Article

H5N1 clade 2.3.4.4b dynamics in experimentally infected calves and cows

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In March 2024, highly pathogenic avian influenza virus (HPAIV) clade 2.3.4.4b H5N1 infections were reported in dairy cows in Texas, USA[1]. Rapid dissemination to more than 380 farms in 14 states followed[2]. Here we provide results of two independent clade 2.3.4.4b experimental infection studies evaluating the oronasal susceptibility to and transmission of a US H5N1 bovine isolate, genotype B3.13 (H5N1 B3.13), in calves, and the susceptibility of lactating cows following direct mammary gland inoculation of either H5N1 B3.13 or a current EU H5N1 wild bird isolate, genotype euDG (H5N1 euDG). Inoculation of the calves resulted in moderate nasal replication and shedding with no severe clinical signs or transmission to sentinel calves. In dairy cows, infection resulted in no nasal shedding, but severe acute infection of the mammary gland with necrotizing mastitis and high fever was observed for both H5N1 isolates. Milk production was rapidly and markedly reduced and the physical condition of the cows was severely compromised. Virus titres in milk rapidly peaked at 10^9 50% tissue culture infectious dose ($TCID_{50}$) per ml, but systemic infection did not ensue. Notably, the adaptive mutation E627K emerged in the viral polymerase basic protein 2 (PB2) after intramammary replication of H5N1 euDG. Our data suggest that in addition to H5N1 B3.13, other HPAIV H5N1 strains have the potential to replicate in the udder of cows and that milk and milking procedures, rather than respiratory spread, are likely to be the primary routes of H5N1 transmission between cattle.

Epidemic occurrence of HPAIV of subtype H5 has recently developed into a panzootic disease with dynamic spread into an expansive number of host species[3–9]. In 2021, A/H5N1 clade 2.3.4.4b virus crossed the Atlantic and rapidly spread through wild bird and commercial poultry populations in the Americas[1,5,10]. Subsequent reports of sporadic mammalian infections have become more frequent, with data suggestive of mammal-to-mammal transmission chains in South American seals since 2023[11,12].

Historically, natural infections of cattle with influenza A virus (IAV) are not well documented[13] despite rare detection of IAV seropositive cattle[14]. However, in March 2024, an outbreak of HPAIV H5N1 was reported in dairy cows in Texas, caused by the novel B3.13 genotype, a reassortant of an ancestral European clade 2.3.4.4b virus and North American wild bird AIVs[1]. Phylogenetic analyses of whole-genome sequences recovered from wild birds, poultry and mammals suggest a single spillover event into cattle, with the time to the most recent common ancestor indicating that introduction occurred in late 2023 or early 2024[15,16]. Current epidemiological data suggest that subsequent inter-farm spread is mainly associated with unknowingly transporting infected cows[1]. As of 29 October 2024, 388 dairy cattle farms in 14 US states have been affected[2].

In the field, high level H5N1 B3.13 replication has been reported in the mammary gland of infected cows, resulting in high-titre virus shedding in milk accompanied by mastitis, a massive drop in milk production and limited reports of respiratory disease[1,17]. The susceptibility and rapid viral replication of HPAIV in the mammary gland are consistent with the evidence of highly abundant α2,3-linked sialic acid receptors in the bovine udder[18]. A novel PB2 substitution (M631L) accompanied the switch from avian to bovine hosts as a marker mutation[1,15].

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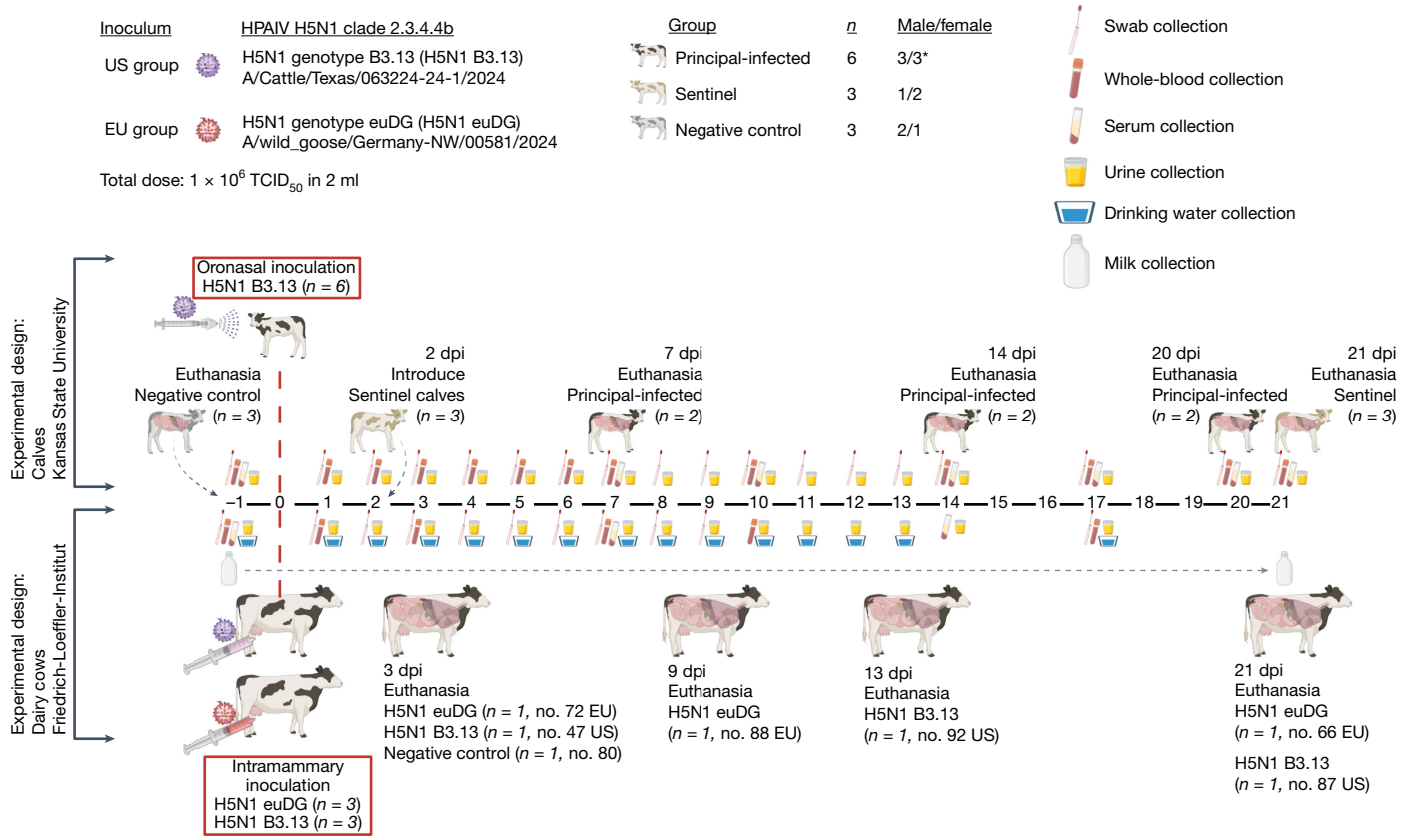

Fig. 1 | Experimental design for infection with HPAIV H5N1 clade 2.3.4.4b isolates. Timeline of the experimental study. Top, 12 Holstein calves of mixed sex (the asterisk indicates that one calf was hermaphroditic) were allocated to three experimental groups: (1) principal-infected ($n = 6$; 2 female, 3 male, 1 hermaphrodite); (2) sentinel ($n = 3$; 2 female, 1 male); (3) negative control ($n = 3$; 1 female, 2 male). Negative control calves were euthanized prior to experimental infection and tissues were collected for baseline comparison. Principal-infected calves were oronasally inoculated with 1×10^6 $TCID_{50}$ per calf of H5N1 B3.13. Sentinel calves were introduced 48 h post infection. Rectal temperatures and clinical samples, including whole blood and urine, and nasal, oral and rectal swabs, were collected daily for 7 (whole blood) or 14 dpi and every 3 days thereafter. Serum was collected at 0, 7, 10, 14, 17 and 20 or 21 dpi. Postmortem examinations and extensive tissue collections were performed on days 7 ($n = 2$, principal-infected), 14 ($n = 2$, principal-infected) and 20 or 21 ($n = 2/3$, principal-infected/sentinel) post infection. Bottom, 7 Holstein–Friesian multiparous lactating dairy cattle were used in this experiment. Three cows were inoculated in the mammary gland with $10^{5.9}$ $TCID_{50}$ per cow of H5N1 B3.13 (A/Cattle/Texas/063224-24-1/2024 (US group), $n = 3$) and three cows were inoculated in the mammary gland with $10^{6.1}$ $TCID_{50}$ per animal of H5N1 euDG (A/wild_goose/Germany-NW/00581/2024 (EU group), $n = 3$). One cow served as a negative control. Swab samples (nasal, conjunctival and rectal) were taken daily until 9 dpi. EDTA blood samples were taken from individual cattle at 1, 3, 7 and 10 dpi. Urine was taken regularly until 14 dpi. Serum samples were obtained at 7 and 14 dpi and on the day of euthanasia. One cow of each group (no. 47 US and no. 72 EU) reached the humane endpoint at 3 dpi, one further cow reached it at 9 dpi (no. 88 EU) and one additional cow reached it at 13 dpi (no. 92 US); two cows (no. 66 EU and no. 87 US) survived until 21 dpi; All of these were analysed by necropsy. Figure created with BioRender.com under agreement number YH275PUF4T.

Spillover of bovine-origin B3.13 into several mammalian hosts (such as racoons and cats) has been reported[1,19], and spillback into domestic and wild avian species with maintenance of bovine adaptations has been sporadically observed[15]. Recent human cases of H5N1 have also been directly linked to workers following contact with affected cattle or poultry farms, causing conjunctivitis and conjunctival haemorrhage[20]. Accordingly, the current series of outbreaks in US cattle presents several urgent and unanswered questions: (1) Is the B3.13 genotype able to replicate in the bovine respiratory tract with viral shedding capable of onward transmission? (2) At what timepoint after infection do cattle produce IAV-specific neutralizing antibodies? (3) Is the mammary gland also permissive for infection with other H5N1 clade 2.3.4.4b strains? (4) What is the clinical presentation, and what is the duration of virus shedding in milk? And (5) does an H5N1 infection of the mammary gland lead to systemic spread?

Here we performed two independent in vivo experiments to investigate the clinical outcome, pathogenicity, transmission and tissue tropism of H5N1 clade 2.3.4.4b in calves and multiparous lactating cows. Calves ($n = 6$) were oronasally inoculated with H5N1 B3.13[9] and co-housed with sentinel calves ($n = 3$), with additional calves serving as negative controls ($n = 3$). The same virus isolate was used for an intramammary inoculation of lactating cows ($n = 3$). For comparison, three additional lactating cows were inoculated with an EU genotype euDG H5N1 clade 2.3.4.4b wild bird virus isolate (H5N1 euDG). One lactating cow served as a negative control.

Mild clinical presentation in calves

Twelve healthy Holstein calves were enrolled in this study and allocated into three experimental groups: principal-infected calves ($n = 6$; 3 male, 2 female and 1 hermaphrodite); sentinel calves ($n = 3$; 1 male, 2 female); and negative control calves ($n = 3$; 2 male and 1 female). Six principal-infected calves were oronasally inoculated with 1×10^6 50% tissue culture infectious dose ($TCID_{50}$) per calf of a virus suspension of H5N1 B3.13 (A/Cattle/Texas/063224-24-1/2024, GISAID accession number: EPI_ISL_19155861). Two days post infection, sentinel calves were co-mingled with principal-infected calves (Fig. 1). All calves were monitored daily for clinical signs and clinical samples were collected at regular timepoints (Fig. 1).

Throughout the 21-day study period, signs of mild respiratory illness were occasionally observed in calves, including nasal mucus secretions (no. 712 at 2 days post infection (dpi); no. 754 at 8 and 9 dpi; and no. 6772 at 2 and 6 dpi) and coughing (no. 6772 at 2 dpi; and no. 754, persistent

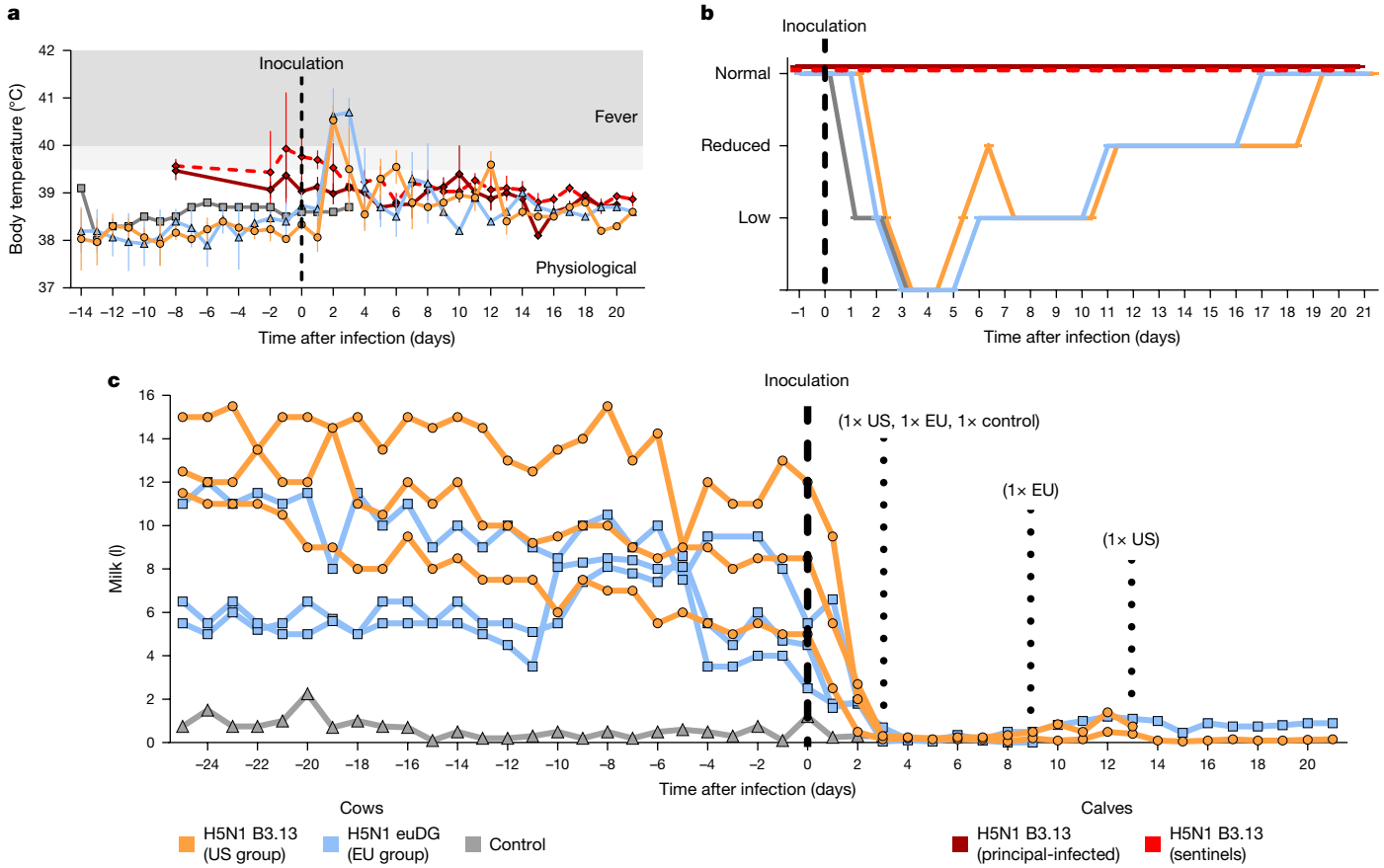

Fig. 2 | Clinical features of calves and lactating cows infected with influenza A/H5N1 clade 2.3.4.4b virus. a, Rectal temperature of principal-infected ($n = 6$) and sentinel ($n = 3$) calves and lactating cows (EU group, $n = 3$; US group, $n = 3$; control, $n = 1$) prior to and following inoculation. Data are shown as mean ± s.d. **b**, Average feed intake of each group of calves and cows following H5N1 infection. **c**, Individual milk production of lactating cows prior to and following experimental infection. Milk production of individual cows was tracked daily from −25 dpi until the end of the experiment at 21 dpi. The control animal never produced high amounts of milk. Dotted lines indicate when cows were euthanized.

from 2 dpi until euthanasia). Rectal temperatures generally remained within normal range (Fig. 2a), and no other clinical signs consistent with acute illness or consistent with clinical signs reported in affected dairy cattle in the US were observed. All calves maintained normal appetite (measured by feed intake) and normal activity levels (Fig. 2b).

Severe clinical presentation in lactating cows

Three multiparous Holstein–Friesian cows late in lactation were inoculated by the intramammary route with 2 ml (0.5 ml per teat) of a virus suspension of H5N1 B3.13 (US group), containing $10^{5.9}$ $TCID_{50}$. Three additional cows were similarly inoculated with $10^{6.1}$ $TCID_{50}$ per 2 ml (0.5 ml per teat) of a virus suspension of H5N1 euDG[21] (EU group). One cow was inoculated with 2 ml NaCl and served as a negative control (Fig. 1).

Intramammary inoculation induced clinical disease as early as 1 dpi with impaired general condition, postural abnormalities and lethargy. All 6 inoculated cows developed fever (over 40 °C) starting at 2 dpi, further exceeding 40.5 °C in both groups (Fig. 2a). Moreover, drastically reduced feed intake was observed in both groups of H5N1-infected cows (Fig. 2b). One cow per group (no. 47 US and no. 72 EU) displayed clinical signs that met criteria for humane euthanasia at 3 dpi. These included postural and motion disorders, refusal of feed and water intake, dehydration and severe lethargy. For direct comparison, the control cow (no. 80) was also euthanized at 3 dpi. Over the next days, one additional cow from each group (no. 88 EU at 9 dpi and no. 92 US 13 dpi) deteriorated into clinical conditions meeting humane endpoint criteria (severe lethargy, postural instability, staggering and signs of respiratory distress).

