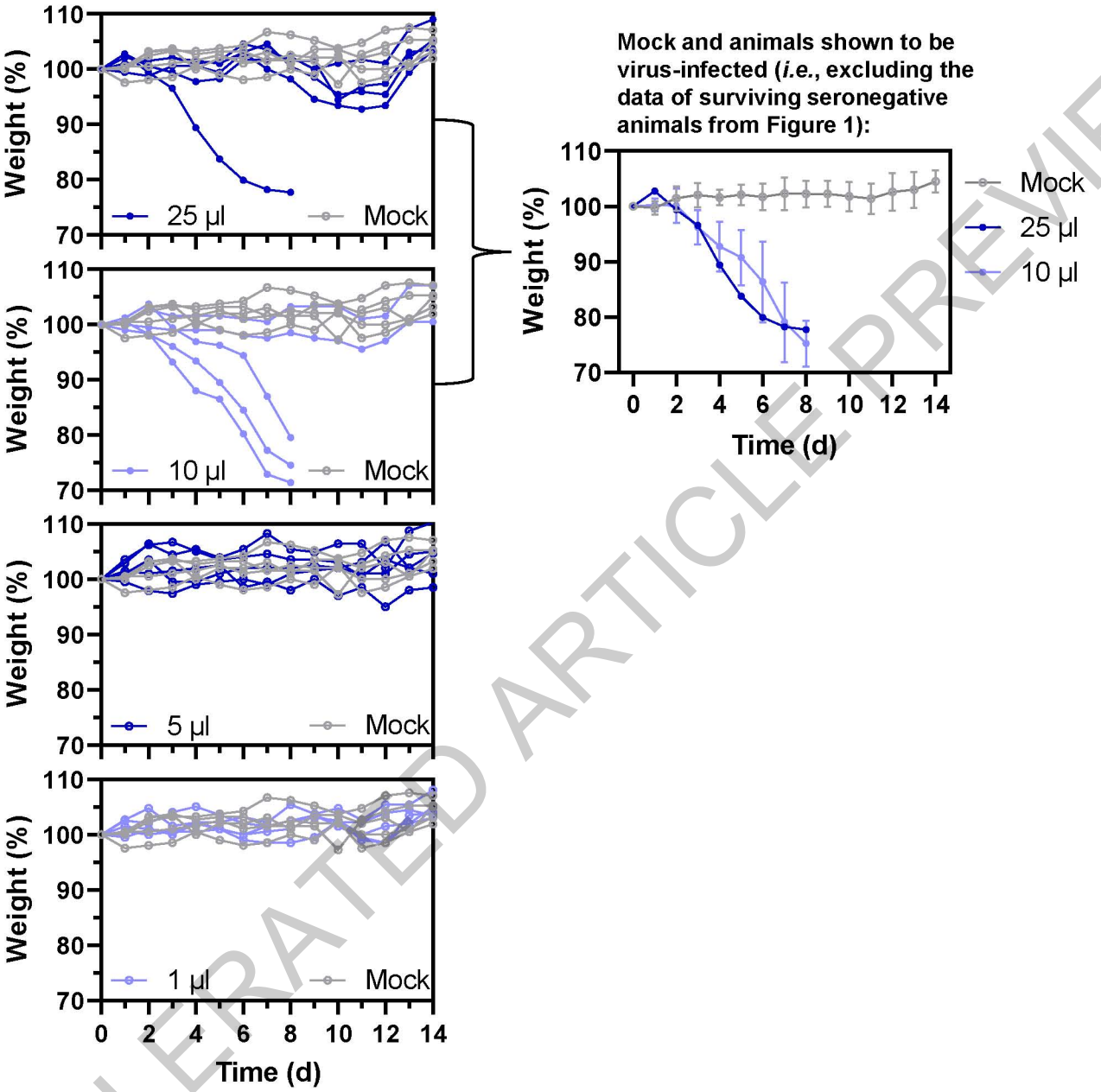




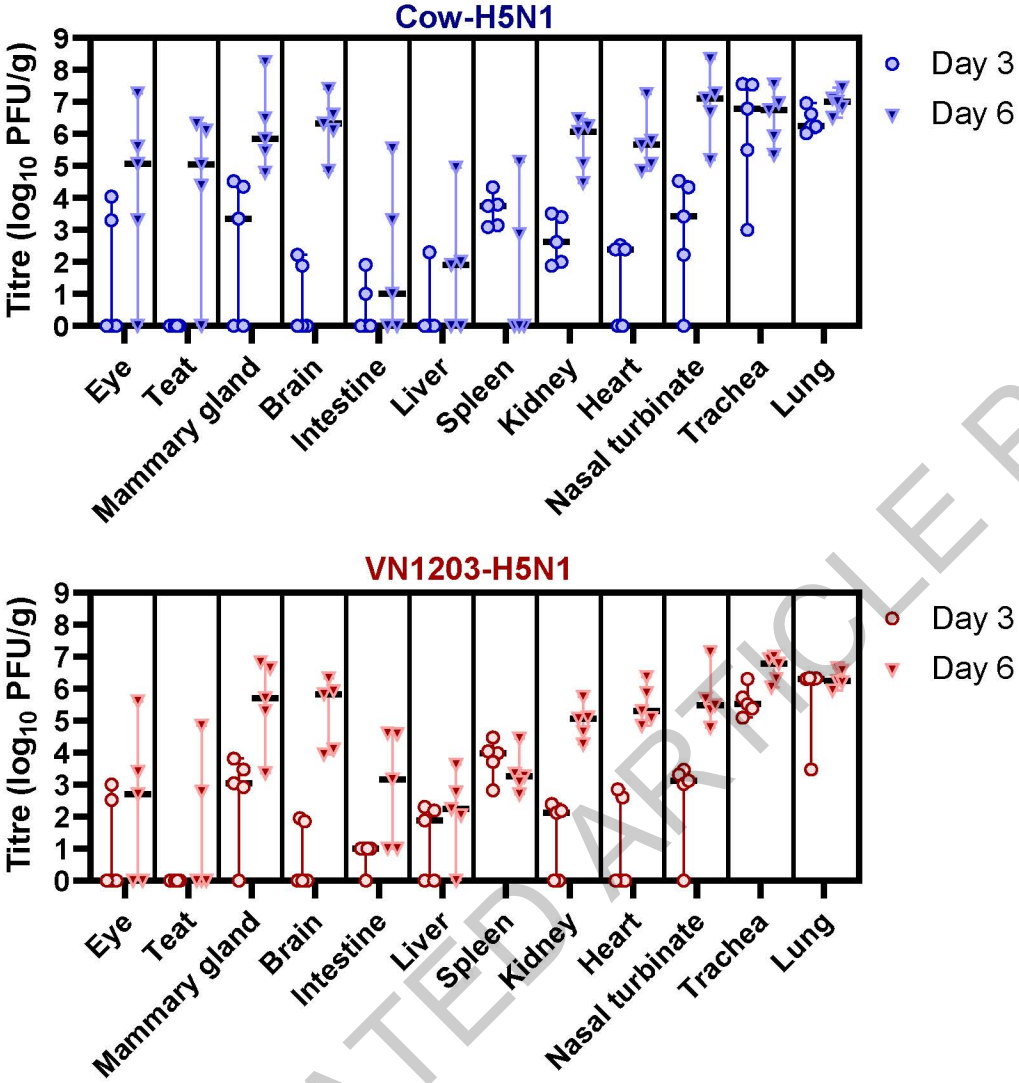




