Title: Surveillance on California dairy farms reveals multiple sources of H5N1 transmission

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Abstract (118 WORDS)

Transmission routes of highly pathogenic H5N1 between cows or to humans remain unclear due to limited data from affected dairy farms. We performed extensive air, farm wastewater, and milk sampling on 14 H5N1 positive dairy farms across two different California regions. Virus was detected in the air in milking parlors and from exhaled breath of cows. Infectious H5N1 virus was detected in the air and water streams; sequence analysis revealed viral variants on a farm in these locations. Longitudinal analysis of milk from the individual quarters of cows revealed a high prevalence of subclinical H5N1 positive cows and a heterogeneous distribution of infected quarters that maintained a consistent pattern over time. Our data highlight potential modes of H5N1 transmission on dairy farms.

Main text

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 clade 2.3.4.4b B3.13 virus was first detected in dairy cattle in March of 2024 and has since spread to 16 states [1–3]. California, the largest dairy producing state in the US, detected positive herds in August of 2024 [4]. As of June 3, 2025, 767 herds have tested positive in California [5]. High viral loads in milk from H5N1-infected cows, the persistence of H5N1 on milking equipment, and reports of infection following milk-based eye splashes in dairy workers has led to the general consensus that direct contact with unpasteurized milk is a predominant mode of H5N1 transmission between cows and to humans on dairy farms[6–9]. Additionally, environmental sampling conducted on dairy farms with H5N1 infected cows detected the presence of viral RNA on multiple surfaces within the milking parlor, where both cows and humans have closest contact with unpasteurized milk [10].

While direct contact with contaminated milk may contribute to H5N1 spread on dairy farms, aerosol-based transmission cannot be ruled out. Aerosolization of unpasteurized milk in dairy milking parlors is likely generated during expression of the first milk, termed forestripping, and other milking procedures. Activities within dairy parlors increase the risk of transmission via direct contact with mucus membranes and contamination of surfaces as particles settle out of the air[3]. Both naturally infected and experimentally infected cows have been reported to harbor low levels of virus in the nasal cavity [1,7,11–13], which could produce air particles that are capable of spread to other cows or humans. Whether such H5N1 exist in the air on dairy farms and promote transmission is currently unclear, but understanding their role is critical to developing strategies that mitigate all the modes of virus spread on a farm.

Another potential source of H5N1 transmission between cows could be the use of reclaimed farm water contaminated with both bucket-collected milk from sick animals and clean water used to spray down the milking parlor between milking sessions. Milk from cows with mastitis or other clinical signs, which does not enter the commercial milk production system, is often disposed of in the same system. In this manuscript, any reference to 'wastewater' streams refers to the reclaimed farm water collected through these various processes and held in large manure lagoons on farm premise or sent to fields. This reclaimed wastewater can be further used at multiple sites on a farm, such as irrigation of fields and flushing of housing pens. Both processes could increase the presence of H5N1 on surfaces and generate virus-laden aerosols. Wastewater in lagoons can also be a water source for migratory birds and peridomestic animals that may become infected through exposure to the virus. Given the many uses of farm wastewater, determining viral loads in these waste streams is vital.

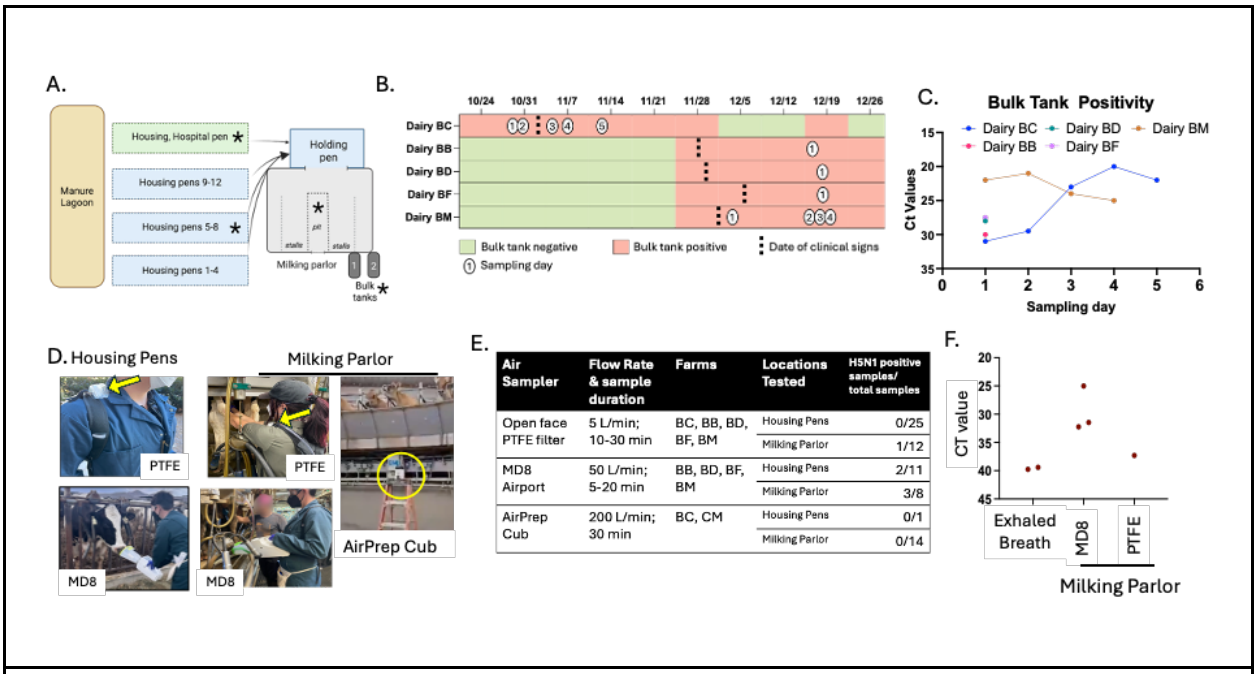
In this study, we describe the results of extensive sampling efforts at fourteen H5N1-positive dairy farms. We report detection of virus in the air during milking of suspected H5N1-infected cows on multiple farms and on multiple days, and in the exhaled breath of cows, suggesting that airborne transmission may serve as a mode of H5N1 spread between cows and to humans in dairy parlors. We also detected H5N1 virus in farm wastewater, which could contribute to the continued spread of H5N1 between cows to people and other wildlife. Finally, we sampled