Prior to infection, daily milk production from individual infected cows ranged from 3 l to 15 l (Fig. 2c). After infection, milk yields rapidly decreased by more than 90%, with only partial recovery observed in the cows remaining at 21 dpi (recovery less than 3% in no. 87 US, and up to a maximum of 25% in no. 66 EU) (Fig. 2c). Starting at 2 dpi, the milk became mucilaginous and viscous and rapidly separated into a serous and a solid fraction with visible curds (Extended Data Fig. 1a,b). Milk yields in the control cow were low prior to infection, probably owing to drying off of this cow (involution in 3 quarters) (Fig. 2c). Onset of severe mastitis was confirmed by California mastitis test (CMT) performed daily in both groups (Extended Data Figs. 2, 3 and 4a–c). Clearly positive CMT in infected cows was seen as early as 1 dpi (Extended Data Figs. 3a–c and 4a–c).

In conclusion, calves inoculated oronasally presented signs of mild respiratory illness including nasal mucus secretions and coughing, although these cannot be unequivocally associated with outcomes of H5N1 inoculation, whereas intramammary infection of dairy cattle with both clade 2.3.4.4b isolates resulted in severe clinical disease in both groups, requiring early euthanasia in some cases. Severe disease in lactating cows was accompanied by a marked reduction in milk production and obvious changes in milk quality.

Viral shedding dynamics in calves and cows

Shedding of IAV RNA was observed in 5 out of 6 principal-infected calves for a maximum of 8 days, primarily in nasal swabs (Fig. 3a). Generally, low-to-medium levels of IAV RNA were detected, with peak shedding

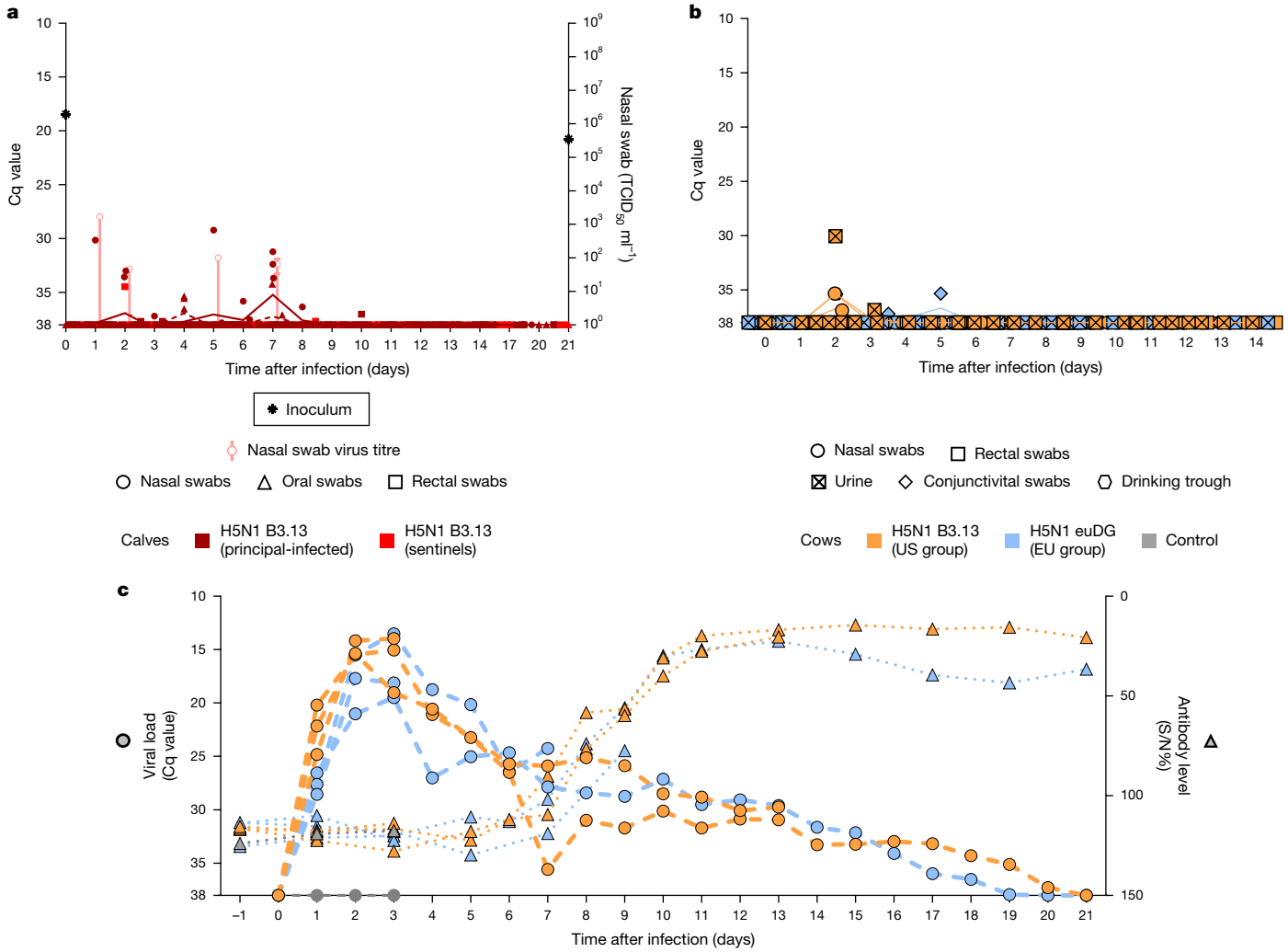

Fig. 3 | Viral shedding of influenza AH5N1 clade 2.3.4.4b virus isolates in experimentally infected calves and lactating cows. a, RT–qPCR was used for the detection of the influenza A *M* gene (left axis; scatter plot) in nasal, oral and rectal swabs collected from H5N1 B3.13 oronasally infected calves post-inoculation and sentinel calves. Viral titres of nasal swabs (right axis; bars) are represented as the average and standard deviation of positive nasal swabs on each day ($n = 3$ calves at 7 dpi). The Cq value and titre of the inoculum are shown as asterisks on their respective axes. **b**, RT–qPCR results from nasal, rectal and conjunctival swabs of lactating cows following intramammary infection with H5N1 B3.13 or H5N1 euDG. **c**, H5N1 viral genome load (left axis) and corresponding H5-specific antibody titre (right axis) in milk samples over time. All cattle were milked daily and individual pooled milk samples were analysed by RT–qPCR to detect H5N1 viral RNA. Detection of H5-specific antibodies in selected milk samples was achieved using an H5-specific enzyme-linked immunosorbent assay (ELISA) and reported as sample OD_{450} as a percentage of negative control OD_{450} (S/N%).

occurring in nasal swabs between 5–7 dpi in 3 principal-infected calves. IAV RNA was also detected in oral swabs, most frequently at 4–7 dpi, and only seldom detected as suspect-positive (cycle quantification (Cq) ≥ 35, single-replicate positive) in rectal swabs. Vaginal and preputial swabs, conjunctival swabs collected at necropsy, urine and whole blood were negative for IAV RNA throughout the study period. All clinical samples collected from sentinel calves were negative for IAV RNA except for two suspect-positive rectal swabs, attributed to environmental contamination during sample collection, suggesting that no transmission of IAV to sentinel calves occurred throughout the study period. There was no difference observed between male and female calves. Virus isolation and titration were attempted on samples with Cq ≤ 36 (Fig. 3a and Supplementary Table 1). Successful recovery of virus was achieved primarily from nasal swabs of 3 different calves at 1, 2, 5 and 7 dpi. Titres ranged from 4.64×10^1 $TCID_{50}$ ml^{-1} to 1.7×10^3 $TCID_{50}$ ml^{-1} (Fig. 3a).

In lactating cows, quantitative PCR with reverse transcription (RT–qPCR) analyses of nasal, conjunctival and rectal swabs and urine samples of cows from the US and EU groups were negative for viral RNA except for 2 nasal swab samples of no. 87 US and no. 92 US (Cq values 35 and 36, respectively) and 2 urine samples of no. 47 US and no. 92 US at 2 and 3 dpi (Cq values of 30 and 36, respectively) (Fig. 3b). By contrast, milk samples from all cows in both groups tested positive, starting at 1 dpi with peak viral genome loads in milk samples at 3 dpi, revealing Cq values ranging from 13 to 21 (Fig. 3c). Viral RNA was detectable in milk samples until 20 dpi and by 9 dpi, antibodies directed against the H5 virus were present in milk samples from each cow, increased to a maximum on 11 dpi and stayed at this level until the end of the study period (Fig. 3c and Extended Data Table 1).

Virus titration from milk samples was performed from 1–13 dpi, but was only successful (that is, infectious virus could be isolated) from 1–8 dpi, with peak titres of greater than 10^9 $TCID_{50}$ ml^{-1} (Fig. 4a). Owing to the milk composition, it was not possible to determine accurate virus titres from 9 dpi onwards. Virus titration from mammary gland homogenates reached peak titres of around 10^6 $TCID_{50}$ ml^{-1} in cows euthanized at 3 dpi (Fig. 4b). Nevertheless, infectious virus could still be isolated from the udder of cows euthanized at 9 or 13 dpi (Fig. 4b). Sequencing of viral RNA from mammary gland tissue and milk samples

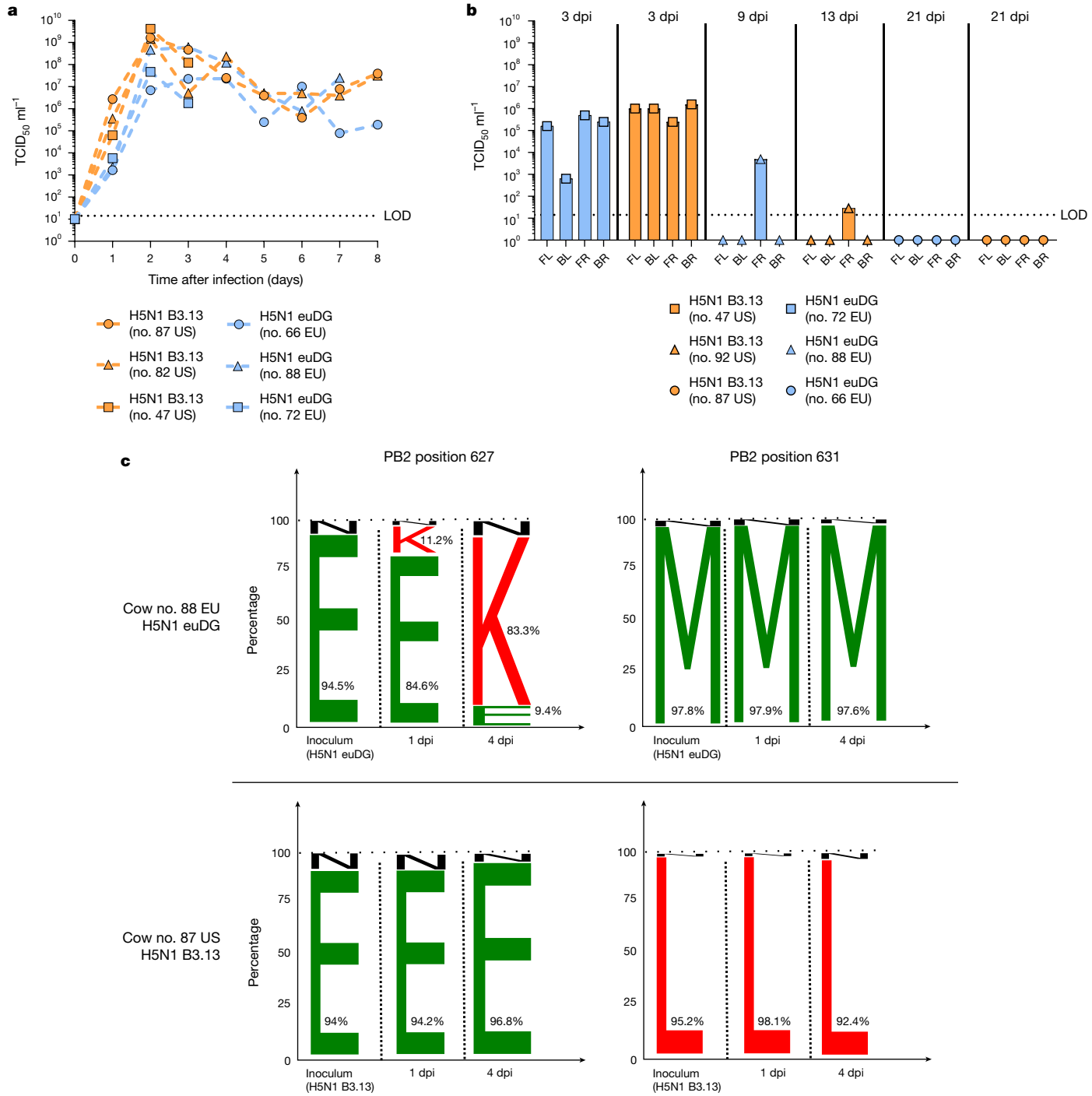

Fig. 4 | Infectious virus yields in milk and udder tissue of H5N1-infected lactating cows and genetic adaptation over time. a, H5N1 viral titres recovered from milk samples of individual H5N1 B3.13- and H5N1 euDG-infected lactating cows. LOD, limit of detection. **b**, Viral titre of H5N1 infectious viral particles from individual udder quarters (FL, front left; BL, back left; FR, front right; BR, back right) collected at their euthanasia timepoint. **c**, Genetic adaptation at position 627 and 631 in PB2 of H5N1 B3.13 and H5N1 euDG. The sequence logo plot displays the relative proportion of amino acids present at positions 627 and 631 of PB2 from H5N1 B3.13 and H5N1 euDG in milk sampled from cows no. 88 EU and no. 87 US at indicated timepoints.

revealed the emergence of the amino acid substitution E627K in the viral PB2 protein in all three cows after infection with the H5N1 euDG (milk sample of no. 66 EU on day 4, 20% PB2(E627K); milk sample of no. 88 EU on day 4, 83.3% PB2(E627K); udder organ sample of no. 72 on day 3, 89% PB2(E627K)) (Fig. 4c). Detection of minor variants at this position from samples of milk from day 1 to day 4 post infection showed that this mutation was acquired early after infection, as it was not detected in the inoculum (Fig. 4c). By contrast, in the H5N1 B3.13-infected cows, the marker mutation PB2(M631L) was maintained and PB2 E627 remained unaltered (Fig. 4c).

The high viral loads in milk samples provided an additional opportunity to validate H5-antigen detection using rapid antigen tests (RATs) (Extended Data Fig. 5). Two out of three H5N1 B3.13-infected cows (no. 87 US and no. 92 US) tested positive in an AIV-specific RAT at 1 dpi (Extended Data Fig. 5a). In RATs, all H5N1-inoculated cows tested positive by 2 dpi (Extended Data Fig. 5b,c) and negative at 10 dpi (Extended

Data Fig. 5d), consistent with increasing antibody levels in milk from 7 dpi onwards (Fig. 3c).

In summary, oronasal H5N1 B3.13 inoculation in calves resulted in moderate levels of nasal shedding in five out of six principal-infected calves for a maximum of eight days independent of sex, without any evidence for transmission to sentinels. In lactating cows, milk samples obtained from both experimental groups contained high infectious viral loads with viral shedding up to 8 dpi and viral genome detected up to 20 dpi, providing the first evidence for susceptibility of dairy cows to two H5N1 clade 2.3.4.4b viruses belonging to different genotypes from separate continents.

Localized viral replication in calves and cows

Out of nearly 40 organ samples collected from oronasally inoculated calves euthanized at 7, 14 or 20 dpi, IAV RNA was detected only in mucosa-associated lymphoid tissue (retropharyngeal lymph node, palatine tonsil, nasopharyngeal tonsil and suppuration from palatine tonsil) of one principal-infected calf (no. 712) at 7 dpi (Supplementary Table 1). All other liquid, swab and tissue samples (visceral and lymphoid tissues), including lung tissues and bronchoalveolar lavage fluid collected from principal-infected and sentinel calves at 7, 14, 20 and 21 dpi were negative for IAV RNA (Supplementary Table 2). Infectious virus was recovered only from the palatine tonsil suppuration collected postmortem at 7 dpi.

RT–qPCR analysis of tissues collected from lactating cows euthanized at 3 dpi revealed peak viral RNA loads in mammary glands, with Cq values of 20 (no. 47 US) and 25 (no. 72 EU) (Extended Data Fig. 6a). At 9 (no. 88 EU) and 13 (no. 92 US) dpi, viral genome loads in mammary glands were slightly lower than in mammary gland samples collected at 3 dpi and were undetectable at 21 dpi (no. 66 EU and no. 87 US) (Extended Data Fig. 6b and Fig. 4b). H5N1 viral RNA was also detected at low levels in both groups in neuronal and other tissues at 3 dpi (for example, no. 47 US: spinal cord, Cq = 28; cerebrum, Cq = 36; nervus genitofermoralis Cq = 31; no. 72 EU: nervus genitofermoralis Cq = 34) (Extended Data Fig. 6c,d). Organ samples of the respiratory tract were negative in all cows at their euthanasia timepoints (Extended Data Fig. 6e). Additionally, there was no IAV RNA detected in whole blood or peripheral blood mononuclear cells collected from oronasally inoculated calves or cows inoculated in the mammary gland.

In summary, low levels of IAV RNA were detected only in mucosa-associated lymphoid tissue of the upper respiratory tract in 1 out of 2 oronasally infected calves at 7 dpi. No IAV RNA was detected in regular ante-mortem whole blood samples or in samples collected post-mortem from major organs, lymphoid tissue, swabs or fluids from principal-infected and sentinel calves, suggesting the absence of a viremic phase of the infection. Similarly, intramammary infection of lactating dairy cattle with two distinct genotypes of clade 2.3.4.4b viruses remained restricted to the mammary gland, and no evidence of systemic spread was observed.

Pathology confirms localized infections

Gross pathology for calves is presented in Extended Data Fig. 7. Histological changes in calves oronasally infected with bovine H5N1 at 7 and 14 dpi are depicted in Fig. 5. At 7 dpi, there was a suppurative tracheitis in one calf (no. 6760) with degenerate neutrophils filling the tracheal lumina. The second calf (no. 712) displayed discrete foci of fibrinous interstitial pneumonia with fibrin filling regional alveolar spaces and mild numbers of neutrophils, macrophages and lymphocytes expanding alveolar septa and minimal peribronchiolar inflammatory cells of associated terminal bronchioles at 7 dpi. No viral antigen was detected by immunohistochemistry (IHC) in these samples (Fig. 5b,d). At 14 dpi, the bronchioles of one calf (no. 754) were lined by hyperplastic epithelium, filled with degenerate neutrophils and partially occluded by papillary projections composed of a core of fibrous connective tissue with few inflammatory cells and lined by bronchiolar epithelium (bronchiolitis obliterans). Bronchioles were also frequently delimited by prominent lymphoid aggregates (bronchus-associated lymphoid tissue (BALT) hyperplasia); however, no viral antigen was detected (Fig. 5f).