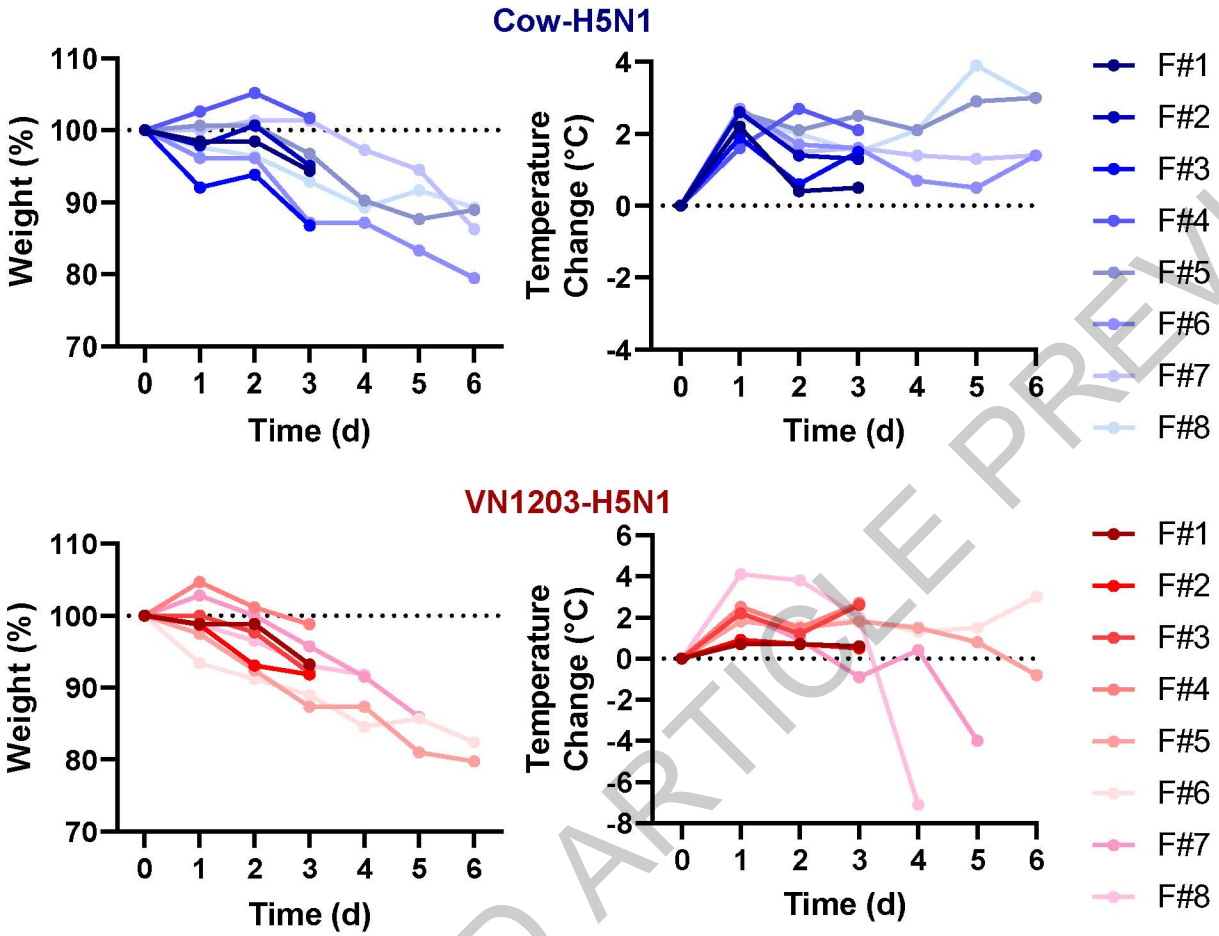
Extended Data Figure 1



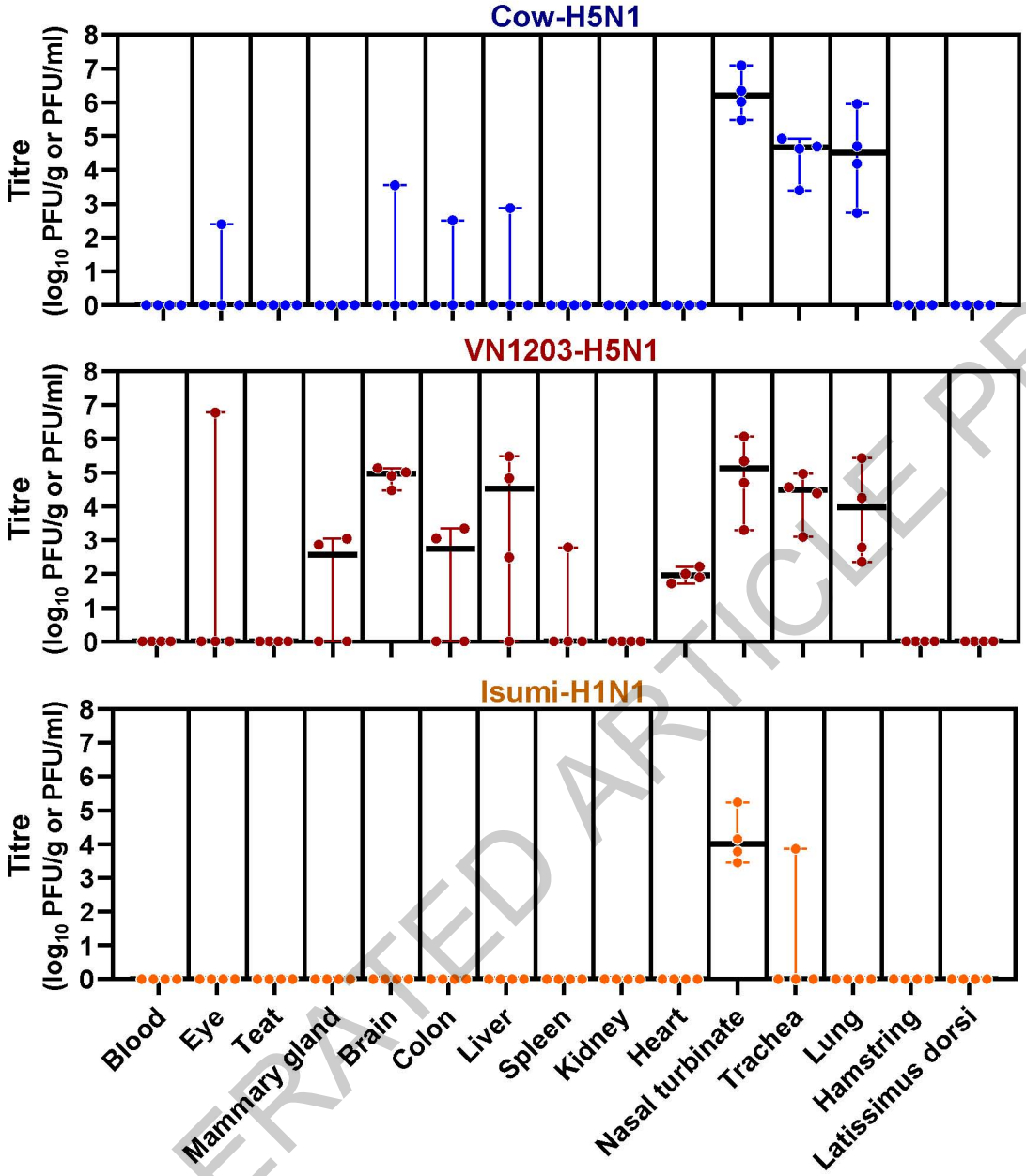