milk from individual quarters (also referred to as udders) of cows on a single farm over several days to determine the distribution of H5N1 positivity over time. We found that the presence of mastitis, a noted clinical sign associated with H5N1 infection[2], did not always correlate with H5N1 positivity. In addition, mastitis presented in a predominant quarter of the cows while H5N1 quarter positivity varied by animal, but all no single quarter was over-represented with presence of H5N1 virus. Together our data identify multiple potential modes of cow-to-cow transmission of H5N1 on dairy farms.

Results

Detection of H5N1in the air

During the winter of 2024, five dairy farms in the Central Valley of California identified as having HPAI H5N1 positive cows were enrolled in our initial air sampling studies (Supplemental Table 1 and Figure 1A for general dairy farm layout). In the first phase of our study, we compared a variety of air sampling strategies during the active outbreak on dairy farms to define the optimal methodology that could be implemented in future sampling efforts. Aggregated milk from cows, referred to as bulk tank milk, was tested frequently for the presence of H5N1 viral RNA from late October 2024 until January 4, 2025 per federal recommendations [14]. The date that each farm tested positive and first observation of clinical signs are depicted in Figure 1B. H5N1 was detected in bulk tanks on farms prior to the observation of clinically ill cows. Ct values from bulk tank on the day of sampling performed in this study are shown in Figure 1C. Three different air sampling devices were tested in this initial phase (Figure 1D and 1E and Methods): 1) open-face PTFE filter cassettes connected to an air sampling pump; 2) Sartorius MD8 Airport; and 3) AirPrep Cub 210. Open-face PTFE filter cassettes were worn on a backpack to model exposure to facility workers. A cone around the MD8 airport or open-face PTFE filter was used to minimize dilution of aerosols and droplets from the source (Figure 1D). On each farm, aerosols and droplets were collected from exhaled breath from individual cows or rows of cows, within milking parlors during milking, and within housing areas using a variety of these aerosol sampling devices. Seventy-one air samples were collected and analyzed for the presence of H5N1 viral RNA (Figure 1E and Supplemental Table 2). Six samples tested positive: five with the MD8 Airport sampler and one from an open face PTFE sampler worn during milking in the milking parlor (Figure 1E and Extended Table 2). Based on these observations, further studies were conducted primarily using the MD8 Airport sampler on additional dairy farms. Four positive samples were collected in the milking parlor and two, very weakly positive samples, were obtained from the exhaled breath of rows of 15-30 cows in head stanchions (Figure 1F),



Air Sampler	Flow Rate & sample duration	Farms	Locations Tested	H5N1 positive samples/ total samples
Open face PTFE filter	5 L/min; 10-30 min	BC, BB, BD, BF, BM	Housing Pens	0/25
			Milking Parlor	1/12
MD8 Airport	50 L/min; 5-20 min	BB, BD, BF, BM	Housing Pens	2/11
			Milking Parlor	3/8
AirPrep Cub	200 L/min; 30 min	BC, CM	Housing Pens	0/1
			Milking Parlor	0/14

Figure 1: **Successful detection of H5N1 viral RNA in the air in milking parlors in the Central Valley of California using the MD8 Airport sampler.** (A) Top-down schematic of a representative dairy farm layout with multiple pens housed together under open-air structures, a separate hospital pen area, and a holding area where cows are moved prior to milking in the parlor. Areas with asterisks denote spaces where air samples were collected. Arrows indicate the movement of cows from housing areas to holding pen, then to the milking parlor. Milk is aggregated into bulk tanks. 'Sick' cows, with clinical signs such as reduced appetite, fever, mastitis, or nasal discharge, were moved to a hospital pen and milked last into individual buckets; their milk was excluded from the bulk tank (also commercial supply) and entered the farm wastewater stream. Schematic created with BioRender.com. (B) Timeline of sampling during initial phase on dairy farms, including date the bulk tank tested positive, bulk tank status, onset of clinical signs in cows, and sampling days numbered 1-5. (C) Ct values for H5N1 viral RNA from bulk tank samples on each sampling day. (D) Photos depicting each type of air sampler. Left to right, open-face PTFE filters in use on the backpack while in a housing pen and milking parlor as indicated (yellow arrows); the MD8 Airport with a cone, to reduce dilution of the air, sampling the breath of a cow in a housing pen and during milking in the parlor; the AirPrep Cub (yellow circle) was placed either on the ground or was elevated to about six feet above the ground in milking parlors. (E) Summary of air sampling methods and H5N1 positive samples from the five dairy farms. (F) Ct values (average of duplicates) from the H5N1 positive air samples. Exhaled breath samples also collected using the MD8 Airport sampler.