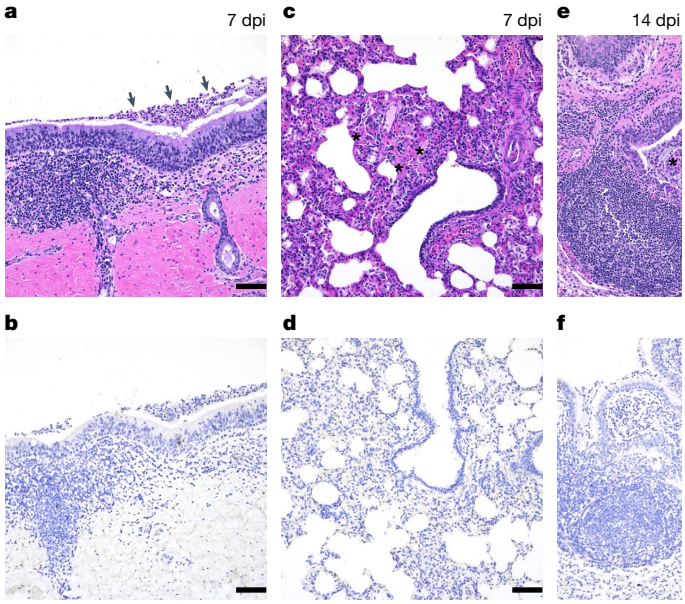

Fig. 5 | Histological changes observed in respiratory tissues of oronasally infected calves. Histological changes in calves oronasally infected with H5N1 B3.13 at 7 dpi (**a**–**d**) and 14 dpi (**e**,**f**). **a**,**b**, Haematoxylin and eosin (H&E) staining (**a**) shows a segmental region of suppurative tracheitis at 7 dpi (no. 6760). Degenerate neutrophils filled the tracheal lumen (arrows). No viral antigen was detected by IHC (**b**). **c**,**d**, In calf no. 712 (7 dpi), there were multiple small and discrete foci of interstitial pneumonia (**c**) with fibrin filling regional alveolar spaces (asterisks) and small numbers of neutrophils, macrophages and lymphocytes expanding alveolar septa. No viral antigen was detected (**d**). **e**,**f**, In calf no. 754 (14 dpi), bronchioles were frequently lined by hyperplastic epithelium (**e**), filled with degenerate neutrophils and partially occluded by papillary projections composed of a core of fibrous connective tissue with few inflammatory cells and lined by bronchiolar epithelium (bronchiolitis obliterans, asterisk). Bronchioles were also frequently delimited by prominent lymphoid aggregates (BALT hyperplasia). No viral antigen was detected (**f**). Scale bars, 100 µm.

During postmortem examinations of the lactating cows, 45 tissue locations per cow were sampled for histological examination and virus antigen detection (Supplementary Table 3). At 3 dpi, H5N1 B3.13 and euDG induced acute mastitis presented with flocculent material in minimal amounts of milk (no. 72, EU and no. 47, US). The character of the histologic changes did not differ between H5N1 B3.13 and euDG. Owing to the small number of cows used, a quantitative comparison of the two isolates with regard to the abundance of the virus antigen was not possible (representative images are shown in Extended Data Fig. 8). Up to 90% of the histological sections of secretory alveoli in the mammary gland evaluated showed acute epithelial necrosis with intraluminal cellular debris admixed with many degenerate neutrophils and intralesional antigen detection (Fig. 6a,b). The basal laminae with lining basal–myoepithelial cells remained largely intact (Fig. 6c). Intralesional virus antigen was confined to the secretory alveolar epithelium and intraluminal cellular debris (Fig. 6d). The teat canal was less prominently affected by necrosis and inflammation, exhibiting IAV nucleoprotein in the remaining lining epithelium (Fig. 6e,f) and debris. The enlarged, draining supramammary lymph node exhibited acute lymphadenitis lacking antigen detection. At 9 dpi (no. 88 EU), in addition to the acute necrotic lesions, interstitial, mainly lymphocytic

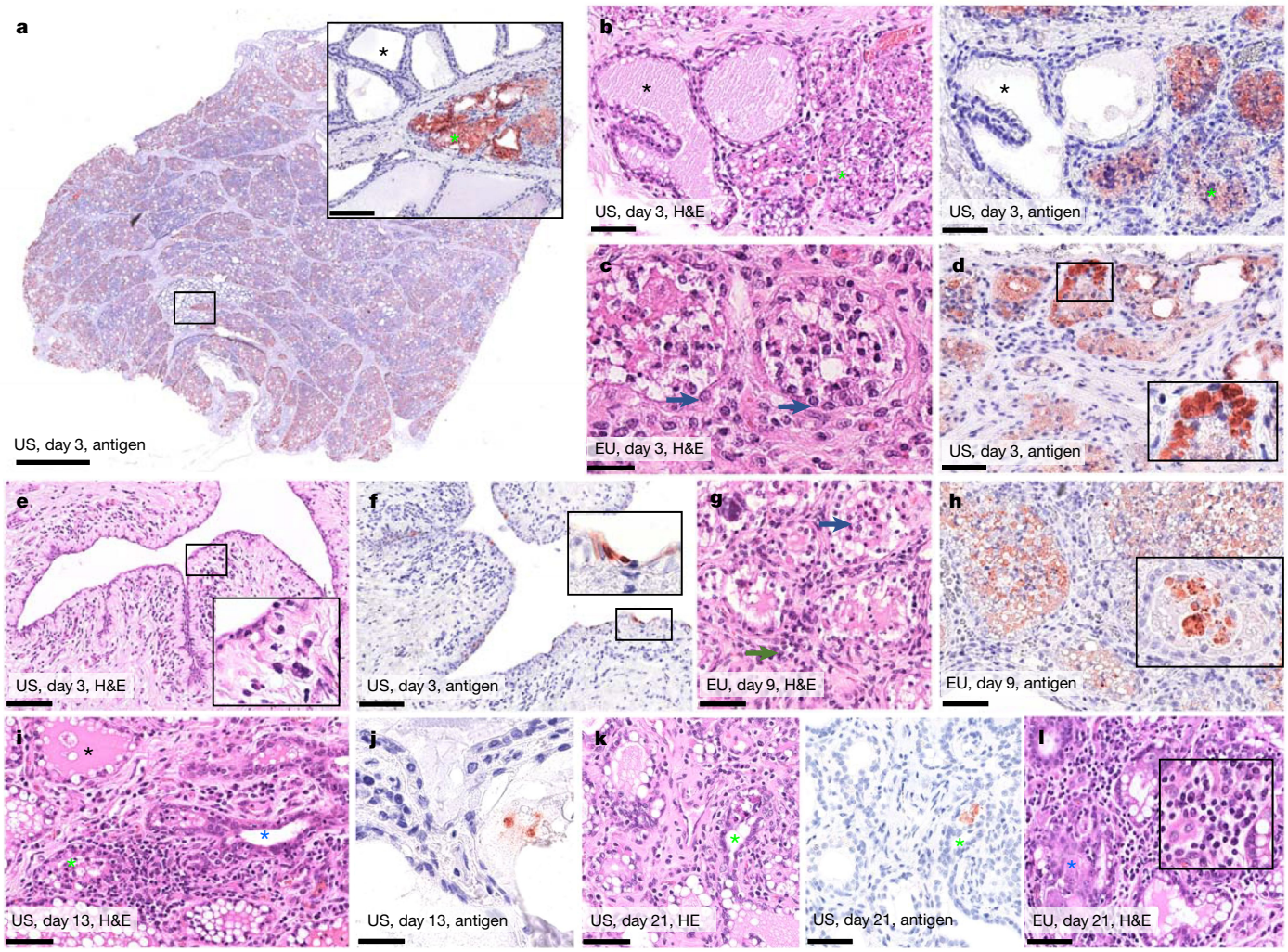

Fig. 6 | Histopathology and IAV NP detection in the mammary gland of multiparous cattle after intramammary infection with H5N1 B3.13 and H5N1 euDG. a, IAV NP detection at 3 dpi. Inset, enlarged view of the outlined region showing juxtaposition of intact, lactating alveoli lacking antigen (black asterisk) and affected areas (green asterisk) in a lobular pattern. IHC using AEC chromogen and Mayer's haematoxylin counterstain. Scale bars: 2.5 mm, main image; 100 µm, inset. **b**, Full necrosis of the alveolar epithelium, with cellular debris filling the lumen (left) and intralesional detection of IAV antigen (green asterisk) on a consecutive section (right). Some adjacent alveoli remain unaffected (black asterisk). Scale bars, 50 µm. **c**, Alveoli affected by necrosis with mostly intact basal lamina lined by basal and myoepithelial cells (blue arrows). Scale bar, 25 µm. **d**, Target cells identified on the basis of morphology following IHC include alveolar secretory epithelium. Scale bar, 50 µm. **e**, Teat with diffuse degeneration and necrosis of the lining epithelium, subepithelial oedema and mainly neutrophilic infiltrates (inset). Scale bar, 100 µm. **f**, Target cells identified on the basis of morphology following IHC include teat canal epithelium (inset). Scale bar, 100 µm. **g**, Necrotic alveoli filled with cellular debris admixed with degenerate neutrophils (blue arrow) in acute lesions and many lymphocytes, and fewer macrophages, neutrophils and plasma cells in the interstitium (green arrow). Scale bar, 50 µm. **h**, Intralesional IAV NP detection in secretory alveoli, mainly within cellular debris (inset). Scale bar, 50 µm. **i**, Simultaneous occurrence of intact, lactating alveoli (black asterisk), disruption of alveolar epithelium by necrosis (green asterisk) and beginning of regeneration (blue asterisk). Scale bar, 50 µm. **j**, Late stage IAV NP detection is restricted to scattered cellular debris. Scale bar, 25 µm. **k**, Left, mammary alveolus with intraluminal sloughed epithelium and cellular debris (green asterisk). Right, intralesional detection of IAV antigen on a consecutive slide (green asterisk). Scale bar, 50 µm. **l**, Regenerating alveoli (blue asterisk) with lack of IAV antigen labelling (not shown). Interstitial immune cell infiltrates include many lymphocytes and plasma cells (inset). Scale bar, 50 µm.

infiltrates were present (Fig. 6g) with antigen detection present but limited to up to 50% of the alveoli evaluated histologically, mostly within cellular debris (Fig. 6h) as well as single teat canal lining epithelial cells. At 13 dpi (no. 92 US), there was still evidence of acute necrosis (Fig. 6i) with scattered antigen detection within cellular debris (Fig. 6j). However, the predominant feature observed at this timepoint consisted of regenerative and non-suppurative interstitial inflammatory infiltrates (Fig. 6i). Cellular debris in affected mammary alveoli at 21 dpi remained positive for virus antigen (H5N1 B3.13 only; Fig. 6k, right). The majority of the examined alveoli were in a regenerative state at 21 dpi (Fig. 6l), with mainly lymphoplasmacytic, interstitial infiltrates (Fig. 6l). All other tissues tested negative for IAV antigen, including those identified to contain low levels of viral RNA in individual cows (spinal cord, cerebrum and genitofemoral nerve, cervix, vestibulum vaginae and the urinary bladder; details included in Extended Data Fig. 9a–e). The relevance of the intralobular or interlobular fibrosis of the inflamed mammary tissue to HPAIV infection could not be established, since it was found in varying degrees in the mammary gland, both in the uninfected control cow (no. 80, all quarters) and in individual quarters of all infected cows. In accordance with the clinical findings, a smaller amount of dry, but otherwise normal ingesta was found in the gastrointestinal tract on 3 and 9 dpi, which was interpreted as a sequela of the HPAIV infection. Additional changes were considered as not being associated with infection but were attributed to the age and lactation status of these multiparous cows (details included in Supplementary Table 4).

Table 1 | IAV-specific serological response in calves and lactating cows following inoculation with HPAIV H5N1 clade 2.3.4.4b

			dpi						
Calf ID		**Assay**	**−1**	**7**	**10**	**14**	**17**	**20**	**21**
H5N1 B3.13 Principal-infected calves									
697	Serum	NP	−	−	+	+	+	+	
		$H5_{V3}$	−	−	−	+	−/+	−/+	
		VNT_{50}	<5	<5	20	80	80	40	
712	Serum	NP	−	−					
		$H5_{V3}$	−	−					
		VNT_{50}	<5	<5					
754	Serum	NP	−	−	+	+			
		$H5_{V3}$	−	−	−	−			
		VNT_{50}	<5	<5	20	20			
6760	Serum	NP	−	−					
		$H5_{V3}$	−	−					
		VNT_{50}	<5	<5					
6768	Serum	NP	−	+	+	+			
		$H5_{V3}$	−	−	−	−			
		VNT_{50}	<5	<5	<5	5			
6772	Serum	NP	−	−	+	+	+	+	
		$H5_{V3}$	−	−	−	−/+	−/+	−/+	
		VNT_{50}	<5	<5	10	20	20	20	
Sentinel calves									
718	Serum	NP	−	−	−	−	−		−
		$H5_{V3}$	−	−	−	−	−		−
		VNT_{50}	<5	<5	<5	<5	<5		<5
748	Serum	NP	−	−	−	−	−		−
		$H5_{V3}$	−	−	−	−	−		−
		VNT_{50}	<5	<5	<5	<5	<5		<5
6770	Serum	NP	−	−	−	−	−		−
		$H5_{V3}$	−	−	−	−	−		−
		VNT_{50}	<5	<5	<5	<5	<5		<5

			dpi					
Cow ID		**Assay**	**0–6**	**7**	**9**	**13**	**14**	**21**
H5N1 B3.13 lactating cows								
87	Serum	NP	−	+			+	+
		H5	−	−			+	+
		VNT_{100} (B3.13)	<16	<16			406	323
		VNT_{100} (euDG)	<16	<16			645	323
92	Serum	NP	−	+		+		
		H5	−	−		+		
		VNT_{100} (B3.13)	<16	<16		813		
		VNT_{100} (euDG)	<16	<16		813		
47	Serum	NP	−					
		H5	−					
		VNT_{100} (B3.13)	<16					
		VNT_{100} (euDG)	<16					
H5N1 euDG lactating cows								
66	Serum	NP	−	+			+	+
		H5	−	−			+	+
		VNT_{100} (B3.13)	<16	<16			406	256
		VNT_{100} (euDG)	<16	<16			323	406
88	Serum	NP	−	−	+			
		H5	−	−	+			
		VNT_{100} (B3.13)	<16	<16	<16			
		VNT_{100} (euDG)	<16	<16	25			
72	Serum	NP	−					
		H5	−					
		VNT_{100} (B3.13)	<16					
		VNT_{100} (euDG)	<16					

Nucleoprotein (NP) and hemagglutinin H5-subtype (H5) specific cELISA; positive (+), doubtful (−/+), negative (−); virus neutralization assay (VNT). Serum collected from oronasally inoculated calves (left) and intramammary inoculated lactating cows (right) was evaluated using commercially available competitive ELISA (cELISA) kits targeting the influenza A virus nucleoprotein (NP) and H5 subtype hemagglutinin protein as well as virus neutralization tests (VNT) against H5N1 B3.13 (calves and cows) and H5N1 euDG (cows). Results of cELISA are calculated as sample OD_{450}/negative control OD_{450} percentage (S/N%) represented here as positive (+; NP, S/N% ≤ 45%; H5, S/N% ≤ 50%; H5-V3, S/N% ≤ 40%), doubtful (−/+; NP, 45% < S/N% < 50%; H5, 50% < S/N% < 60%; H5-V3, 40% < S/N% < 50%), and negative (−; NP, S/N% ≥ 50%; H5, S/N% > 60%; H5-V3, S/N% ≥ 50%), per manufacturer's instructions. The relative titers of H5N1-specifc neutralizing antibodies are reported as the reciprocal of the final dilution of neutralizing dose 50 (VNT_{50}) or 100 (VNT_{100}) virus neutralization. Serological data for milk samples collected from lactating cows is shown in Extended Data Table 1.

Early detection of antibodies in milk and serum

IAV-specific antibodies in serum collected from oronasally inoculated calves were first evaluated using a NP-specific cELISA. All four principal-infected calves were seropositive by 10 dpi (Table 1). Subsequent evaluation of serum using the H5-subtype-specific cELISA indicated that only 2 calves were seropositive by 14 dpi. However, neutralizing antibodies were detected in 3 out of 4 remaining principal-infected calves at 10 dpi, with all 4 testing positive by 14 dpi (Table 1). Neutralizing titres ranged from 1:5 to 1:80 and were maintained in calves until they were humanely euthanized at 14 or 20 dpi (Table 1). There was no IAV-specific antibody response detected in the sentinel or negative control calves throughout the study period (Extended Data Table 1).

IAV-specific antibodies of infected lactating cows were analysed by ELISA and VNTs in serum and milk samples throughout the experiment. At 7 dpi, serum samples from 2 out of 2 cows inoculated with H5N1 B3.13 and 1 out of 2 cows inoculated with H5N1 euDG were positive in both the NP-specific and the H5-specific ELISAs (Fig. 3c and Table 1). Neutralizing antibodies against H5N1 clade 2.3.4.4b were detectable in milk samples of infected cows from 9 dpi and neutralizing titres ranged from 1:25 to 1:813 (Extended Data Table 1). IAV-specific antibody detection in the milk was delayed by approximately two days in comparison to sera (Table 1 and Extended Data Table 1).