Extended Data Figure 2



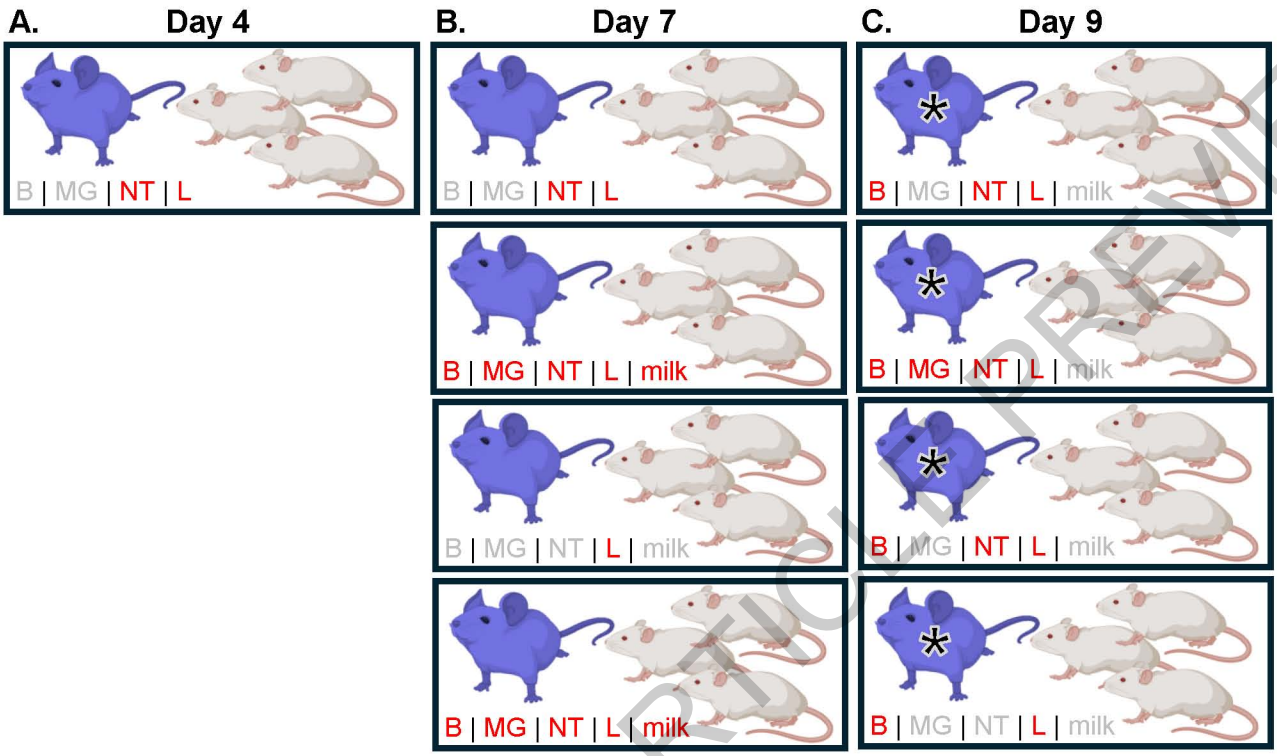
Extended Data Figure 3



Extended Data Figure 4