Infectious aerosols in milking parlors

Based on the successful initial sampling phase, additional surveillance was performed on nine farms, with in-depth focus on three farms in southern California during their outbreaks in

February and March 2025 (Supplemental Tables 1, 4-6 and Figure 2A). On these farms, air samples from the milking parlor and exhaled breath cows in housing pens was collected as previously described, using the MD8 Airport sampler fitted with a cone (Figure 1D). Sampling was conducted within days of each farm testing positive in the California state bulk tank testing program. Additional, bulk tank milk samples from two of the three farms was collected on multiple days by research staff during the sampling time frame, highlighting the viral load of the herd during sampling (Figure 2B; farms EC, EG). The number of cows per herd was recorded for each farm (Supplemental Table 1), but the number of infected cows per herd was unknown so sampling was targeted to milking of pens containing cows with known clinical signs.

H5N1 viral RNA was detected in 21 of 35 air samples (Figure 2C and Extended Tables 5 and 6). Samples are presented as genome copies per liter of air and range from 1 to 10,000. Exhaled breath was collected from rows of cows within the hospital pen on farm EC, and two samples tested positive with 4-41 genome copies / L of air (C_{air}) (Figure 2C). For comparison, other studies of influenza A virus in air in around livestock reported concentrations ranging from 7 to 440 gc/L in poultry barns or markets[15–19] and an average of 142-320 gc/L in swine barns[20,21]. Particle sizes generated in milking parlors were quantified in two methodologies to capture small aerosols (0.3µm to 25µm) and larger particles by qualitative analysis of droplets collected on the MD8 gelatin filter (Supplemental Figure 1), and reveal that both submicron aerosols and larger aerosols are likely produced during milking and could contain infectious virus. All positive samples collected on EC and EG were subject to viral titration, and infectious virus was detected in four samples with C_{air} values >1000 collected with the MD8 Airport in a milking parlor (Figure 2D). Together, these results indicate that H5N1 viral RNA can be found in the air on farms and conclusively demonstrates that infectious H5N1 virus is present in the air during the milking process.

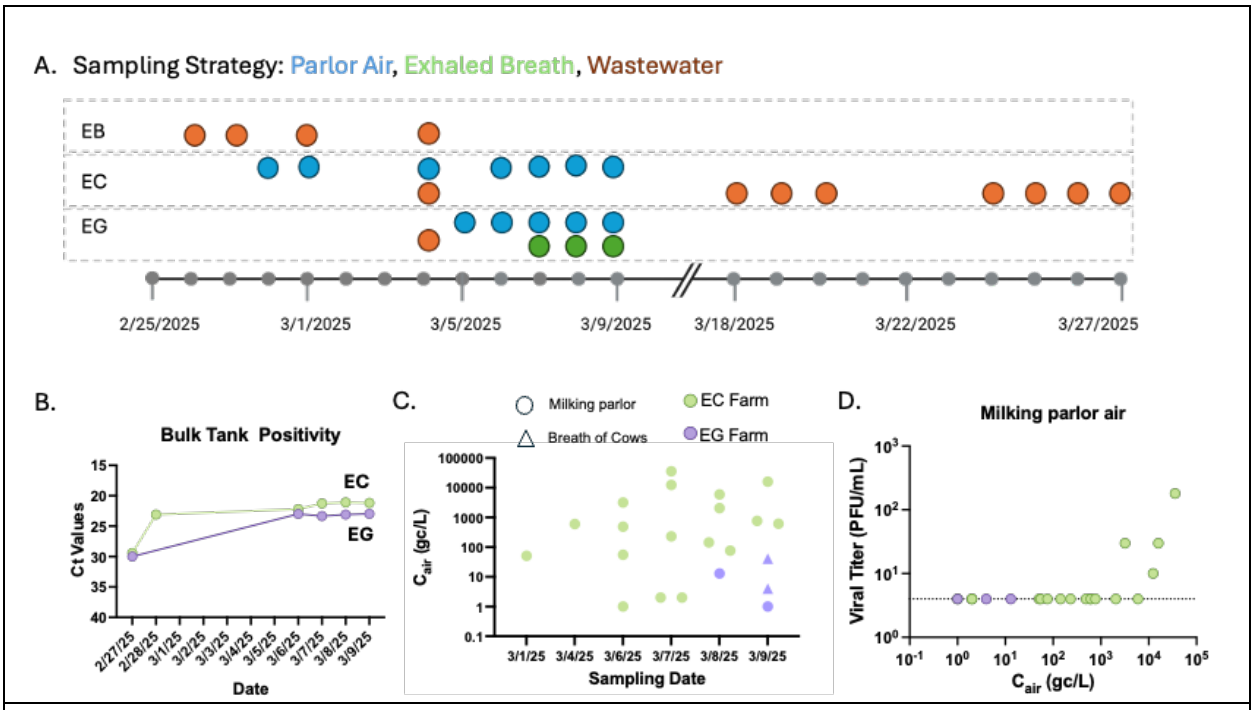
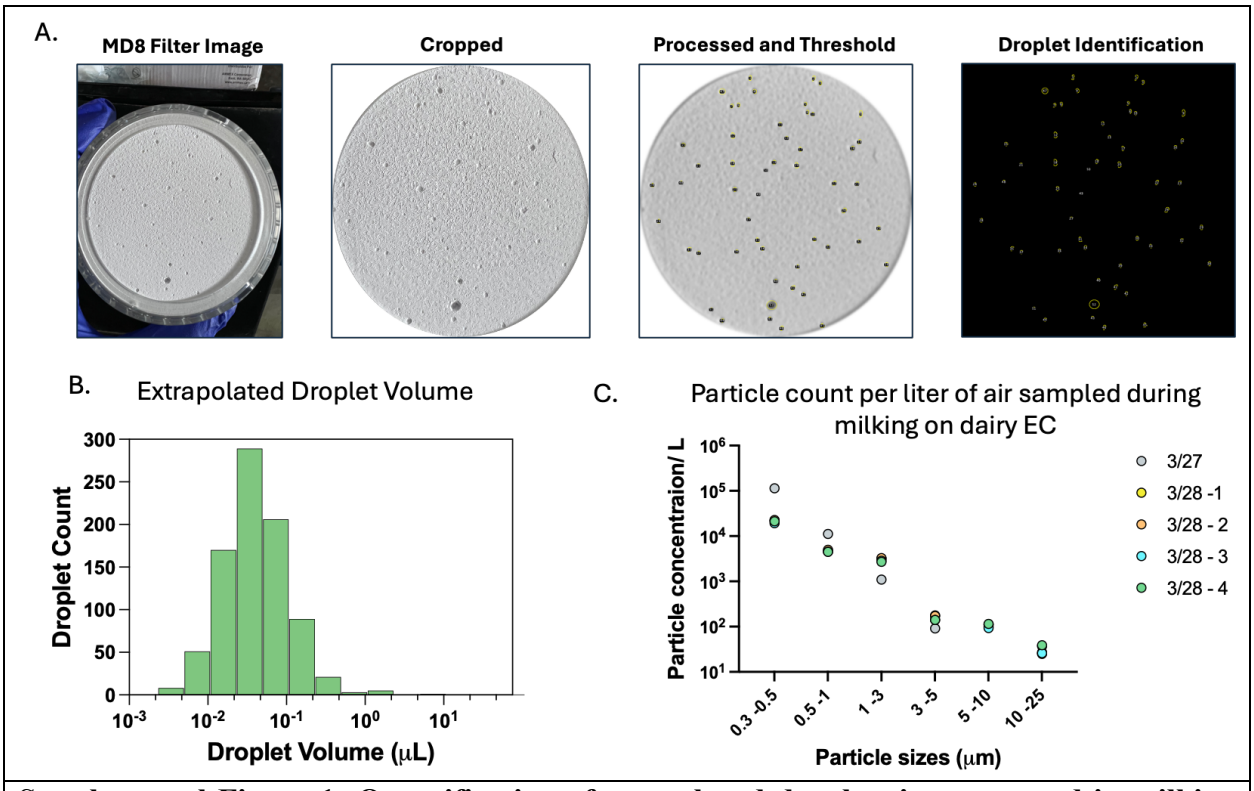