Discussion

H5N1 B3.13 was the first IAV reported to circulate efficiently among dairy cattle, with widespread dissemination between US farms and onward transmission to various avian and mammalian species, including humans[1,17]. This highlights the promiscuous nature of avian influenza viruses, greatly expanding the range of potential hosts and clearly demonstrating their potential to spillover and adapt to new environments. Nevertheless, the capacity for cows to serve as a host supportive of productive HPAIV infection was unexpected, as previous experiments have shown a very low susceptibility of young calves to intranasal inoculation with HPAIV H5N1[13]. In this study, we present detailed data on cattle

susceptibility to different HPAIV H5N1 genotypes within the broadly circulating clade 2.3.4.4b, providing insights into pathogenesis, potential transmission routes and mammalian adaptation. We demonstrate that high-dose oronasal infection of clinically healthy calves with an early H5N1 B3.13 isolate derived from an infected US dairy cow results in low-to-moderate replication confined to the upper respiratory tract and low-to-moderate oronasal viral shedding, presenting clinically with only mild signs, and IAV-specific antibody production from 7 dpi onwards. However, modest replication and shedding in principal-infected calves were not sufficient to infect direct contacts despite recovery of live virus from some of these samples up to 7 dpi. Of note, in a previous study using a mammalian H5N1 isolate from 2006, one sentinel calf out of two seroconverted upon H5N1 infection[13]. Our results provide evidence that systemic spread or replication in the lower respiratory tract is limited and transmission to sentinels is inefficient following oronasal inoculation of calves under these experimental conditions; H5 A/Goose/Guangdong/1/1996 pathogenicity in male and non-lactating female calves appears to have remained unchanged over the past decades. However, the interaction between suckling calves and cows, and the reciprocal transmission that could occur at this interface were not evaluated here.

Recently, Baker et al.[22] reported results in four heifers inoculated by an aerosol respiratory route. Clinical disease was mild and infection was confirmed by virus detection, lesions and seroconversion, as in the present study. However, transmission was not evaluated in these aerosol-infected heifers, but viral antigen was detected in the lung[22]. In our study, pathological alterations in the respiratory tract were limited in calves oronasally infected with H5N1 B3.13. Although the changes noted at 7 dpi and 14 dpi (1 calf each) could be consistent with an acute to late stage of IAV infection, lack of intralesional antigen detection precluded unequivocal confirmation. However, the window for detection of IAV antigen following infection is usually narrow, and antigen could have been present at low abundance in these calves. This probably explains the lack of detection of viral antigen at 7 dpi. The overall histologic alterations and their distributions in both calves are not consistent with bacterial bronchopneumonia associated with the bovine respiratory disease complex despite detection of *Pasteurella multocida*, *Bibersteinia trehalosi* and *Mannheimia haemolytica* by PCR with high Cq values. The extent to which active or previous infection with influenza D virus (IDV) might influence the course of IAV infection was not evaluated in this study. We believe that prior infection of some calves with IDV did not affect the outcome of the experiment, as there is no known cross-reactivity between IDV and IAV and no difference was found between the IDV-positive and IDV-negative calves.

Drastically different outcomes were observed following direct intramammary inoculation of lactating dairy cows with H5N1 B3.13 or H5N1 euDG. Acute presentation of severe clinical signs including lethargy, fever and impaired general condition were accompanied with abrupt reduction of feed intake and clinical mastitis with immediate and persistent milk losses of more than 90% in all cows, irrespective of the H5N1 virus isolate. Histopathology identified a severe, acute, diffuse, necrotizing mastitis with intralesional virus antigen detection in the secretory epithelium and teat canal lining epithelium, but no evidence of systemic spread of the pathogen. In our study, humane euthanasia of four individual cows was required prior to the initially planned end points owing to the severity of clinical signs (Extended Data Fig. 1f). Although there is currently a lack of evidence of increased mortality events in dairy farms across the USA, a recent field observation study demonstrates that two dairy farms reported mortality associated with H5N1 infections[1]. Conversely, the severe clinical presentation observed in our study may also be related to the age and late lactation phase of these cattle (4–8 years of age, just before dry-off). It is possible that confounding co-morbidities that are common in older dairy cows contributed to the severity of the disease, and that H5N1-associated disease may be milder in younger, monoparous cows at a different stage of lactation or when individual udder quarters are affected.

It also seems likely that udder manifestation is not a unique feature of genotype B3.13, but rather a particular intrinsic ability of the bovine udder to be readily susceptible to H5N1 clade 2.3.4.4b viruses, as similar tropism and disease were demonstrated in this study with H5N1 euDG. Susceptibility of cattle to IAVs other than H5 is speculative, but there are historic reports of productive intramammary infection in dairy cows by an ancestral human H1N1 (A/PR8)[23–25]. Mammary infections of cattle by avian influenza viruses are supported by the recently described expansive expression of α2.3 sialic acid receptors in the udder tissue, but not in the upper respiratory tract of cattle[18]. We isolated high levels of infectious virus only from milk and udder samples of H5N1-infected lactating cows, clearly demonstrating that replication of H5N1 clade 2.3.4.4b is restricted to the mammary glands after intramammary inoculation. In addition, RT–qPCR analysis of environmental samples collected daily from a communal water trough, urine samples and nasal, conjunctival and rectal swabs from infected lactating cows revealed only trace amounts of viral RNA, indicating that non-milk-related transmission routes appear to be less relevant; however, modes of transmission in adult cows remain to be evaluated in more detail. Field reports suggest that cow-to-cow transmission is most probably driven by the milking process, and appears to be equipment-related, thus representing a mechanical and anthropogenic event[1,15,17,26]. Milk and milking procedures, in this respect, appear to be the central mediator of spread within holdings. This is further supported by recent research showing that raw milk spiked with HPAIV H5N1 remained infectious on milking machines for several hours[27] and can be detected in environmental samples collected from a milking parlour[26]. In the field, titres in milk from infected cows can exceed 10^8 $TCID_{50}$ ml^{-1}, about two logs higher than the dose we used for intramammary inoculation. The methods used to inoculate cows (intramammary) may not be a perfect representation of how milking equipment would infect an udder; however, given the similarity of our virological and clinical results to field reports, they appear to validate the conditions used in our experiment. With respect to oronasal transmission in calves, there is no information on the infectious dose of potential respiratory transmission. If oral transmission occurs by ingestion of contaminated milk, our inoculation dose is probably lower than what calves would be exposed to in the field. If calves are infected by aerosol transmission, the atomization method that we used for infection might not be the appropriate approach and could explain some of the differences that we observed compared with Baker et al.[22]. In addition, the extensive cleaning and environmental controls of biosafety level 3 agriculture facilities, where airflow is directional and constantly exchanged, must also be considered, as they do not reflect natural barn conditions and could reduce the opportunity for airborne transmission.

Substitutions in the PB2 gene sequence, such as the M631L mutation in H5N1 B3.13, appear to be very favourable for replication in the mammary gland[15]. We found a similar PB2 adaptation, E627K, for H5N1 euDG in our lactating cows, from 1 dpi as a minor variant, with clear presence by 3 dpi. Independent appearance of this substitution in 3 out of 3 cows suggests a strong bottleneck and high evolutionary pressure towards this adaptation. Conclusively, PB2 adaptation to mammalian hosts (at position 627 or 631) seems to be beneficial for replication in the mammary gland, as these genotypes remain stable in any tested milk and udder tissue sample. Notably, H5N1 B3.13 has also acquired the E627K mutation upon replication in a human case[28] and was present as a minor variant (2%) in environmental samples from a dairy farm in Kansas[26]. It remains to be determined whether strain and/or host dependencies drive the selection of the two PB2 mutations and whether they resemble similar phenotypes.

Spillback of bovine H5N1 B3.13 into multiple poultry farms has been reported[1], and this may result in increased environmental contamination in poultry, furthering spillback into wild bird populations and possible zoonotic transmissions. This has recently been demonstrated by an outbreak of H5N1 B3.13 in a large commercial layer hen farm in

Colorado, USA, where several farm workers tested positive for H5N1 after culling the infected hens[29]. The possibility for non-lactating cattle to serve as a virus source for onward transmission to adult dairy cows, poultry or mammalian species such as cats, should be considered, as we observed nasal shedding of infectious virus for seven days. The same scenario also exists for environments contaminated with milk from H5N1-infected cows.

A tailored surveillance strategy is crucial for effective control. We demonstrate here that in addition to RT–qPCR, AIV-specific RATs might provide a simple testing tool for milk from individual cows and are suitable for the detection of H5N1 clade 2.3.4.4b, including genotype B3.13. Influenza mastitis should be considered as a differential diagnosis whenever milk characteristics change. As antibody levels in milk increase and viral loads decrease, antigens are no longer detected by RATs. Infectious yields in the milk also decrease at this point, owing to the neutralizing activity of secreted antibodies. The unique situation that both viral shedding and neutralizing antibody shedding occur in milk may also present an opportunity to control this epizootic and reduce infectious titres in pooled milk from affected herds. In addition to genomic surveillance, serologic surveillance of pooled milk from individual herds may be appropriate to assess the distribution of IAV among dairy herds and facilitate control efforts.

In conclusion, we demonstrated that: (1) H5N1 B3.13 had only a moderate capacity for respiratory replication in young calves; (2) H5N1 B3.13 was not transmitted to sentinel calves; (3) dairy cattle were readily susceptible to two distinct and geographically separated H5N1 clade 2.3.4.4b isolates of mammalian or wild bird origin following intramammary inoculation; (4) viral replication was localized to the udder with subsequent high-titre shedding in milk; (5) the clinical picture of severe disease in dairy cattle was identical for both strains, with severe mastitis; and (6) high-titre infectious virus was shed in milk for at least 8 days. Finally, the manifestation and main replication site for both tested H5N1 isolates following intramammary inoculation was the mammary gland, and systemic spread and infection of other organ systems, including the respiratory tract, was not observed in lactating cows.

No human-to-human transmission has been reported so far for these strains, supporting the notion that they have not yet overcome critical barriers to enable human-to-human transmission, such as improved receptor binding, pH stability and escape from MxA restriction[30]. The frequent interfaces between humans and affected animals (cattle, poultry and wild birds) provide opportunities for reassortment of the bovine B3.13 with human seasonal influenza viruses or other avian and maybe swine influenza viruses in circulation. Thus, effective mitigation strategies must be urgently outlined and implemented to: (1) prevent continuous replication and spread of this pathogen in cattle; (2) avoid any further mammalian adaptation; and (3) stop spillover and spillback infections to other livestock, wild birds, other mammals and humans. Focused efforts to better characterize transmission pathways and H5N1 ecology in the dairy industry worldwide are critically needed.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-024-08063-y.

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Methods

Ethics statement and biosafety

All experiments conducted at Kansas State University were approved and performed under the Kansas State University (KSU) Institutional Biosafety Committee (IBC, protocol no. 1758) and the Institutional Animal Care and Use Committee (IACUC, Protocol no. 4992) in compliance with the Animal Welfare Act. All animal and laboratory work involving infectious highly pathogenic avian influenza virus were performed in biosafety level 3+ and 3Ag laboratories and facilities in the Biosecurity Research Institute (BRI) at KSU in Manhattan, KS, USA. Further evaluation of inactivated samples was conducted in biosafety level (BSL)-2 laboratories using enhanced biosafety practices. The calf transmission studies mimicked 'natural' transmission events that could happen daily in US dairy operations and are not considered passaging or adaptation experiments, since no further passaging and no forced transmission was attempted.

The lactating dairy cattle experiment was evaluated by the responsible ethics committee of the State Office of Agriculture, Food, Safety, and Fishery in Mecklenburg–Western Pomerania (LALLF M-V) and gained governmental approval under the registration number 7221.3-2-010/23. At the Friedrich-Loeffler-Institut (FLI), the experiments with lactating dairy cattle were performed in the licensed high containment BSL-3 laboratories and BSL-3 large animal facilities of the institute with the required personal protective measures in accordance with the respective permits. The experiments were in compliance with the relevant requirements of the German Infection Protection Act, the Biological Agents Ordinance and the Animal Pathogens Ordinance.

Virus

The European HPAIV isolate A/wild_goose/Germany-NW/00581/2024 (H5N1), genotype DE-23-11-N1.3 euDG (H5N1 euDG)[21], was supplied by the German National Reference Laboratory for avian Influenza (T. Harder) at FLI. It was propagated in embryonated specific pathogen-free chicken eggs for 5 days at 37 °C, followed by collection of the allantoic fluid, which in turn served as the virus stock. In post-inoculation sterility testing of the original undiluted H5N1 euDG virus stock, the marginal presence of *Enterococcus* spp. was confirmed on sheep blood agar. This was confirmed by next-generation sequencing analysis, which revealed a close genetic relationship to *Enterococcus casseliflavus*, a commensal to the normal bacterial flora. There was no evidence that this had any effect on the results obtained in the study.

The North American HPAIV H5N1 isolate A/Cattle/Texas/063224-24-1/2024, genotype B3.13 (H5N1 B3.13, GISAID accession number: EPI_ISL_19155861) administered to calves and cattle in this study was isolated from the milk of infected dairy cattle in Texas, USA, kindly provided by D. Diel[1]. Virus stock was propagated using bovine uterine epithelial cells (CAL-1; in-house) for three passages. A single passage on MDCK cells was used to propagate viral stocks for application in virus neutralization assays. The titres of both virus preparations were determined by endpoint dilution titration on MDCK type II cells.

Cells

FLI Riems: Madin-Darby Canine Kidney (MDCK, RIE-1061) type II cells originating from the Collection of Cell Lines in Veterinary Medicine (CCLV) were used. Cells were incubated at 37 °C under a 5% CO_2 atmosphere. Cultivation medium is composed of a mixture of equal volumes of Eagle Minimum Essential Medium (MEM) (Hank's balanced salts solution) and Eagle MEM (Earle's balanced salts solution), 2 mM L-glutamine, nonessential amino acids, adjusted to 850 mg l^{-1} $NaHCO_3$, 120 mg l^{-1} sodium pyruvate, pH 7.2 with 10% fetal calf serum (FCS) (Bio & Sell).

Kansas State University: Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Corning), supplemented with 5% fetal bovine serum (FBS; R&D Systems) and 1% antibiotic-antimycotic solution (Gibco). Medium used during virus cultivation (VNT, virus isolation) was similar, but deprived of FBS and supplemented with 0.3% bovine serum albumin (BSA; Sigma-Aldrich) and 1% minimum essential medium vitamin solution (Gibco), in addition to 1 µg ml^{-1} of TPCK-treated trypsin.

Experimental design for calf study

Twelve Holstein calves (5–6 months of age) of mixed sexes were transported from an Iowa livestock operation to Kansas State University College of Veterinary Medicine (KSU-CVM) in Manhattan, Kansas.

Upon arrival, blood and swabs were collected and screened for current or recent IAV infection as well as various pathogens associated with bovine respiratory disease complex (BRD), including IDV. Calves were negative for any active or recent infections with IAV based on RT–qPCR and IAV NP-specific cELISA results. Results of the RT–qPCR based BRD panel revealed some calves were qPCR positive for common bovine respiratory pathogens (Supplementary Table 3)[31]. Calves were semi-randomly (sorted according to sex; one (female) calf determined to be hermaphroditic; then randomized intro groups) allocated into three experimental groups: principal-infected (n = 6; 2 female, 3 male, 1 hermaphrodite); sentinel (n = 3; 2 female, 1 male); negative controls (n = 3; 1 female, 2 male). The control animals were humanely euthanized prior to the day of infection (−2 and −3 dpi). Calves were in good health prior to virus infection based on health examinations conducted by KSU veterinarians.

On the day of virus infection, sentinel calves were physically separated and placed up-current of the room's directional airflow from principal-infected calves. Principal-infected calves were administered a total dose of 1×10^6 $TCID_{50}$ (5×10^5 $TCID_{50}$ ml^{-1}) in 2 ml of H5N1 B3.13 applied as follows: 0.5 ml per nostril using an atomization device (MAD Nasal atomization device, Teleflex) to deliver a fine mist of particles 30–100 µm in size and 1 ml orally using a syringe. Forty-eight hours post infection, sentinel calves were co-mingled with principal-infected calves (Fig. 1).

Clinical observations and rectal temperatures were recorded daily (Fig. 1). One calf (no. 6770; sentinel) developed a high fever prior to the start of the experiment, which resolved following treatment with florfenicol. Baseline samples, including swabs and blood, were collected from all calves upon arrival (−8 dpi) and again after an acclimation period outdoors (−1 dpi). Clinical samples, including urine (when possible), nasal, oral, rectal, vaginal and preputial swabs (collected in 2 ml of DMEM containing 1% antibiotic/antimycotic solution) were collected at −1, 1–14, 17, 20/21 dpi (Fig. 1). Whole blood samples were collected on −1, 1–7, 10, 12, 14, 17, 20/21 dpi (Fig. 1). Serum samples were collected on −1, 7, 10, 14, 17, 20/21 dpi (Fig. 1). Thorough post-mortem examinations were conducted on days 7 (n = 2; principal-infected), 14 (n = 2; principal-infected), 20 (n = 2; principal-infected) and 21 (n = 3; sentinel) post infection (Fig. 1). Apparent gross lesions were documented prior to extensive sampling of tissue as to determine the scope and extent of impacted tissues (tissue tropism) and any correlation with subsequent IAV detected. Lungs were macroscopically evaluated and scored (a report was generated) according to the percent of lung affected (individual lobes and left/right) with gross lesions including congestion with atelectasis or oedema, pneumonia, haemorrhage and plural fibrosis when present[32] (Extended Data Figs. 4 and 10).