Extended Data Figure 5

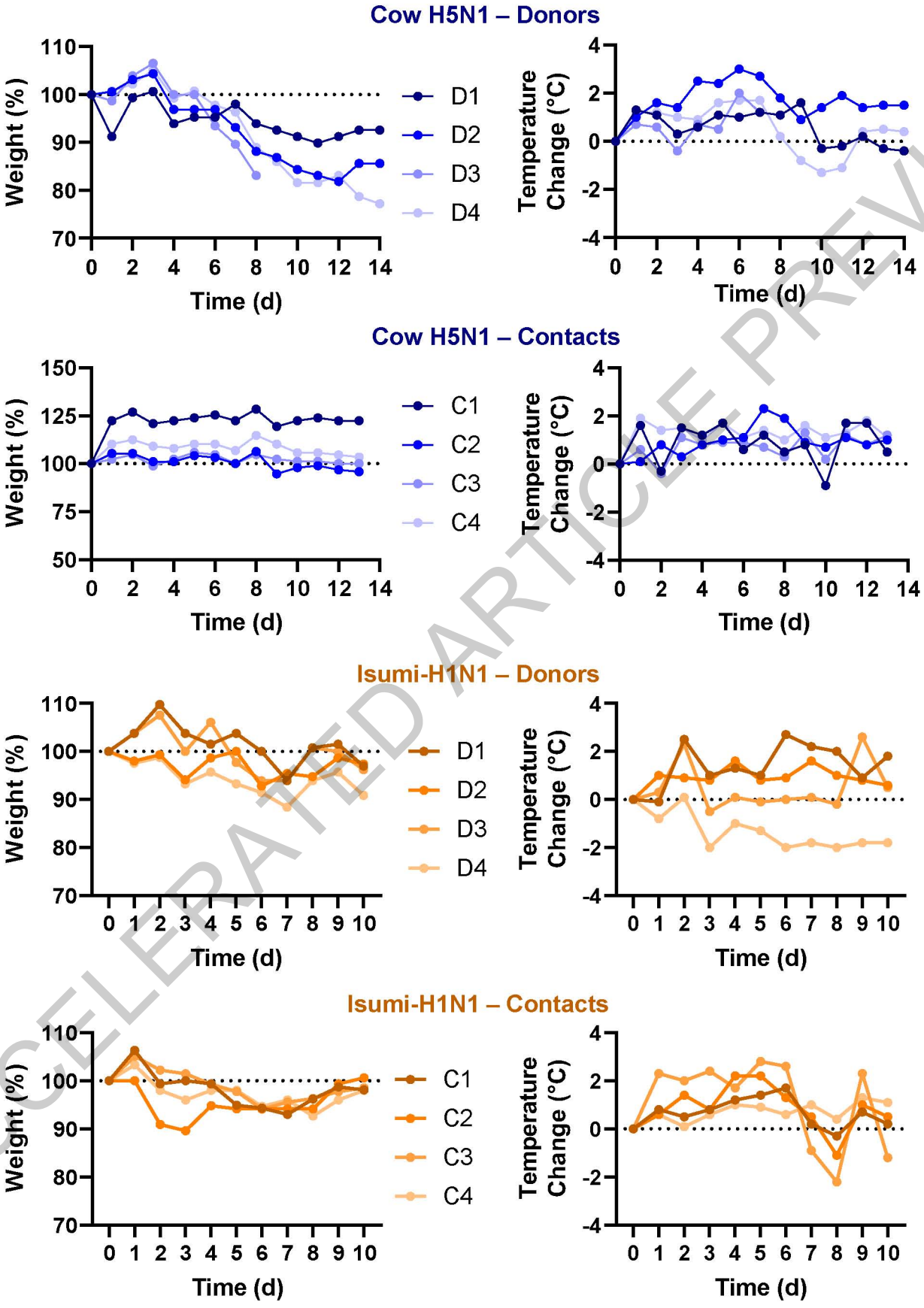


Cow-H5N1-positive