Figure 2: Presence of infectious H5N1 virus in air samples from dairy farms in southern California. (A) Timeline of sampling strategy for farms EB, EC, and EG. Blue dots indicate milking parlor air samples, green dots indicate exhaled breath samples from cows, red dots indicate water samples, and small grey dots indicate sampling dates. Detailed sampling information available in Supplemental Tables 4-6 (B) H5N1 positivity in bulk tank milk samples over time from farms EC and EG as determined by viral RNA levels. (C) Viral genome concentration in the air (Cair) calculated as the genome copies (gc) per liter of air (L) from H5N1 positive air samples from the milking parlor (circles) or breath of cows (triangles) for farms EC (green, N=19) or EG (purple, N=4) over time. (D) Infectious virus from all positive air samples from farms EC and EG was assessed by plaque assay. Dotted black line indicates the limit of detection. For B-E, each dot is the average of technical duplicates.



Supplemental Figure 1: Quantification of aerosol and droplet sizes captured in milking parlors. (A) A representative MD8 gelatin filter with aerosol and droplet marks. Images from milking parlor sampling were collected on multiple days and processed to quantify the aerosol sizes. Processing included cropping of images, blurring, and thresholding of pixel intensities to identify individual spots. (B) Analysis of 14 distinct MD8 filters provided 843 unique spots that were analyzed for the volume of each droplet using an equation to estimate the spread factor (see methods). (C) Quantitation of number of aerosol particle counts of different sizes within the milking parlor at farm EC on the indicated sampling dates. Four different measurements were collected on 3/28.

Detection of infectious H5N1 in farm wastewater sources

We collected samples from reclaimed wastewater sources on multiple farms. This includes water used to clean milking equipment and dairy parlors as well as milk from sick cows collected into buckets. We confirmed that both milking equipment and milk from sick cows contained infectious H5N1 virus on these farms during this sampling period (Supplemental Figure 2). Samples were collected along the reclaimed water stream at each available point such as 1) water moving down the drain in the milking parlor, 2) at the sump pump pit adjacent to the dairy parlor, 3) fields where captured water is used, and 4) at manure lagoons where reclaimed water is stored (Figure 3A). H5N1 viral RNA was detected at each point of the waste stream, including in manure lagoons that are widely used by migratory birds and in fields with grazing cows (Figure 3B and Supplemental Tables 3-6). Selected samples with an H5N1 RNA concentration exceeding 650 genome copies/mL were subject to viral titration, and two samples contained detectable infectious virus (Figure 3C). These results demonstrate that H5N1 is prevalent in reclaimed farm wastewater

sites across dairy farms and may serve as another source of H5N1 spread between cows, to humans, and to peri-domestic animals.