Experimental design for lactating dairy cows study

Seven female multiparous lactating Holstein–Friesian dairy cattle in an age range between four and eight years, at a state of decreasing milk production, and around 12 months after last calving were obtained from a local dairy farm. The animals were kept in three separate animal rooms (3 × 3 × 1 animals per stable) in the BSL-3 animal facility of the FLI, Greifswald, Isle of Riems, Germany. During the 25-day acclimatization period, the animals were milked once per day using a can milking system (Minimelker, Melktechnik-Discount) and the amount of milk

produced per cow was documented to have a reliable baseline for each individual (Fig. 1). Additionally, a CMT was performed on each udder quarter from each cattle daily from −1 dpi onwards until each individual endpoint. In brief, CMT reagent was mixed with an equal amount of raw milk received directly from the respective cow teat on a special CMT plate (Extended Data Fig. 1a), followed by gentle swivelling. The CMT was interpreted according to a comparative picture showing either: (1) unchanged colour and consistency (negative, −); (2) low mucus formation (altered, +); (3) strong mucus formation (positive, ++); or (4) intense clumpy and gelatinous mucus formation (strongly positive, +++). Milk production percentages were calculated by generating a mean value of −2 to 0 dpi for each individual animal, which was set as 100%. Prior to infection, all animals tested negative for IAV RNA in nasal swabs and milk samples via RT–qPCR, as well as seronegative in an IAV-specific ELISA targeting the viral NP (ID-Vet). Prior to inoculation, the udder epidermis was cleaned and the teat and teat orifice were disinfected using an alcohol-based disinfectant. A teat drainage cannula was employed to evacuate all residual milk from the cistern and for inoculation into teat a/o gland cistern. Three animals were inoculated in the mammary gland with $10^{5.9}$ $TCID_{50}$ per cow by equally administering 0.5 ml per teat (~2 ml total volume, $10^{5.31}$ $TCID_{50}$ per teat, all four teats) of H5N1 B3.13, and three animals were inoculated in the mammary gland with $10^{6.1}$ $TCID_{50}$ per cow by equally administering 0.5 ml per teat (~ 2 ml total volume, $10^{5.49}$ $TCID_{50}$ per teat, all four teats) of H5N1 euDG. One additional animal, kept in a separate unit, served as negative control and received 0.5 ml sodium chloride solution per teat. The infectious virus titres of both inocula were determined by back-titration on MDCKII cells. Cattle were milked daily, and nasal swab samples as well as samples from the drinking trough of the animals were taken daily until 9 dpi (Fig. 1). Swab samples from conjunctiva and rectal swabs were taken from 4 dpi until 9 dpi (Fig. 1). Urine was collected until 14 dpi. EDTA blood samples were taken at 1, 3, 7, 10 and 17 dpi (Fig. 1). Serum samples were generated before inoculation, 7, 14 dpi, and at the day of euthanasia (Fig. 1). The control cow had a bacterial infection, most probably in the uterus, prior to the start of the experiment. This necessitated the use of antibiotics, which partially resolved the clinical conditions. In addition, this animal did not produce large amounts of milk as it was very close to dry-off. It was therefore chosen as a control. The reduction in feed intake and the low milk yield of this cow were therefore most likely due to these overall conditions.

RNA extraction and RT–qPCR analysis for calf study

To document the presence of H5N1 infection, clinical samples (swabs, EDTA blood) and clarified 10% (weight:volume) tissue homogenates were combined with equal volumes of RLT lysis buffer (Qiagen) prior to total nucleic acid extraction using an automated magnetic bead-based extraction system (Taco Mini, GeneReach; BioSprint 96, Qiagen) in combination with associated reagents (GeneReach), according to previously established protocols[26]. Subsequently, samples were run in duplicate reactions using a one-step RT–qPCR assay targeting the matrix gene segment of IAV employing a modified M + 64 probe and qScript XLT 1-Step RT–qPCR ToughMix (QuantaBio) on the Bio-Rad CFX96 Optics Module C1000 Touch (Bio-Rad) to determine the quantity of IAV RNA, with thermocycling conditions as described previously[26]. A positive Cq cut-off of 38 cycles was established for samples where both wells were positive.

Sample collection, RNA extraction and RT–qPCR analysis for lactating dairy cows study

Raw milk samples were collected individually per quarter and directly used for RNA extraction. In addition, bulk milk samples generated via milking machine were also collected and analysed. Swabs were taken using rayon swabs (DRYSWAB Standard Tips, MWE) and were immediately transferred into 2 ml of cell culture medium containing 1% Baytril (Bayer), 0.5% Lincomycin (WDT) and 0.2% Amphotericin/Gentamycin (Fisher Scientific). Blood samples were taken using the Kabevette G system and disposable needles. Organ samples 2 × 2 × 2 mm in size were transferred into a 2 ml collection tube containing 1 ml of cell culture medium containing 1% penicillin-streptomycin (Biochrome) and one stainless steel bead per sample. Homogenization was established by rough shaking of the samples in a TissueLyser II instrument (Qiagen) for 2 min. at 300 Hz. Viral RNA of all samples was extracted using 100 µl of raw milk or sample medium or supernatant in the NucleoMag Vet-kit (Macherey-Nagel) on a BioSprint 96 platform (Qiagen). Detection of H5N1 B3.13 and H5N1 euDG via RT–qPCR was established as recommended and described[33,34]. Relative quantification was performed using the Bio-Rad CFX Maestro 1.1 Version 4.1.2433.1219 software.

Virus isolation and virus titration for calf study

Clinical samples and tissue homogenates with C_t values < 36 were subjected to virus isolation and virus titration. Viral titration and immunofluorescence assays (IFAs) were performed as described previously[32]. In brief, tenfold serial dilutions of syringe filtered (0.45 µm) samples, tested in four replicates, were transferred onto 96-well plates containing confluent monolayers of MDCK cells. After infecting cells, 96-well plates were incubated at 37 °C and observed daily (light microscope) to monitor the conditions of the cellular monolayer and cytopathic effects (CPEs) and cell morphology. After 48 h, plates were washed with PBS prior to fixation with ice-cold 100% methanol for 10 min at −20 °C; then cells were washed with 1× PBS and incubated for 1 h with influenza A-specific H16-L10-4R5 primary antibody (ATCC HB-65[35]; undiluted) at room temperature. Subsequent washes with PBS containing 0.05% Tween-20 (PBS-T) were conducted prior to incubation with goat anti-mouse IgG (H + L) secondary antibody (Alexa-488, Fisher Scientific, A11001; 1:1000) and incubation at room temperature for 30 min. Plates were washed and dried prior to observation of fluorescent signal using an EVOS fluorescent microscope (EVOS FL Color, AMEFC4300, Fisher Scientific). Viral titres were calculated using the Reed–Muench method[36] with a limit of detect at 46 $TCID_{50}$ ml^{-1}.

In parallel, attempts for virus isolation were conducted utilizing 25 cm^2 flasks (T-25) of confluent MDCK cells. Cells were first washed with 1× PBS to remove any residual FBS and subsequently incubated with 500 µl diluted (1:10) sample and 2 ml of infection medium for 2 h, gently rocked every 15 min, prior to addition of 2.5 ml of infection medium. After two-three days of incubation at 37 °C, supernatants were collected from T-25 flasks. Flasks were then subjected to IFA protocols as described above and reported as positive or negative for viral presence based on fluorescent signal.

Virus isolation and virus titration for lactating dairy cows study

Virus titres of selected milk and udder organ samples were determined by a $TCID_{50}$ endpoint dilution assay on MDCKII cells. In brief, tenfold serial dilutions of respective samples were prepared and transferred onto 96-well plates containing confluent monolayers of MDCKII cells (duplicates). Plates were incubated for 72 h at 37 °C. Virus titre was evaluated by the presence of a specific CPE and was calculated according to the measure of infectious dose in specific infections (midSIN) method[37].

Serology and host immune responses for calf study

Serum, collected from all calves upon arrival (−8 dpi), prior to infection (−1 dpi), and at defined timepoints (7, 10, 14, 17, 20/21 dpi) throughout the study, were evaluated for the presence of influenza A-specific antibodies using several methods.

Neutralizing antibody titres were determined according to previously established protocols[38] with slight modifications. In brief, heat-inactivated serum was combined with an equal volume of H5N1 B3.13 stock virus (propagated additionally once on MDCK cells), diluted to 100 $TCID_{50}$ per 50 µl, in duplicate wells on 96-well plates and incubated at 37 °C for 1 h. Subsequently, the serum/virus mixture

was transferred to 96-well plates containing confluent monolayers of MDCK cells. After 48 h of incubation, IFA was preformed (similar to described above for virus titration and isolation) and neutralizing antibody titres were recorded as 50% inhibition of virus growth per well.

Additionally, commercially available enzyme-linked immunosorbent assay (ELISA) kits, validated for application with bovine-origin serum, targeting: (1) the conserved IAV nucleoprotein (NP-ELISA; ID Screen Influenza A Antibody Competition Multi-species, Innovative Diagnostics); and (2) the H5-specific haemagglutinin protein (H5-ELISA; ID Screen Influenza H5 multi-species competitive ELISA V3, Innovative Diagnostics) were used according to manufacturer's instructions on a Varioskan LUX (Thermo Scientific, N16044). Results were extracted from Thermo Scientific SkanIt Software for Microplate Readers (version 6.0, N16243) and further analysed using Microsoft Excel 2016 (version 16.0.5188.1000).

Serology for lactating dairy cows study

Serological analysis of blood samples or milk samples from all animals was performed by using a commercial IAV-specific enzyme-linked immunosorbent assay (ELISA) detecting NP- and H5-specific antibodies (ID-Vet) according to the manufacturer's instructions. Results were quantified via the Tecan i-control 2014 1.11 software and analysed via Microsoft Excel 2016 (version 16.0.5188.1000). Heat-inactivated serum was used for serum samples, but not for milk samples. There was no indication for any non-specific neutralization or reactivity with the milk samples. The recommended cut-off values in the test kit were used for both serum and milk samples. It is noteworthy at this point, that the cut-off values are not fully validated for milk samples yet.

Virus-neutralizing antibodies were investigated via a VNT_{100} on MDCKII cells. In brief, serum samples of respective timepoints were serially diluted in DMEM on a 96-well plate (log2 steps) and mixed with 100 $TCID_{50}$ of H5N1 B3.13 or H5N1 euDG followed by incubation for 1 h at 37 °C. Subsequently, 100 µl of MDCKII cells were added to each well, followed by another incubation period for 72 h at 37 °C. Neutralizing antibodies were evaluated and recognized by light microscopy in the absence of a CPE. The last serum dilution with intact cells and no visible CPE was considered as neutralization titre against the respective virus.

Macroscopic and microscopic pathology for calf study

Postmortem examinations were conducted at 7, 14 and 20/21 dpi. Macroscopic pathology was determined and scored in toto and the percentage of lung lesions were reported (Supplementary Table 5), based on previously published protocols[32]. Tissue samples were fixed in 10% neutral buffered formalin for a minimum of 7 days and subsequently transferred to 70% ethanol and processed using standard histological techniques, and stained with H&E. Collections included paired collections (fresh tissue and formalin-fixed) of representative sections (and lesions) obtained from respiratory, gastrointestinal, and reproductive tract, lymphoid tissue including spleen, various lymph nodes and mucosa-associated lymphoid tissue, brain, eyes and eye lids, and other tissues including adrenal gland, heart and pancreas. Bronchoalveolar lavage fluid was collected from the right side of the lung. Fluids collected included aqueous humour, cerebral spinal fluid and bile. Transudates from the pericardial sac, thoracic cavity and abdominal cavity and urine were also collected when present. Conjunctival swabs and swabs from the reproductive tract were also collected at necropsy. Selection of animals for necropsy was based on sex to ensure representative samples from each sex, as well as on data that was available regarding viral RNA shedding in nasal and oral swabs. Four-micrometre sections from the lower respiratory tract tissues were stained with routine H&E staining after standard automated processing and paraffin embedding. Routine H&E staining and IHC for the calf study was performed at the Louisiana Animal Disease Diagnostic Laboratory (LADDL), an AAVLD-accredited and National Animal Health Laboratory Network level 1 veterinary diagnostic laboratory that operates with highly standardized procedures and meets high quality standards. Routine H&E staining and IHC were performed using fully automated stainers that yield consistent staining results. For H&E staining, tissue blocks from the study were stained in three independent runs, with comparable staining results. For IHC, the primary antibody was first tested and antibody dilution and antigen retrieval optimized following standardized procedures at LADDL and using influenza virus A H5N1-infected tissues from a recent study[32]. IHC was performed on selected respiratory tract and lymphoid tissues of infected calves in 3 independent staining runs, all of which included a positive control tissue slide that yielded consistent results. All evaluations and interpretations were performed by a double board-certified veterinary pathologist and virologist.

IAV-specific IHC for calf study

IHC for detection of IAV H5N1 NP antigen was performed on the automated BOND RXm platform and the Polymer Refine Red Detection kit (Leica Biosystems). Following automated deparaffinization, 4-µm formalin-fixed, paraffin-embedded tissue sections on positively charged Superfrost Plus slides (VWR) were subjected to automated heat-induced epitope retrieval (HIER) using a ready-to-use EDTA-based retrieval solution (pH 9.0, Leica Biosystems) at 100 °C for 20 min. Subsequently, tissue sections were incubated with the primary antibody (rabbit monoclonal anti-IAV NP (Cell Signaling Technology, 99797/F8L6X) diluted 1:1,200 in Primary Antibody diluent (Leica Biosystems)) for 30 min at ambient temperature followed by a ready-to-use (pre-diluted) polymer-labelled goat anti-rabbit IgG coupled with alkaline phosphatase (30 min). Fast Red was used as the chromogen (15 min), and counterstaining was performed with haematoxylin for 5 min. Slides were dried in a 60 °C oven for 30 min and mounted with a permanent mounting medium (Micromount, Leica Biosystems). Lung sections from a pig experimentally infected with swine influenza virus A/swine/Texas/4199-2/1998 H3N2 and mink-derived clade 2.3.4.4b H5N1 isolate, A/Mink/Spain/3691-8_22VIR10586–10/2022 were used as positive assay controls (Extended Data Fig. 10).

Pathology for lactating dairy cows study

Full autopsy was performed on all animals under BSL-3 conditions and macroscopic diagnoses were recorded. In total, 45 samples (Supplementary Table 3) were fixed in 10% neutral buffered formalin. Tissues were paraffin-embedded and 2- to 4-µm-thick sections were stained with H&E. Consecutive slides were processed for IHC according to standardized procedures of avidin-biotin-peroxidase complex-method as described[39]. The primary antibody against the IAV nucleoprotein was applied overnight at 4 °C (ATCC clone HB-64, 1:200), the secondary biotinylated goat anti-mouse antibody was applied for 30 min at room temperature (Vector Laboratories, 1:200). Colour was developed by incubating the slides with avidin-biotin-peroxidase complex solution (Vectastain Elite ABC Kit; Vector Laboratories), followed by exposure to 3-amino-9-ethylcarbazole substrate (AEC, Dako). The sections were counterstained with Mayer's haematoxylin and coverslipped. As negative control, the non-infected cow was tested with the primary antibody, and consecutive sections of infected animals were labelled with an irrelevant antibody (anti Sars clone 4F3C4, 1:45)[40]. A positive control slide from an IAV infected chicken was included in each run (details included in Extended Data Fig. 9f–h). All sides were scanned using a Hamamatsu S60 scanner, evaluation was done using the NDPview.2 plus software (Version 2.8.24, Hamamatsu Photonics) by a trained pathologist (T.B.) and a board-certified pathologist (A.B.). H&E-stained sections were evaluated and described. Following IHC the distribution of virus antigen was graded on an ordinal scale with scores as follows: 0, no antigen; 1, focal, affected cells/tissue <5% or up to 3 foci per tissue; 2, multifocal, 6%–40% affected; 3, coalescing, 41%–80% affected; 4, diffuse, >80% affected. The target cell was identified based on the morphology. Routine staining (H&E) was performed in six independent runs, yielding comparable staining results. For virus antigen

detection, IHC was performed on up to 45 tissues per cattle ($n = 3$ per group; Supplementary Table 3). Five independent IHC runs gave comparable results for the negative and positive controls. Evaluation and interpretation were performed by a board-certified pathologist with more than 17 years of experience.

Next-generation sequencing for calf study

Samples with high quality RNA extracts were subjected to previously established next-generation sequencing methods[32] in order to evaluate the presence/frequency of genomic variants and their potential relation to host-adaptation (in reference to/changes compared to inoculum). The whole-genome sequence of the cattle -derived clade 2.3.4.4b H5N1 virus was determined using the Illumina NextSeq sequencing platform (Illumina). In brief, viral RNA was extracted from the infection inoculum and virus-positive clinical samples (inactivated in RLT lysis buffer; Qiagen) using the QIAamp viral RNA mini kit (Qiagen). Viral gene segments for infection inoculum and clinical samples were amplified using SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Thermo Fisher Scientific) with a universal influenza primer set[41,42]. All samples were normalized to 20 ng μl^{-1} (100–300 ng) prior to library preparations. Sequencing libraries were prepared using the Illumina DNA Prep kit (Illumina). Libraries were sequenced using pair-end chemistry on the Illumina NextSeq 550 (Illumina, SY-415-1002) platform with the NextSeq 500/550 Mid Output Kit v2.5 (300 cycles). Sequencing reads were demultiplexed and parsed into individual FASTQ files and imported into CLC Genomics Workbench version 23.0.5 (Qiagen) for analysis. Reads were trimmed to remove primer sequences and filtered to remove low quality and short reads. The trimmed reads were mapped to the reference sequence (A/Cattle/Texas/063224-24-1/2024; GISAID: EPI_ISL_19155861). Following read mapping, all samples were run through the low frequency variant caller module within CLC Genomic Workbench with a frequency cut-off greater than 2%.

MinION sequencing for lactating dairy cows study

MinION-based sequencing of avian influenza positive samples with Cq values < 28 was carried out as described[43,44]. In brief, the RNA was transcribed into DNA using Superscript 60 III One-Step and Platinum Taq (12574026, Thermo Fisher Scientific) Kit with influenza-specific primers (Pan-IVA-1F_BsmF (26mer wobbel) TATTCGTCTCAGG GAGCRAAAGCAGG; Pan-IVA-1R_BsmR (26mer wobbel) ATATCGTCTC GTATTAGTAGAAACAAGG). DNA amplificants were purified with Agencourt AMPure XP beads (A63881, Beckmann Coulter) magnetic beads using DNA LoBind Tubes (0030108051, Eppendorf). Approximately 200 ng of DNA per sample was used for sequencing by a transposase-based library preparation approach with Rapid Barcoding (SQK-RBK114.24, Oxford Nanopore Technologies) and a PromethION Flow Cell (FLO-PRO114M) on a PromethION 2 solo device with MinKNOW Software Core (v5.9.12). Live high accuracy base calling of the raw data with Dorado (v7.3.11, Oxford Nanopore Technologies) was followed by demultiplexing, a quality check and a trimming step to remove low quality, primer and short (<20 bp) sequences. For analysing, the bioinformatic software suite Geneious Prime (Biomatters, version 2024.0.5) was used. The sequences were trimmed, to remove the primer sequences. Consensus sequences were obtained with an iterative map-to-reference approach with Minimap2 (vs 2.24). The H5N1 B3.13 or the H5N1 euDG isolate sequence was used as a reference. Polishing of the final genome sequences and annotation was done manually after consensus generation (threshold matching 60% of bases of total adjusted quality). A total amount of $n = 53$ samples from the animal experiment as well as both virus stocks were sequenced. For the majority (42 samples) only partial assemblies were achieved. The remaining samples were screened for amino acid exchanges. For major variants A threshold of 55% was used to search for specific mutations in the consensus sequences within the sequences. For adaptive mutations (PB2 E627K) also minor variants were determined. Sequence analysis was completed using the Geneious Prime 2024.0.5.