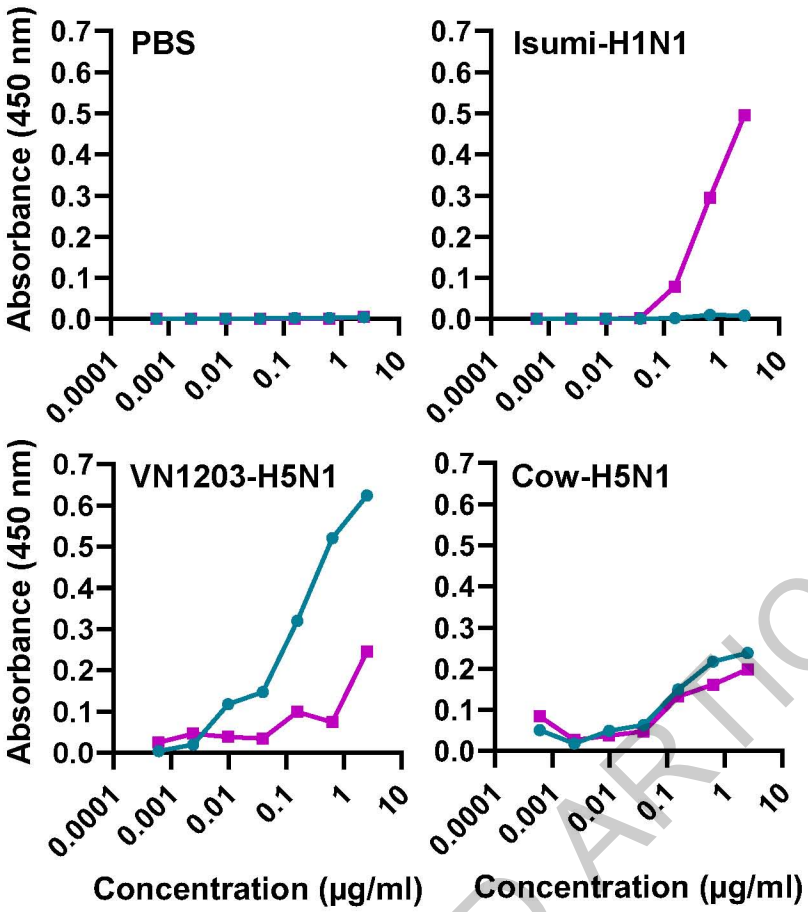
* Lactating female died prior to tissue collection

B, brain
MG, mammary gland
NT, nasal turbinate
L, lung
red text = virus identified in that tissue
gray text = no virus was detected