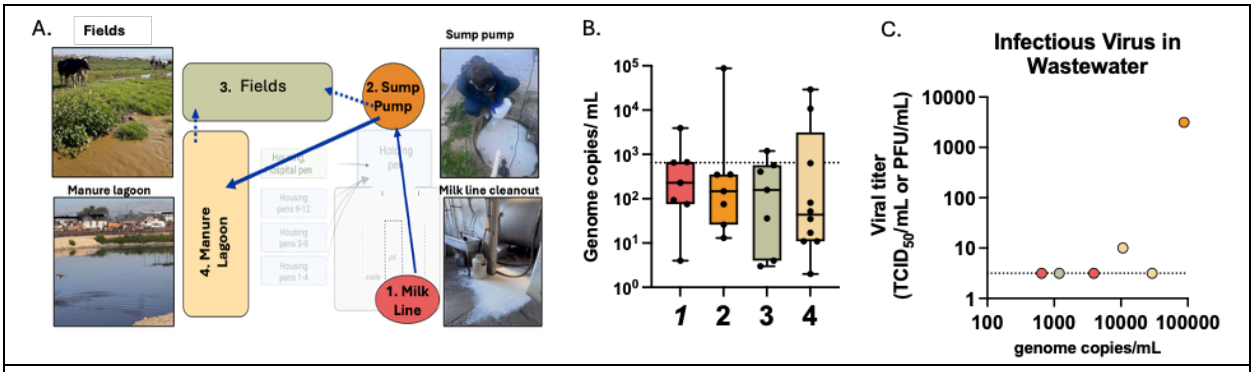
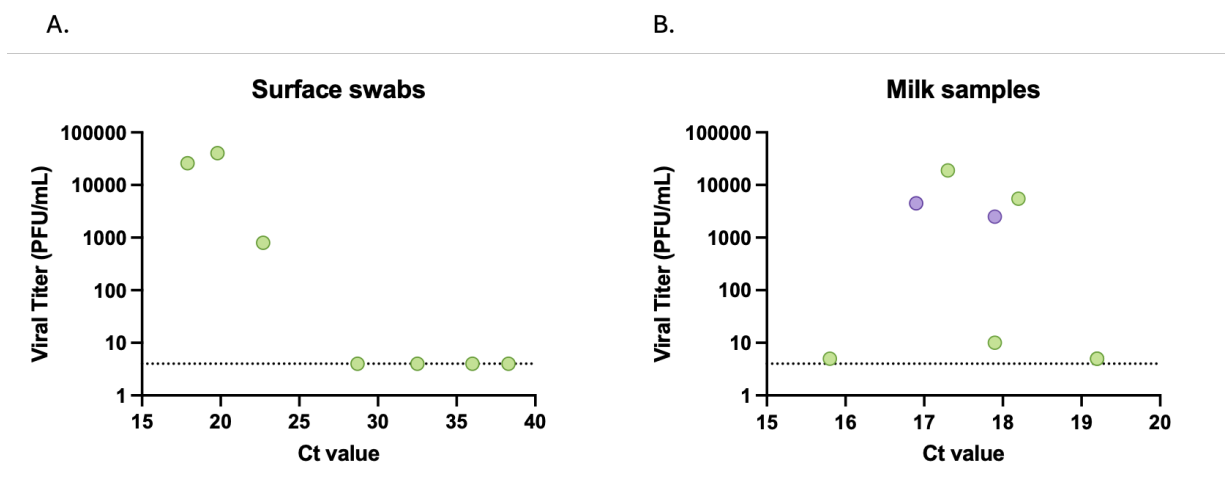


Figure 3: Detection of H5N1 virus in multiple reclaimed wastewater sites on southern California and Central Valley farms. (A) Top-down schematic of a representative dairy farm layout with farm wastewater sources depicted and on-site representative pictures included. Sources tested in the schematic match the colors in part B. Schematic created with Biorender.com. (B) Water sources were screened for H5N1 viral RNA by digital droplet PCR and the data is plotted as a box plot. Each dot represents a single sample, solid horizontal lines represent median value, the lower edge is the first quartile, the upper edge is the third quartile, and max and min values are indicated with error bars. Details on location and date of each sample can be found in Supplemental Tables 3-6. Briefly, milk cleanout (1) n=7, sump pump (2) n=7, field (3) n= 7, manure lagoon (4) n=10. (C) Available samples with genome copies above 650 (indicated by dashed line, n=6) were assessed for infectious H5N1 virus by either TCID50 or plaque assay and infectious titer from either assay is presented.