IAV-specific RAT evaluation

Milk samples from the animal experiment served as samples for validation of this assay with application to bovine milk origin samples. Serial samples of infected animals were analysed in the AIV-specific Megacor test. In brief, the provided swab was dipped into the respective milk sample and afterwards transferred into the assay buffer and mixed according to the manufacturer's protocol. Afterwards, the test strip provided in the kit was dipped into the assay buffer according to manufacturer's instructions. Results were read after 15 min of incubation.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Consensus sequences of both isolates used for inoculation are available in the International Nucleotide Sequence Database Collaboration under accession PQ241097–PQ241104 (calves) and under PQ106994–PQ107009 (cows; H5N1 B3.13: PQ106994–PQ107001; H5N1 euDG: PQ107002–PQ107009). Raw data were filed to the Sequence Read Archive under project number PRJNA1141392. Source data are provided with this paper.

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Author contributions Conceptualization: M.B., J.A.R., N.J.H., L.U., D.H., A.B. and I.M. Data curation: N.J.H., K.C., A.K.A., A.P., T.B., A.B., C.D.M., J.D.T., M.C., L.U., J.S., S.K., M.N. and L.M.C. Methodology: N.J.H., K.C., I.M., L.U., T.B., J.D.T., M.C., T.K., F.M.F., J.S., D.H., R.P., B.C., G.S., S.K., N.N.G., U.B.R.B., L.H., I.M., M.N. and L.M.C. Formal analysis: N.J.H., K.C., A.K.A., L.U., T.B., A.B., C.D.M., J.D.T., M.C., S.K. and A.P. Investigation: L.U., N.J.H., T.B., A.B., K.C., J.D.T., M.C., F.M.F., J.S., R.P., V.P.d.R., T.K. and G.S. Visualization: N.J.H., J.S., A.B. and K.C. Writing, original draft: N.J.H., K.C., L.U., M.B. and J.A.R. Writing, review and editing: M.B., J.A.R., D.H., L.U., N.J.H., K.C., L.H., A.B., J.S., J.D.T., F.M.F., A.K.A., A.P., D.G.D., U.B.R.B. and N.N.G. Supervision: M.B., J.A.R., I.M., A.B., D.H. and L.U. Funding acquisition: M.B., J.A.R. and L.H.

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Additional information

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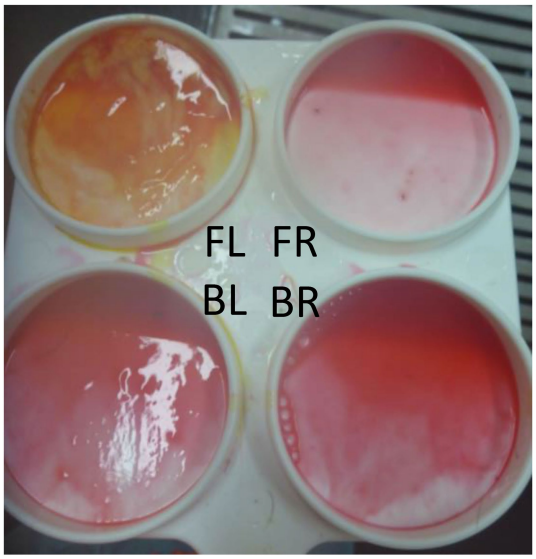
Correspondence and requests for materials should be addressed to Martin Beer or Juergen A. Richt.

Peer review information *Nature* thanks Lars Larsen, Malik Peiris and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer review reports are available.

A



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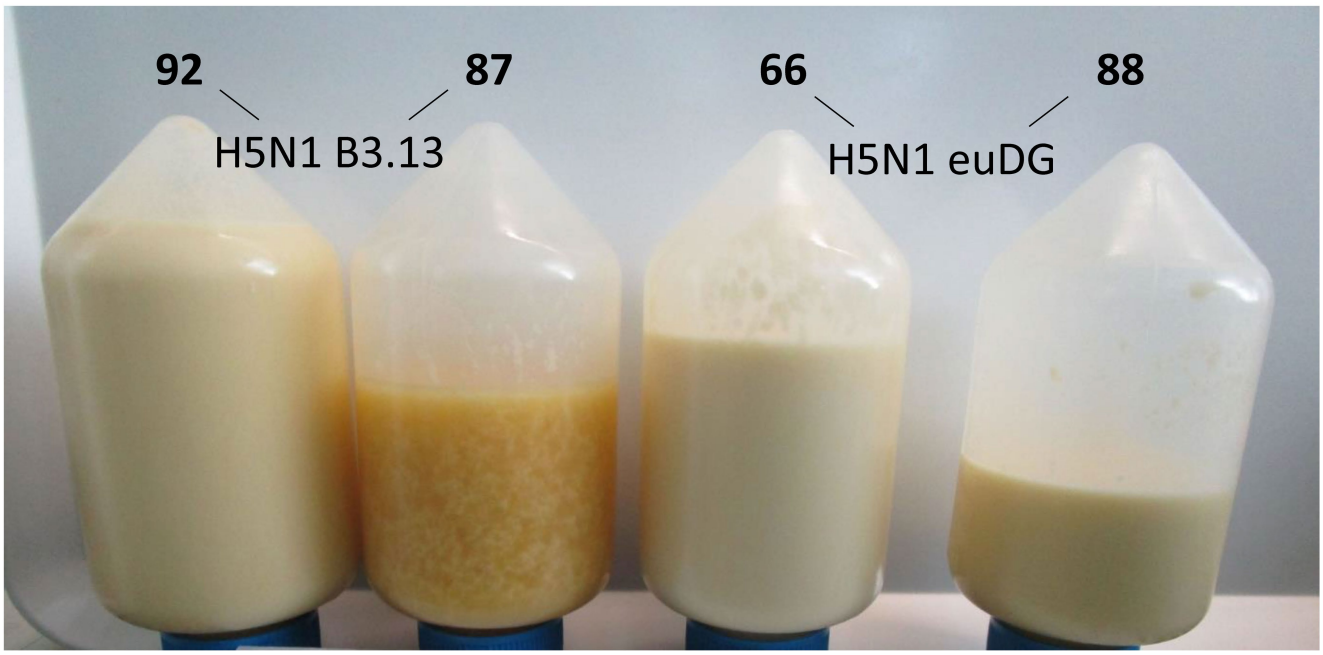
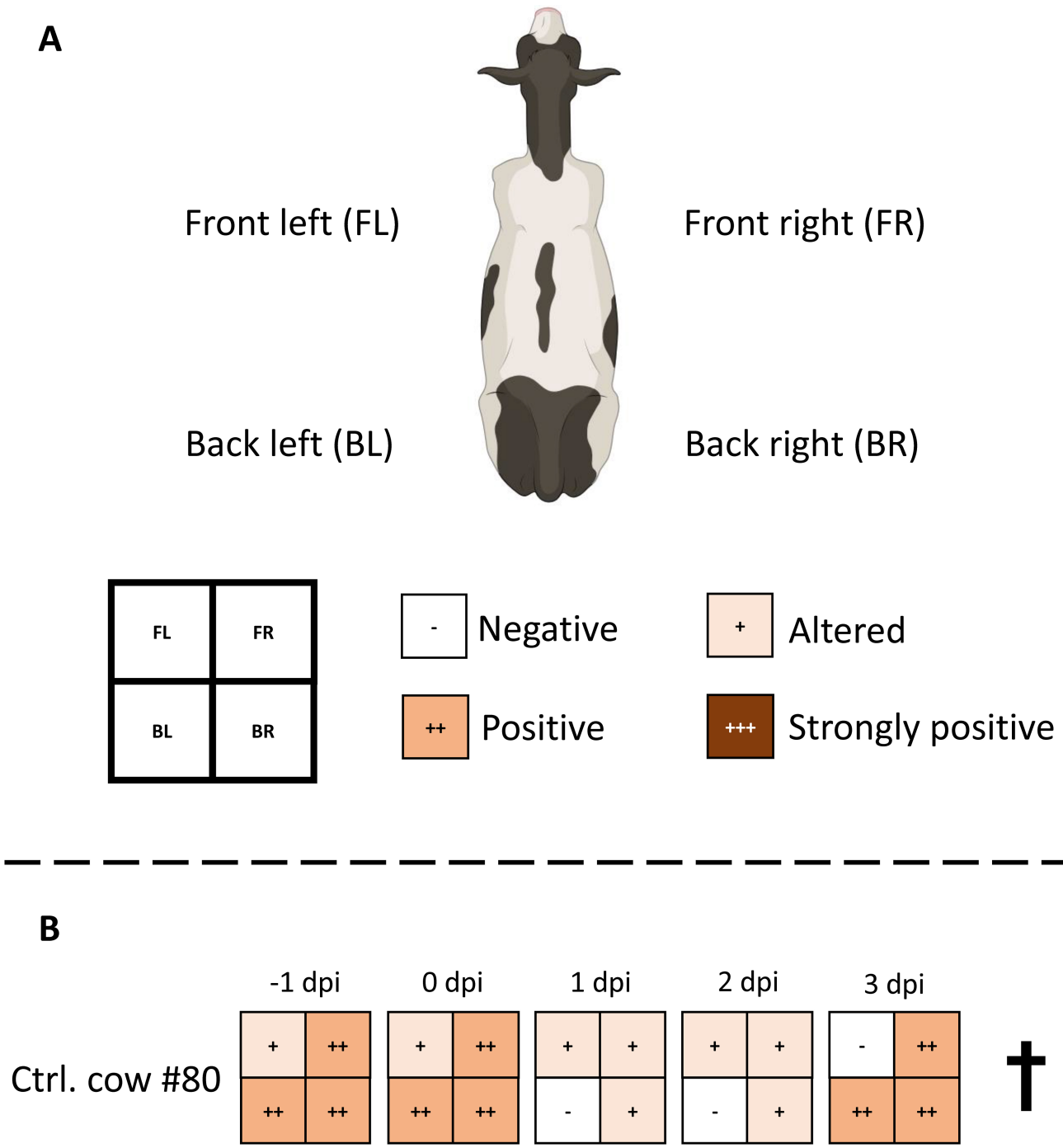
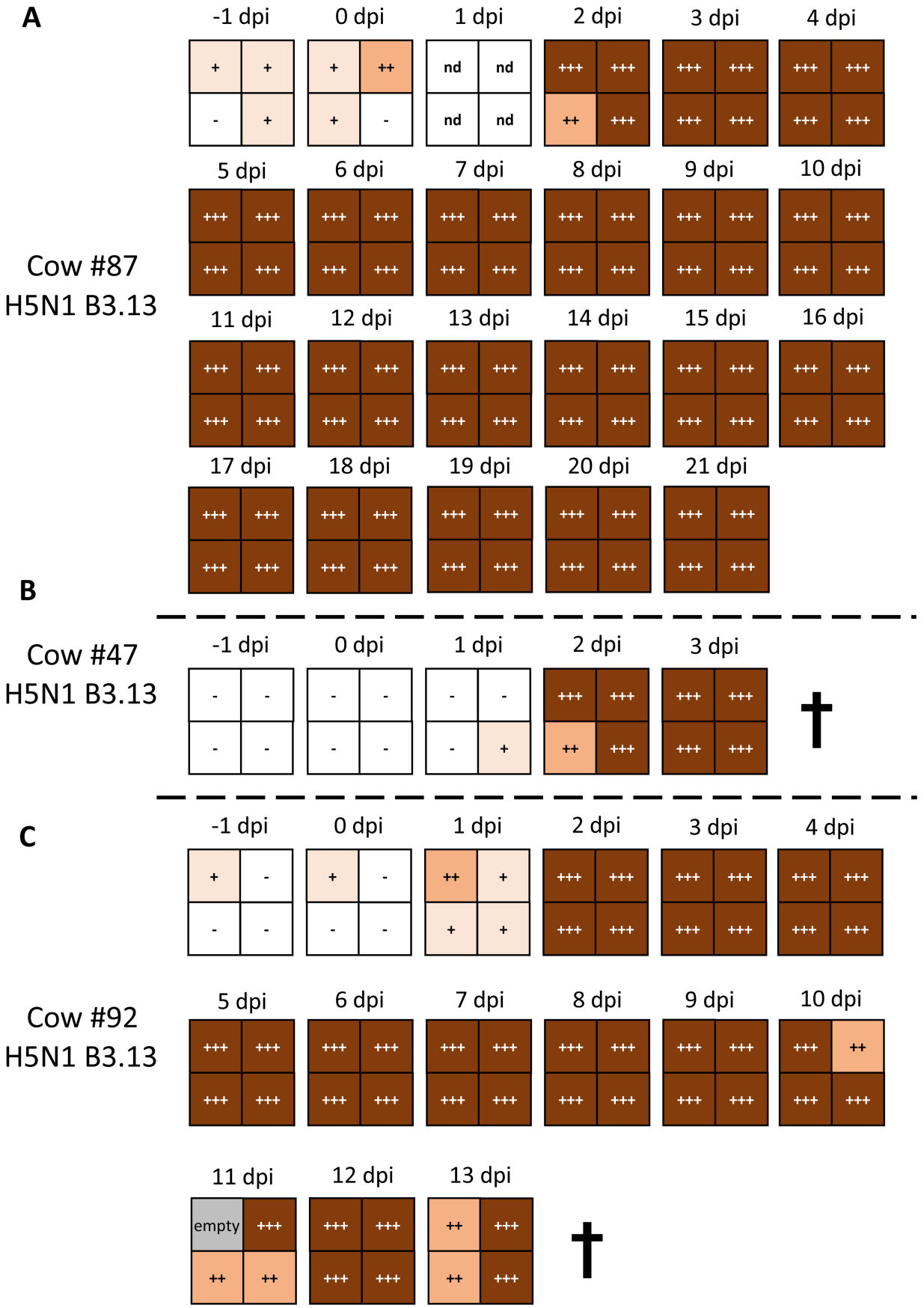


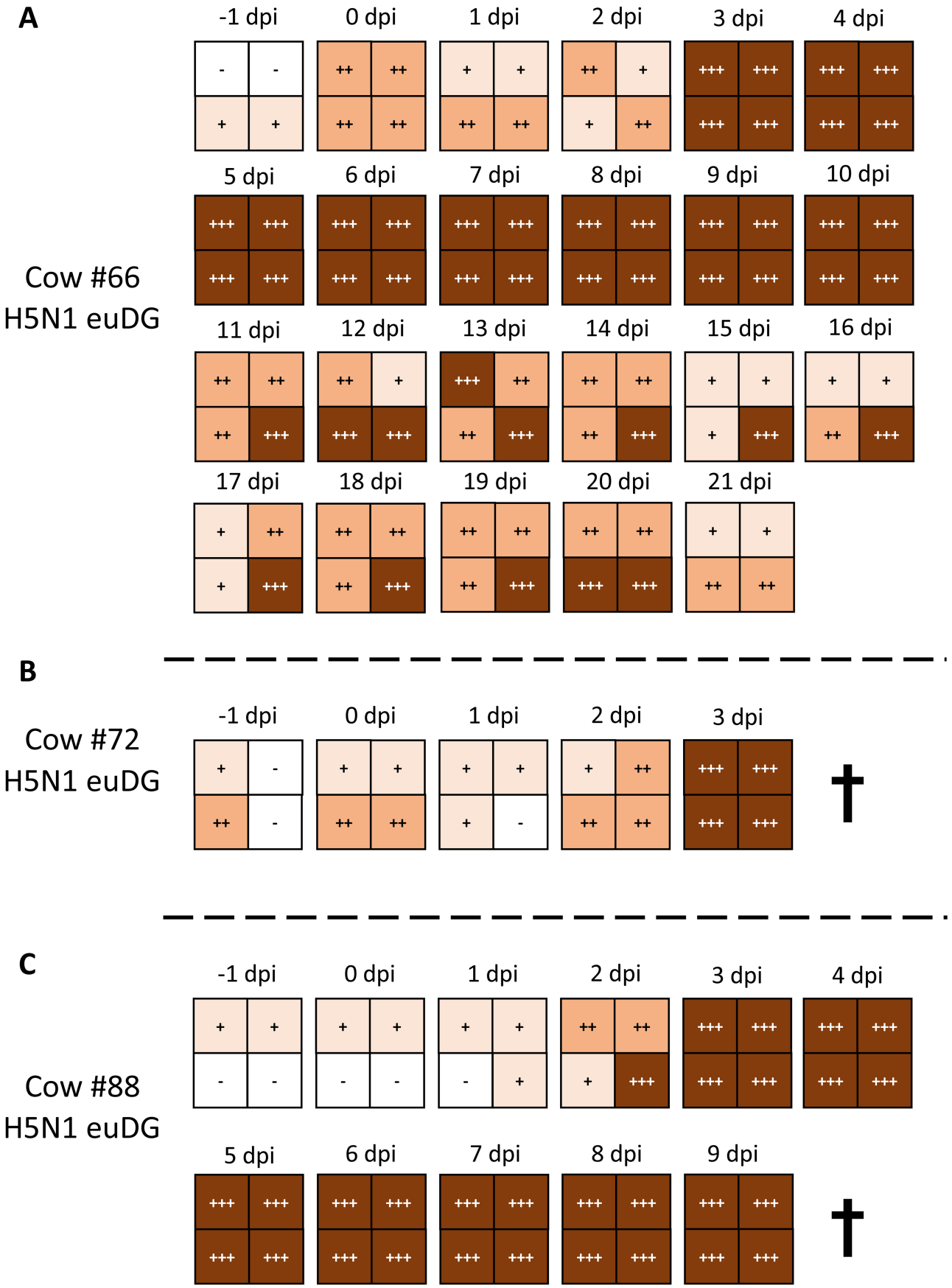
Extended Data Fig. 1 | Exemplary milk consistency and appropriate CMT of H5N1-infected lactating dairy cows during the animal trial. A CMT-picture of an H5N1 euDG-infected lactating dairy cow at 2 dpi. FL = udder front left, FR = udder front right, BL = udder back left, BR = udder back right **B** Milk consistency of H5N1 B3.13 and euDG infected dairy cows during the experiment (4 dpi).