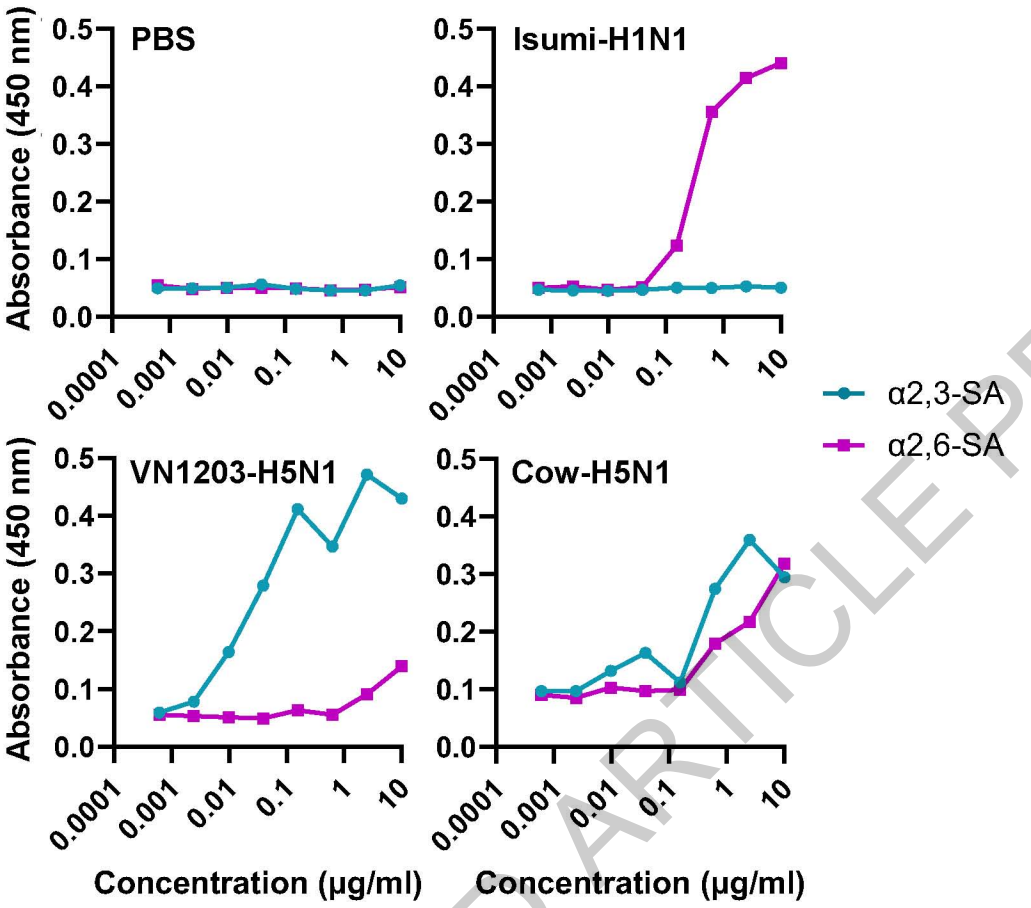
Extended Data Figure 6



Extended Data Figure 7



Extended Data Figure 8



nature portfolio

Corresponding author(s): Dr. Yoshihiro Kawaoka

Last updated by author(s): Jun 26, 2024

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Please do not complete any field with "not applicable" or n/a. Refer to the help text for what text to use if an item is not relevant to your study.
For final submission: please carefully check your responses for accuracy; you will not be able to make changes later.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used for data collection.
Data analysis	GraphPad Prism software, version 9.5.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

Source data for animal experiments is provided in the online version. Receptor binding assay data are available by request from the corresponding author.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Animal study sample size was based on previously published work and limited by space and cost. No statistical methods were used to predetermine sample sizes. In general, group sizes were large enough (n=4-6 animals per group) to enable statistical testing if desired. Small numbers of biological replicates (n=1-2) were used in receptor binding assays; however, three independent experiments were performed, and the results were reproducible across the experiments.
Data exclusions	No data was excluded from the study.
Replication	The mouse oral inoculation, mouse intranasal inoculation for MLD50, lactating mouse, and ferret transmission experiments were each performed one time. Tissue tropism experiments in mice and ferrets after intranasal inoculation were performed two times with some differences in the experimental design (i.e., the included control viruses, the tissues that were assayed, and the time points examined), as described in the Methods section. The receptor binding assay was performed three times. All replicate experiments just described are included in the figures and/or underlying data files.