Presence of Viral Variants in the Air

Whole genome sequencing was performed on a subset of air samples and the reclaimed farm wastewater sample with highest genome copies from farm EC (Supplemental Table 5), and revealed all samples were within the H5N1 2.3.4.4b B3.13 subclade (Supplemental Table 7). Variant analysis was conducted for each sample compared to a consensus sequence from all samples on that farm. Amino acid variants were observed in many samples compared to the farm consensus sequence. Air samples collected from the milking parlor during milking of the same pen of cows on subsequent days revealed variations in the HA, PB2, and PB1 segments (Supplemental Table 7, compare EC36 to EC47 and EC60). Of note HA mutations observed in EC36 identified a change at HA position 189 (193 H3 numbering), which is found within the 190-helix, a critical component of the receptor binding site[22,23]. While position 189 does not directly bind to α2,3-linked sialic acids[24,25], mutations at this site are associated with improved binding to human-like α2,6-linked sialic acid[26]. This observation suggests that viral variants with changes in the HA receptor binding site can emerge on farms but are not maintained in this case. Variants were also observed between the air samples collected during milking of sick vs healthy cows on the same day (Extended Table 7, compare EC47 to EC54 and EC60 to EC66). Sequences from an air sample collected in the milking parlor on March 4, 2025 closely matched the sequence observed in the

water sample from the same farm on the same day (Extended Tables 5 and 7, samples EC22 and EC27). Taken together, analysis of air and water samples highlight that there is genetic variation on a farm and more detailed analysis of sequences could decipher intra-herd transmission patterns.



Supplemental Figure 2: **Viral loads from milking equipment surface samples and from sick cow milk samples collected prior to initiation of the longitudinal study (prior to 3/18/25).** Infectious virus titers as determined by plaque assay compared to viral RNA levels (Ct values) for (A) swab samples collected from milking equipment or (B) milk collected from sick cows, whose milk would go into buckets and not the bulk tank, on farms EC and EG.

H5N1 positivity in individual quarters

Contaminated milking equipment is thought to be a primary mode of H5N1 cow-to-cow transmission[9] though definitive proof from natural or experimental infection scenarios is still lacking. There is a specific orientation of the milking equipment when placed on the cow udder such that each teat cup is almost always placed on the same teat or quarter of the cow. Thus, similar quarters might be predicted to be infected throughout a herd given these constraints if equipment was contaminated. To begin to examine the distribution of H5N1 infection in cow quarters, we initiated a longitudinal study on farm EC (Figure 4A). Twenty-three cows were screened for H5N1 positivity; of these, fourteen cows were selected for longitudinal sampling, including three cows that screened negative. Milk samples from each individual quarter: front left (FL), front right (FR), rear (referred here as back) left (BL), and back right (BR) were collected by a dairy farm worker prior to milking daily over the course of seven days (Figure 4B).

Analysis of individual cows over this timeframe revealed several important features of natural H5N1 infection progression. First, the number of H5N1 positive quarters per animal varied widely, with 5/12 cows having only one quarter positive and the remaining cows having 2-4 quarters positive (Figure 4C and 5). Second, the quarters that tested positive on the first day of sampling generally remained positive throughout the sampling period (Figure 5). This quarter-specific conservation of H5N1 positivity over time is consistent with observations from experimental infections where H5N1 virus remains within the infected quarters and does not

spread to uninfected quarters [11,12]. Third, there was little variation in the viral RNA amount over the 7-day collection period. The absence of H5 viral RNA in specific quarters from infected animals is interesting and may represent quarters that could become infected later during the outbreak, although additional data is needed to confirm this.

H5N1 in dairy cows was identified through investigation of mastitis of unknown origin[2]. Although mastitis (inflammation of the mammary gland that can cause abnormal consistency and discoloration of milk) is often associated with bacterial infections, it has been identified as a common clinical sign in H5N1 infected cows[27]. Since mastitis is usually identified in a single quarter within affected cows [28,29], it is likely that the same quarter would also be positive for H5N1. However, our screening results indicated that mastitis does not always overlap with H5N1 as seven cows were identified as H5N1 positive but lacking clinical signs of mastitis (Figure 6A). We also examined the distribution of mastitis by quarter in a set of cows on farm EC farm a few weeks after initiation of the H5N1 outbreak but prior to the longitudinal sampling. Within these cows there was a predominance of mastitis in the FR quarter, with one animal having mastitis in two quarters (BR and FR) (Figure 6B). However, this pattern was not observed in the cows from our longitudinal study, where most cows had multiple quarters infected and there was a fairly even distribution of infection among the four quarters (Figure 6C). Although only a small number of cows were sampled, these results suggest that H5N1 positivity can occur in quarters that do not present with mastitis, suggesting that some nonclinical animals may be H5N1 positive.

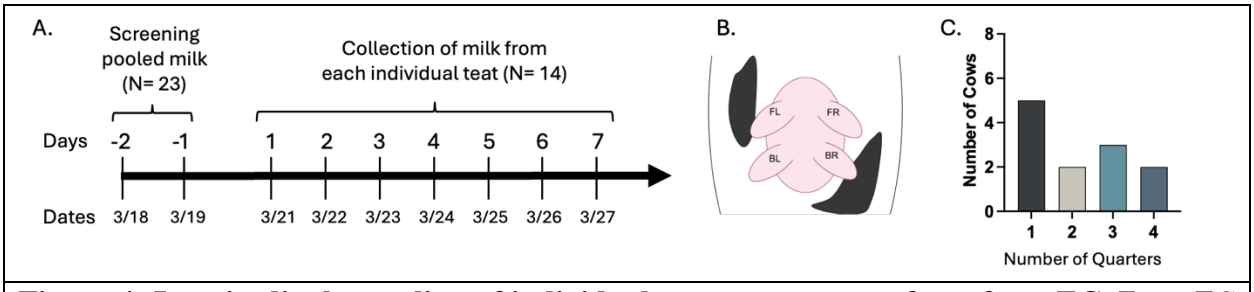


Figure 4: Longitudinal sampling of individual quarters on cows from farm EC. Farm EC tested positive on 2/24/2025. (A) Timeline of screening and longitudinal sampling (for 7 days, starting 3/20 or 3/21) of individual cows. (B) A worm-view schematic identifying the location of each quarter on the udder: front left [FL], back left [BL], back right [BR], and front right [FR]. Schematic created with BioRender.com (C) Number of cows from the longitudinal study with the indicated number of quarters positive for H5N1 viral RNA.