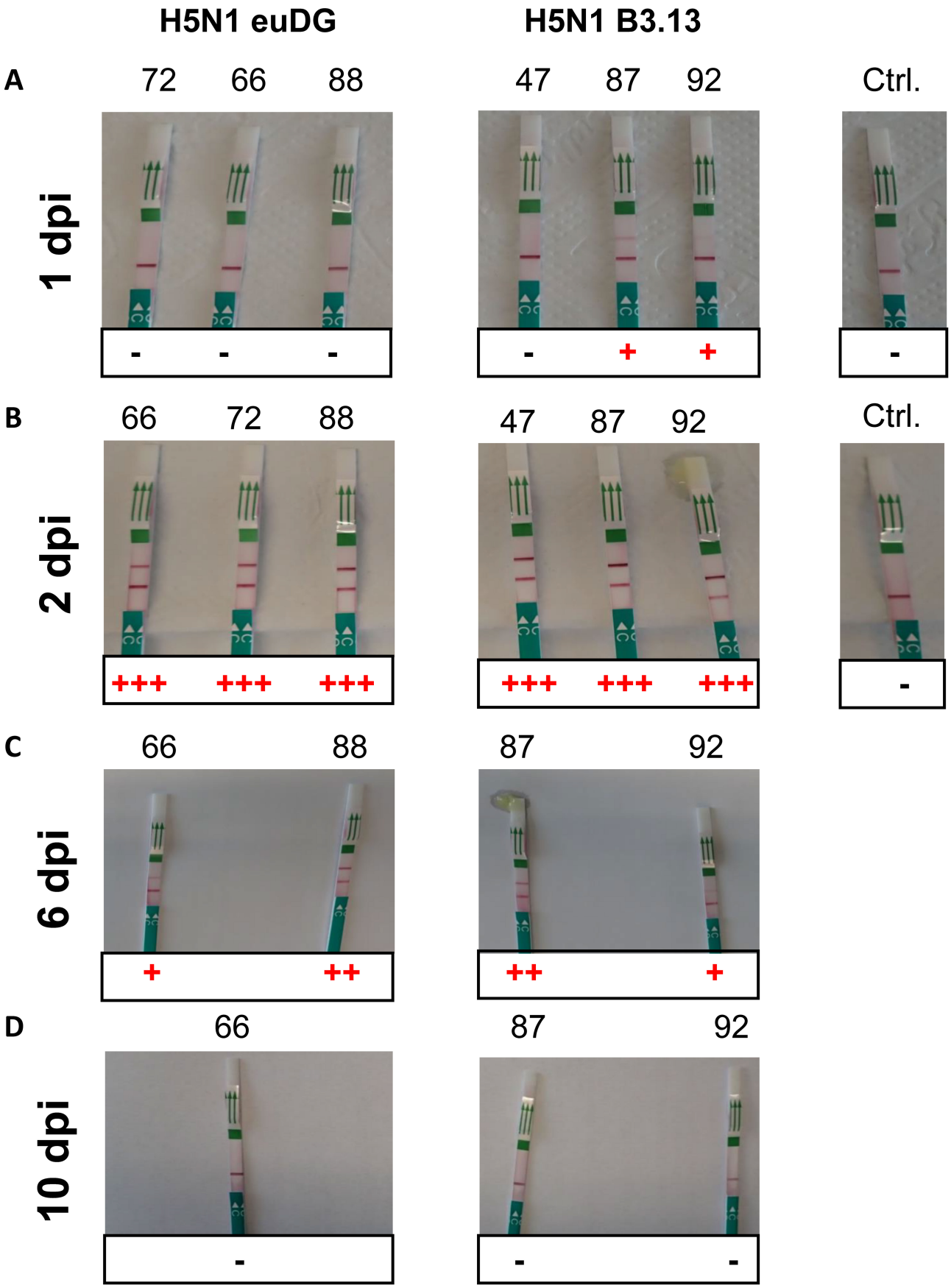
Extended Data Fig. 2 | California Mastitis Test (CMT). A Legend for semi-quantification. Milk samples from individual quarters (front left/right and back left/right were gained and collected on appropriate CMT-plates. CMT-reagent was applied ~1:1 to the milk samples and was graded by eye with the help of a defined template. **B** CMT of milk samples from the uninfected control animal (#80) during the course of the experiment until its euthanasia timepoint.



Extended Data Fig. 3 | California Mastitis Test (CMT) of lactating cows infected with H5N1 B3.13 (US-group). A – C CMT of milk samples from cattle infected with H5N1 B3.13 during the course of the experiment until their respective euthanasia timepoint.

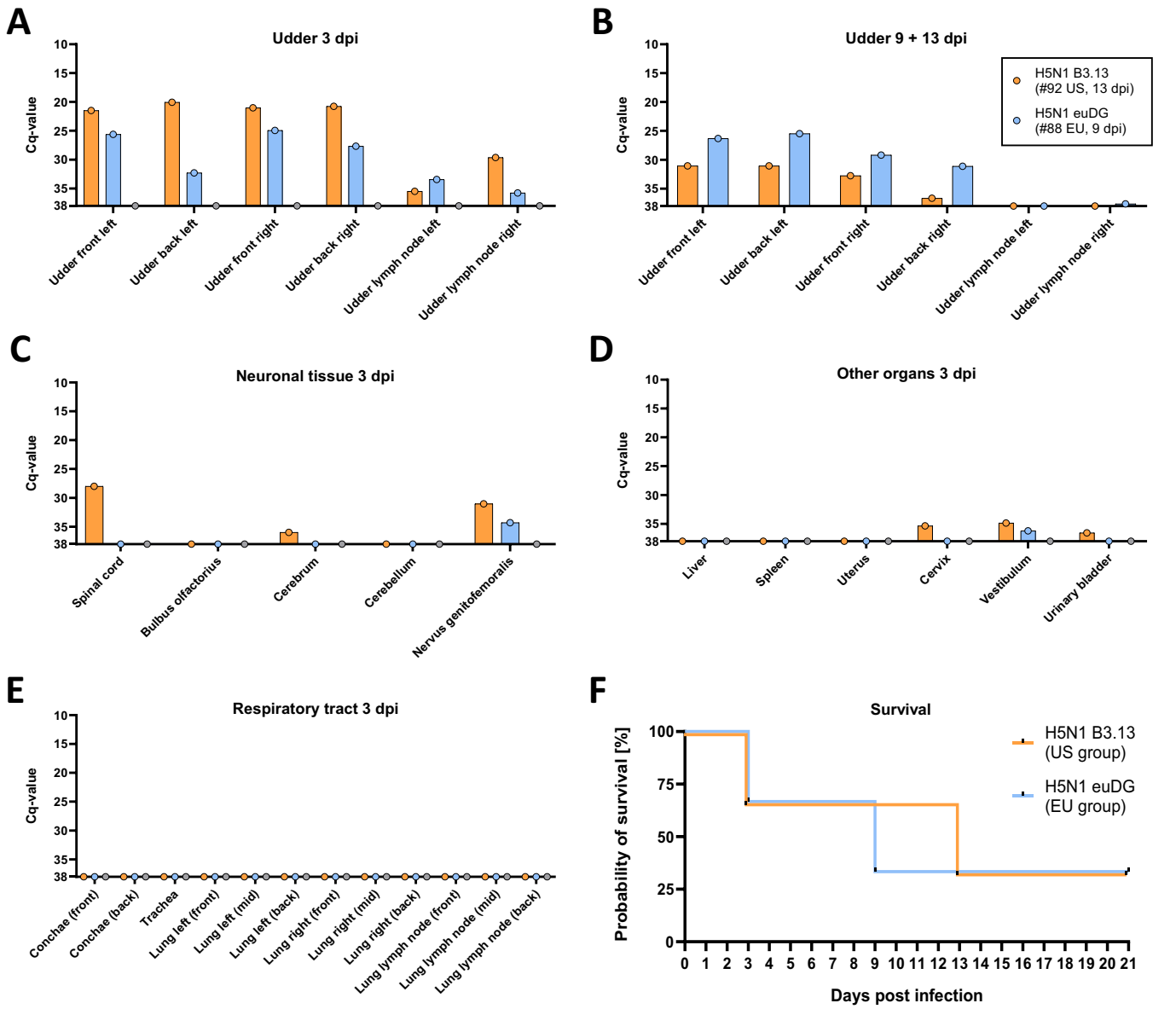


Extended Data Fig. 4 | California Mastitis Test (CMT) of lactating cows infected with H5N1 euDG (EU-group). A – C CMT of milk samples from cattle infected with H5N1 euDG during the course of the experiment until their respective euthanasia timepoint.

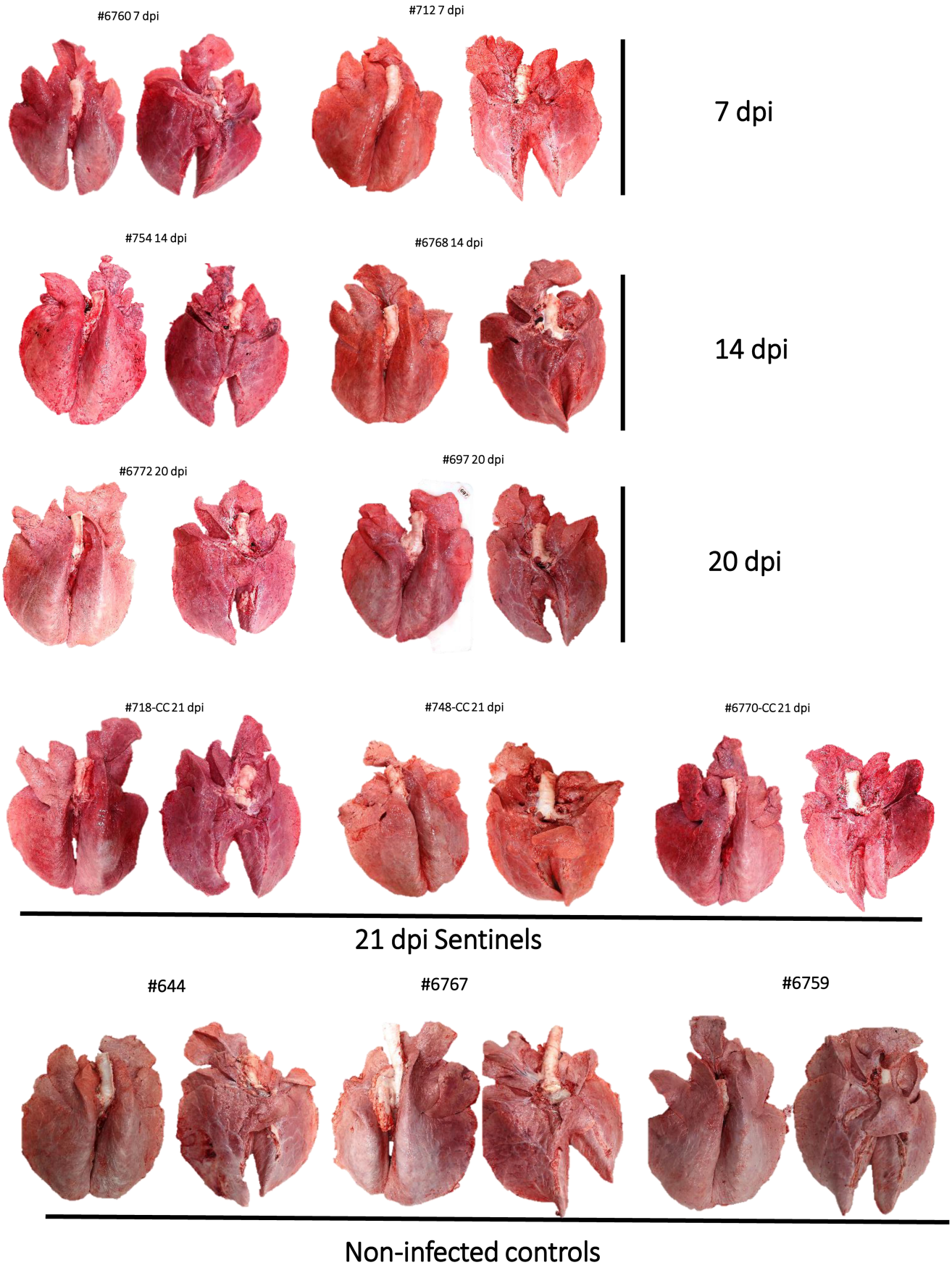


Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Megacor-RAT from milk samples of H5N1 experimentally infected dairy cattle. A H5-specific Megacor-RAT used for milk samples of H5N1-infected cattle at 1 dpi. Positive samples are depicted with a red cross. **B** H5-specific Megacor-RAT used for milk samples of H5N1-infected cattle at 2 dpi. All H5N1-infected lactating dairy cattle have become positive via the H5-specific RAT from Megacor already at 2 dpi, irrespective of the H5N1-virus isolate used. **C** H5-specific Megacor-RAT used for milk samples of H5N1-infected cattle at 6 dpi. **D** H5-specific Megacor-RAT used for milk samples of H5N1-infected cattle at 10 dpi. All cows have become already negative via the RAT at 10 dpi.

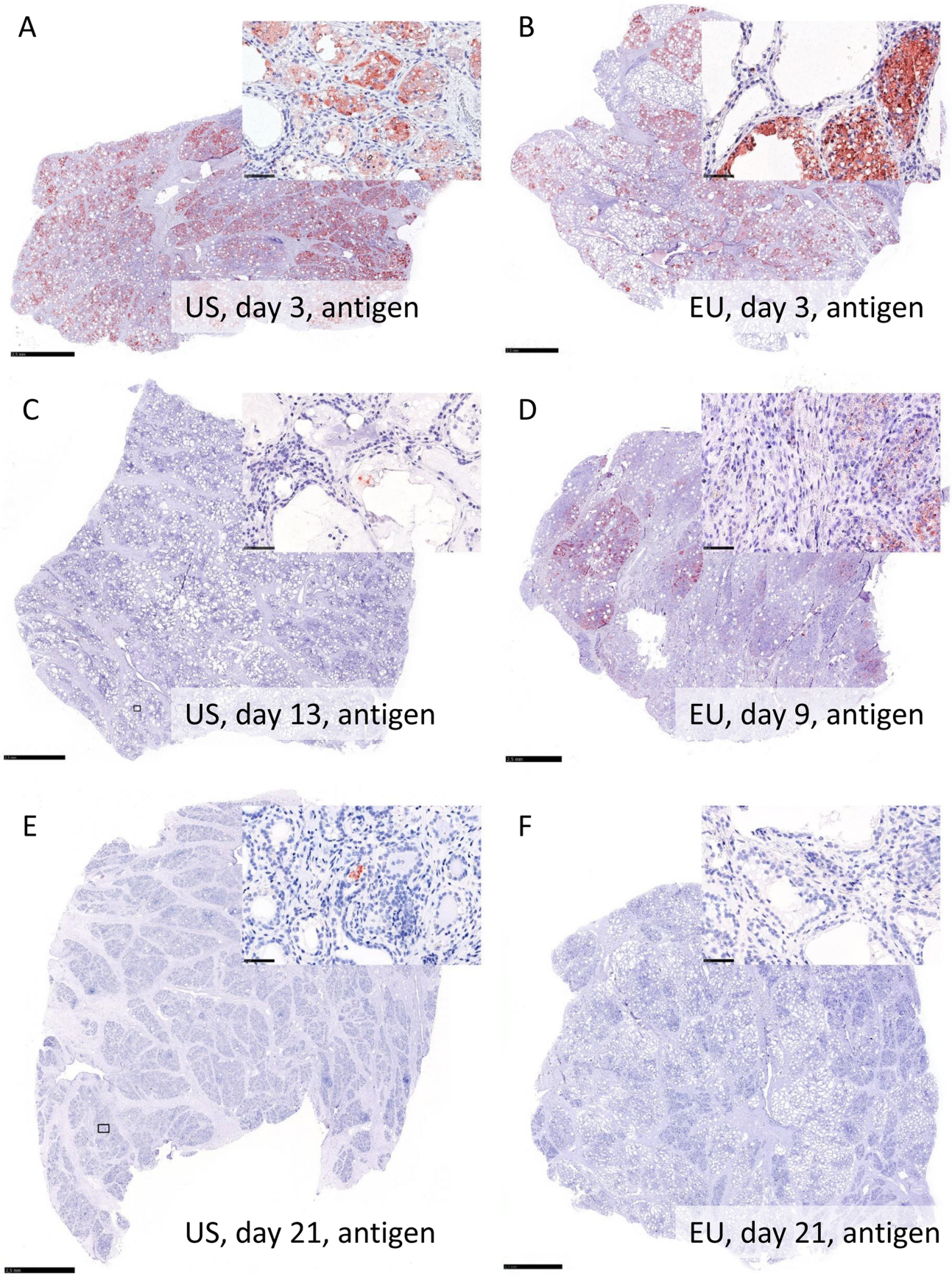


Extended Data Fig. 6 | Viral genome load in organ samples and survival data of lactating cows. Orange: lactating cows infected with H5N1 B3.13. Blue: Lactating cows infected with H5N1 euDG. Grey: Uninfected negative control cow. **A** Viral genome load in udder organ samples of lactating cows euthanized at 3 dpi (#72 EU, #47 US, #80 Ctrl.) **B** Viral RNA load in udder organ samples of lactating cows euthanized at 9 dpi (#88 EU) or 13 dpi (#92 US). **C** Viral genome load in organ samples from neuronal tissues. **D** Viral RNA load in other internal organs of lactating cows. **E** Viral genome load in organ samples of the respiratory tract. **F** Survival curve of lactating cows over the course of the experiment.