Randomization	Allocation of animals was completed at random. Randomization was not employed for receptor binding assays since locations (on 96-well plates) of sialylglycopolymers (both the type and concentration) need to be known to interpret the data. In addition, the experiments were performed under select agent regulations which require separation of agents and clear identification.
Blinding	Blinding was not possible as the experiments were performed under select agent regulations which require separation of agents and clear identification.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used: Broadly Neutralizing Antibodies Against Influenza A And B Viruses, Fully Human, CR9114, HumImmu (catalog no. A90001; 1:1000 dilution); Goat Anti-Human IgG H&L, HRP conjugated, ab6858, abcam.

Validation: CR9114 was characterized by Dreyfus C, et al. (2012). Highly conserved protective epitopes on influenza B viruses.Science. 2012 Sep 14;337(6100):1343-8.
Abcam validated ab6858 for ICC/IF, Dot blot, ELISA, IHC-P, IHC-Fr, Immunomicroscopy, WB. Additional validation can be found in these literature: https://www.citeab.com/antibodies/2361176-ab6858-goat-anti-human-igg-h-l-hrp.

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s): Madin-Darby canine kidney cells, originally sourced from the ATCC, were used in this study

Authentication: None of the cell lines were authenticated

Mycoplasma contamination: All cell lines tested negative for mycoplasma contamination

Commonly misidentified lines (See ICLAC register): No commonly misidentified cell lines were used in the study.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals: Mus musculus (Mouse), Balb/cJ, 6- to 12-week old females.
Mustela furo (Ferret), 4- to 6-month old females
10-day-old embryonated chicken eggs

Wild animals: The study did not involve wild animals.

Reporting on sex: No sex-based analysis was performed as studies were limited to animals capable of lactating.

Field-collected samples: The study did not involve samples collected from the field.

Ethics oversight: Institutional Care and Use Committees of the University of Wisconsin (UW)-Madison School of Veterinary Medicine.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks: N/A

Novel plant genotypes: N/A

Authentication: N/A