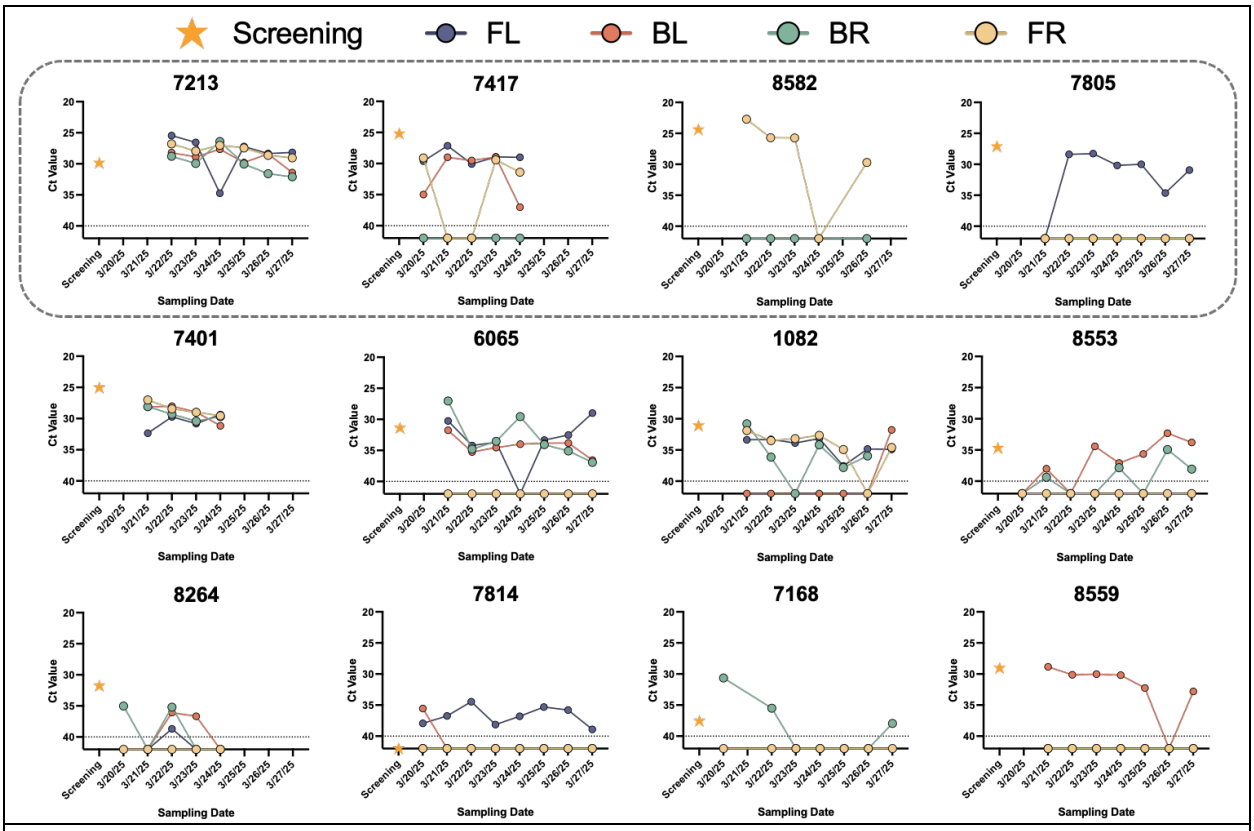


Figure 5: Heterogeneity in H5N1 positive quarters among naturally infected cows. H5N1 viral RNA was measured in milk from individual quarters of 14 cows; 12 PCR-positive cows are shown here, including one cow that had originally screened negative (#7814). Ct values averaged from technical duplicates are shown over time for each individual quarter (FL, FR, BL, BR). Yellow stars represent Ct values detected during screening from milk pooled from all 4 quarters. Cows within the dashed box indicate those that were treated for mastitis during sampling, such that their milk was not put into the bulk tank. Each value is the average of technical duplicates. Not all cows were available to be tested on all dates. Three cows were sent to slaughter during the sampling efforts: cows #7417, 7401, and 8264.