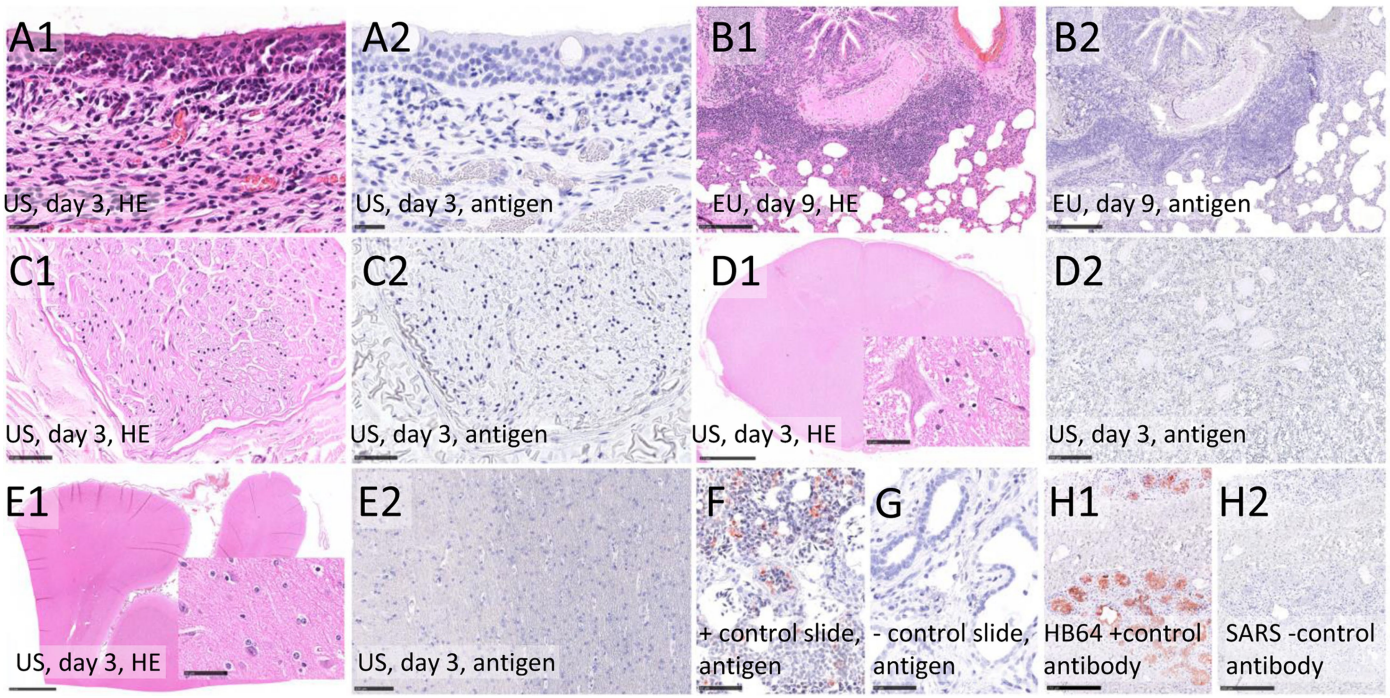
Extended Data Fig. 7 | See next page for caption.

Extended Data Fig. 7 | Gross lung pathology of calves. (**A**) At 7 dpi, multiple well-defined pulmonary lobules were red and slightly depressed on the right cranial lobe (congestion and partial atelectasis) affecting approximately 20% of the cranial and caudal portions of the right cranial lobe extending into the right middle and caudal lobe of one of the two principal-infected calves (#712). There was a focal area of mild subpleural hemorrhage on the ventral surface of the left caudal lung lobe of animal #6760. (**B**) At 14 dpi, one of the two principal-infected calves (#754) had multifocal to coalescing red and depressed foci of congestion and atelectasis on the left and right cranial lobes. Approximately 60% of the caudal portion of the left cranial lobe, 55–60% of both the cranial and caudal portions of the right cranial lobe and <5% of the accessory lobe were affected. There were also multiple pleural adhesions to the thoracic wall. (**C**) At 20 dpi, the two principal-infected calves (#6772 and #697) had either few small red and slightly depressed foci of congestion and atelectasis on the left cranial lobes (#6772), or a focal, similar area on the apical portion of the right middle lobe (#697). (**D**) Postmortem examinations of the three sentinel animals were performed at 21 dpi and revealed scattered red foci of pulmonary congestion/atelectasis. In animal #748, there were multiple, small foci of mild consolidation in the left and right cranial lobes (5% of lung affected) and few pleural adhesions to the thoracic cavity. For animal #6770, congestion and atelectasis were accompanied by mild to moderate edema affecting predominately the right lung lobes. (**E**) One of the three negative control calves (#6767) had a small isolated focus of consolidation of the pulmonary parenchyma at the apical margin of the right middle lobe. Gross lesions were not appreciated in the remaining negative control animals.

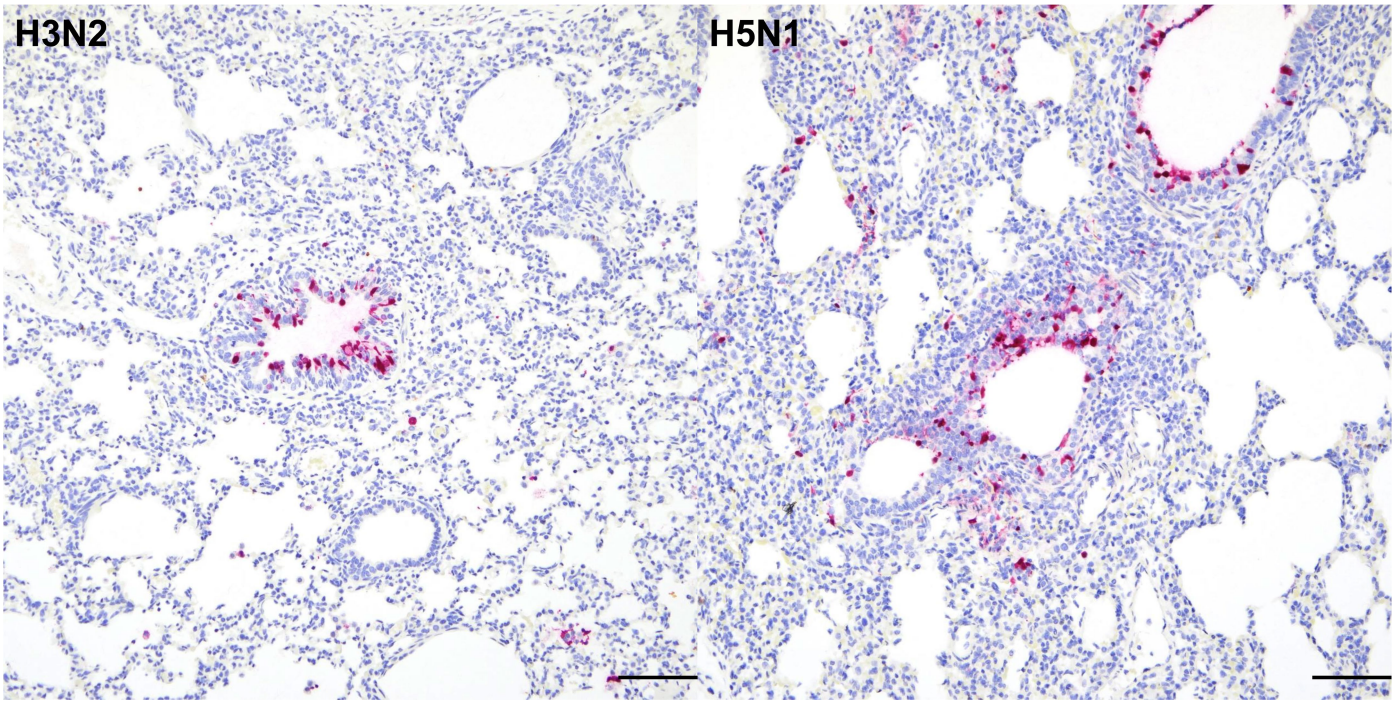


Extended Data Fig. 8 | See next page for caption.

Extended Data Fig. 8 | Influenza A virus nucleoprotein detection using immunohistochemistry in the mammary gland of cattle after intramammary infection with H5N1 B3.13 and H5N1 euDG. The distribution was graded on an ordinal scale with scores 0 = no antigen, 1 = focal, affected cells/tissue <5% or up to 3 foci per tissue; 2 = multifocal, 6%–40% affected; 3 = coalescing, 41%–80% affected; 4 = diffuse, >80% affected. Representative pictures were taken from the most severely affected quarter from each cow. A Score 4, H5N1 B3.13, 3 dpi. B Score 3, H5N1 euDG, 3 dpi. C Score 1, H5N1 B3.13, 13 dpi. D Score 3, H5N1 euDG, 9 dpi. E Score 1, H5N1 B3.13, 21 dpi. F Score 0, H5N1 euDG, 21 dpi. Scale bar 2.5 mm and 50 µm (inlay).



Extended Data Fig. 9 | Histopathology and Influenza A virus nucleoprotein detection (antigen) of cattle after intramammary infection with H5N1 B3.13 and H5N1 euDG including tissue controls. A Nasal concha: Chronic-active rhinitis (A1) lacking IAV antigen (A2). **B**: Lung: Chronic bronchointerstitial pneumonia in convalescence phase (B1), lacking IAV NP (B2). **C** Genitofemoral nerve: No findings (C1), no IAV antigen (C2). **D** Spinal cord: No findings (D1), no IAV antigen (D2). **E** Brain, cortex: No findings (E1), no IAV antigen (E2). **F** Positive control slide, HPAIV infected chicken, lung: abundant IAV antigen. **G** Negative control slide, uninfected cow, mammary gland: no IAV antigen. **H1** Mammary gland: cow infected with H5N1 B3.13, 3 dpi, abundant IAV antigen. **H2** Consecutive slide of H1: an irrelevant antibody (anti Sars clone 4F3C4) yielded no immunopositive reaction. Hematoxylin and eosin (HE) stain (**A1, B1, C1, E1**) and immunohistochemistry (antigen) on consecutive (**A2, B2, C2, E2, H1, H2**) or independent (**F, G**) slides. Scale bar 25 µm (**A1-2**), 50 µm (**C1-2**, inlay **D1, E1, F, G**), 100 µm (**D2, E2, H1-2**), 250 µm (**B1-2**), 2.5 mm (**D1, E1**).



Extended Data Fig. 10 | Tissue controls used for immunohistochemistry. The anti-NP antibody used strongly labels influenza virus A H3N2 and H5N1-infected epithelial cells lining bronchioles.

Extended Data Table 1 | IAV-specific antibodies present in the milk of lactating cows following intramammary inoculation with HPAIV H5N1 clade 2.3.4.4b

	Cow ID		Assay	dpi -1	0-6	7	8	9	10	11	13	15	17	19	21
H5N1 B3.13 lactating cows	87	milk	H5	-	-	-	-/+	-/+	+	+	+	+	+	+	+
			VNT_{100} (B3.13)		<16	<16	<16	<16	<16	<16	256	512	406	323	203
			VNT_{100} (euDG)		<16	<16	<16	<16	<16	128	256	323	406	323	256
	92	milk	H5	-	-	-	-	-/+	+	+	+				
			VNT_{100} (B3.13)		<16	<16	<16	<16	<16	256	203				
			VNT_{100} (euDG)		<16	<16	<16	<16	81	256	406				
	47	milk	H5	-	-										
			VNT_{100} (B3.13)												
			VNT_{100} (euDG)												
H5N1 euDG lactating cows	66	milk	H5	-	-	-	-	-/+	+	+	+	+	+	+	+
			VNT_{100} (B3.13)		<16	<16	<16	51	161	256	512	406	323	128	256
			VNT_{100} (euDG)		<16	<16	<16	<16	128	406	322	256	102	128	128
	88	milk	H5	-	-	-		-							
			VNT_{100} (B3.13)		<16	<16	<16	<16							
			VNT_{100} (euDG)		<16	<16	<16	<16							
	72	milk	H5	-	-										
			VNT_{100} (B3.13)												
			VNT_{100} (euDG)												
Negative control lactating cow	80	milk	H5	-	-										
			VNT_{100} (B3.13)		<16										
			VNT_{100} (euDG)		<16										

Hemagglutinin H5-subtype (H5) specific cELISA; positive (+), doubtful (−/+), negative (−); virus neutralization assay (VNT). Milk collected from H5N1 clade 2.3.4.4b intramammary infected lactating cows was evaluated using a commercially available competitive ELISA (cELISA) kit targeting the H5 subtype hemagglutinin protein. Neutralizing antibody titers for each group were also evaluated using bothB3.13 and H5N1 euDG genotypes. Results of the cELISA were calculated as sample OD450/negative control OD450 percentage (S/N%), represented here as positive (+; S/N% ≤ 50%), doubtful (−/+; 50% < S/N% < 60%), and negative (−; S/N% > 60%). The relative titers of H5N1-specifc neutralizing antibodies are reported as the reciprocal of the final dilution of neutralizing dose 100 (VNT_{100}) was observed.

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Corresponding author(s): Prof. Dr. Martin Beer
Prof. Dr. Juergen Richt

Last updated by author(s): Aug 1, 2024

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☐	☒	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
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☒	☐	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.*
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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	FLI-ELISA: Tecan i-control 2014 1.11 KSU-ELISA: Varioskan LUX (Thermo Scientific, Cat. No. N16044) KSU RT-qPCR: Bio-Rad CFX96 Optics Module C1000 Touch (BioRad Laboratories inc, Hercules, California, USA) KSU NGS: Illumina NextSeq 550 (Illumina, San Diego, CA, USA, Cat No. SY-415-1002)
Data analysis	Relative quantification: Bio-Rad CFX Maestro 1.1 Version 4.1.2433.1219 Relative quantification (KSU): Bio-Rad CFX manager 3.1.1517.0823 Sequence analysis (FLI): Geneious Prime®2024.0.5 Sequence analysis (KSU): CLC Genomics Workbench version 23.0.5 Figures: GraphPad Prism 8.4.2 (679) for Windows, Microsoft PorwerPoint 2016 (16.0.4266.1001) ELISA (FLI): Microsoft Excel 2016 (16.0.5188.1000) ELISA (KSU): Thermo Scientific™ SkanIt™ Software for Microplate Readers (Version 6.0, Cat. No. N16243) and Microsoft Excel 2016 (16.0.5188.1000).

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- 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

Consensus sequences of both isolates used for inoculation are available in the INSDC under accession PQ241097-PQ241104 (calves) and under PQ106994-PQ107009 (cows; H5N1 B3.13: PQ106994-PQ107001; H5N1 euDG: PQ107002- PQ107009). Raw data were filed to the SRA under project number PRJNA1141392.

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	Not applicable
Reporting on race, ethnicity, or other socially relevant groupings	Not applicable
Population characteristics	Not applicable
Recruitment	Not applicable
Ethics oversight	Not applicable

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

Field-specific reporting

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Life sciences study design

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

Sample size	FLI: Sample size was determined using in-house protocols and are stated in the respective Methods section. Sample sizes of animals were approved by a certified statistician (governmental approval under the registration number 7221.3-2-010/23). KSU: The number of calves enrolled in this study was determined by ethical guidelines (IACUC) pertaining to the space alloted per animal in holding facilities. The number of calves allocated to each group was based on priority of study goals while maintaining a minimum number in each group so that statistical comparisons could be made if present.
Data exclusions	No data were excluded from analysis
Replication	Studies performed here involved animals of a defined group size mentioned in the methods section. Therefore, independent replicates of the animal studies were not performed due to ethical considerations (i.e limiting the number of animals used; 3 R's). Experiments were performed according to best practices and as described in the methods.
Randomization	PCR-analysis and ELISA do not require randomization. Samples from individual animals of approximately the same age were collected and measured at specific time points and reported for respective individual animals. Animals were randomly assigned to the respective study groups, or semi-randomly assigned based on sex, as stated in the materials and methods.
Blinding	Blinding was not necessary. This was an observational study to evaluate the clinical and virological progression of individual animals after infection or exposure.

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

H16-L10-4R5 primary antibody (ATCC HB-65, Manassas, VA; undiluted)
goat anti-mouse IgG (H+L) secondary antibody (Alexa-488, Fisher Scientific, Waltham, MA, CAT#A11001; 1:1000)
rabbit polyclonal anti-Influenza A virus NP (Cell Signaling Technology, #99797/F8L6X; 1:1,200)
goat anti-rabbit IgG (Polymer Refine Red Detection kit - Leica Biosystems; ready to use)
primary antibody against the IAV nucleoprotein (ATCC clone HB-64, 1:200)
secondary biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA, BA-9200-1.5)
anti Sars clone 4F3C4 (doi: 10.1016/j.virusres.2006.07.005, 1:45)

Validation

H16-L10-4R5 primary antibody (ATCC HB-65, Manassas, VA) was described and validated previously (doi:10.1128/JVI.05705-11)
goat anti-mouse IgG (H+L) secondary antibody was described and validated previously (doi:10.1080/22221751.2024.2353292)
rabbit polyclonal anti-Influenza A virus NP [ThermoFisher Scientific, PA5-32242] was described and validated previously (doi:10.1080/22221751.2024.2353292) and shown in extended data figure 9.
goat anti-rabbit IgG (Polymer Refine Red Detection kit - Leica Biosystems) was described and validated previously (doi: 10.1080/22221751.2024.2353292) and shown in extended data figure 9.
primary antibody against the IAV nucleoprotein (ATCC clone HB-64) was validated previously (doi: 10.1038/s41426-018-0204-0)
secondary biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) was validated previously (doi: 10.1038/s41426-018-0204-0)
anti Sars clone 4F3C4 was described and validated previously (doi:10.1016/j.virusres.2006.07.005 (2006)

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)

MDCK type II cells: Collection of Cell Lines in Veterinary Medicine CCLV RIE 1061
bovine uterine epithelial cells (CAL-1; In-house, Cornell via Diego Diel)

Authentication

in-house authentication for cell lines was not performed

Mycoplasma contamination

in-house Mycoplasma exclusion is performed regularly

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified lines were used.

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

Twelve Holstein calves, 5-6 months of age, 3 male and 2 female, 1 hermaphrodite
Seven female multiparous lactating Holstein-Friesian dairy cattle in an age range between four and eight years, at a state of decreasing milk production, and around 12 months after last calving

Wild animals

No wild animals were used in this study

Reporting on sex

An equal number of male and female calves were ordered, but sex did not appear to influence the outcome observed in this study. Only female cows were used due to the fact that consequences for milk production in terms of an H5N1 infection were only able to be seen in female lactating cows.

Field-collected samples

Field samples were not collected.

Ethics oversight

All experiments conducted at Kansas State University were approved and performed under the Kansas State University (KSU) Institutional Biosafety Committee (IBC, Protocol # 1758) and the Institutional Animal Care and Use Committee (IACUC, Protocol #

4992) in compliance with the Animal Welfare Act.
The lactating dairy cattle experiment was evaluated by the responsible ethics committee of the State Office of Agriculture, Food, Safety, and Fishery in Mecklenburg–Western Pomerania (LALLF M-V) and gained governmental approval under the registration number 7221.3-2-010/23.

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

Plants

Seed stocks

Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.

Novel plant genotypes

Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.

Authentication

Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.