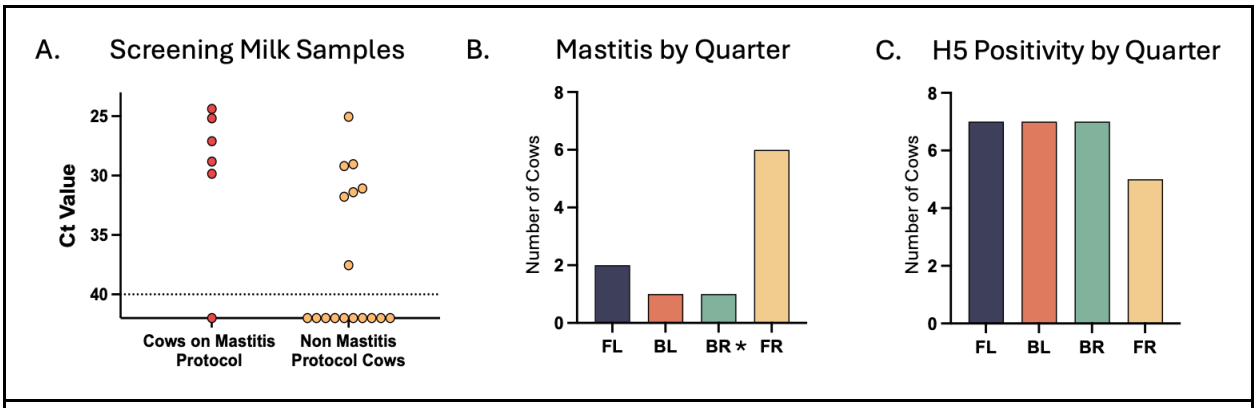


Figure 6. H5N1 infection does not always correlate with clinical mastitis. (A) Cows

screened for the longitudinal study described in Figure 4, were grouped by whether or not they were treated for mastitis. Each dot represents the Ct value of H5N1 RNA from pooled milk from all four quarters of an individual cow, categorized as either having had mastitis (red) or being randomly selected (yellow), and is the average of technical duplicates. Dotted line represents limit of detection. (B) Nine cows on farm EC were noted as having mastitis by dairy parlor staff and the affected quarter was recorded by research personnel. These data were collected prior to screening of cows in part A and do not represent the same cows presented in part A or C. The asterisks denotes that the cow with mastitis in quarter BR was also positive in FR and was the only cow to present with mastitis in 2 quarters on the farm. (C) The 12 cows from Figure 5 were assessed for H5N1 positivity in each quarter.

Discussion

Elucidating the routes of transmission of H5N1 between cows is critical to defining successful mitigation strategies. In this study we successfully detected H5N1 in the air and in reclaimed farm wastewater on separate dairy farms on multiple days. This included infectious air samples from three different milking parlors and viral RNA from the exhaled breath of rows of cows on two distinct farms. Additionally, we detected viral RNA in farm wastewater at multiple sites on various farms and infectious virus at two different sites on the same farm. Together, these results highlight the expansive environmental contamination of H5N1 on affected dairy farms and identify additional sources of viral spread between cows and to humans. Surprisingly, we also observed an HA mutation in a residue (H5 HA 189, H3 numbering 193) known to alter sialic acid specificity[26] in the air during milking of infected cows, suggesting that HA mutations are emerging during this outbreak but are not currently maintained. Notably, mutations at 189 are associated with improved binding to human α2,6-linked sialic acids[26] and a structure of a 2.3.4.4b H5 with an α2,6-linked sialic acid analog shows position 189 helps stabilize receptor binding[24]. Whether the N189D mutation observed in this study alters receptor binding and improved tropism to humans remains to be determined. These data support that emerging viral variants can pose a risk to humans working in the dairy parlors through exposure to viruses in the air and on surfaces.

Given the high viral loads of H5N1 found in milk, milking equipment has been implicated as the likely source for viral transmission. However, data from our longitudinal study revealed a heterogeneous pattern of positivity across the subset of cows analyzed, both in the number and location of positive quarters. If milking equipment alone was the driver of cow-to-cow H5N1 transmission it is likely that a similar pattern of positivity would have been observed between the animals. Experimental transmission studies have also failed to induce H5N1 spread between cows using contaminated milking equipment[30]. Thus, alternative mechanisms of transmission of H5N1 between cows must exist. The variations in H5N1 quarter positivity may reflect that, and animals with all 4 quarters positive may be those with H5N1 viremia as reported in a subset of cows[31]. While animals with fewer quarters H5 positive may have been from contaminated milking equipment or infection in damaged teats that have encountered H5N1 virus through aerosols or contaminated wastewater.

Surprisingly, we observed H5N1 positivity was not always associated with mastitis, suggesting many subclinical infected cows on a farm whose milk will enter the commercial stream. Assessment of mastitis-affected quarters in a distinct set of cows from the same farm done prior to initiation of the longitudinal study identified a clear preference for the FR quarter. In contrast, analysis of the five cows with only a single H5N1 positive quarter revealed two animals with a positive FL quarter, one BL, one BR, and one FR. The lack of a consistent quarter infected across all cows, as observed for mastitis, suggests that these two features are not linked and likely have distinct modes of transmission.

While field surveillance is critical to understanding ongoing infectious disease outbreaks, there are many limitations that are important to note when considering the data presented in this manuscript. First, longitudinal sampling was conducted on a single farm with 14 cows over a short period of time, which may have missed patterns that could be revealed with a larger number of animals. Additionally, sampling across multiple farms, for longer periods of time, or earlier in an outbreak may reveal different patterns. Constraints on time and personnel, shared milking equipment could not be traced in our study, so patterns of the milking equipment usage and order of milking for all 14 longitudinal animals are not known and likely varied each day of sampling. Further examination of antibody load and deep sequencing of virus from individual quarter samples could reveal the phase of the viral infection in the animal and provide a glimpse into the transmission chains using phylogenetic approaches. Second, although previous studies have shown H5N1 influenza virus to remain infectious for days to weeks in aquatic environments[32], the limited number of samples with infectious virus detected in our reclaimed farm wastewater samples could be a consequence of sample fragility, sample complexity, and/or downstream processing inefficiencies. Thus, infectivity in the wild could vary and may be underestimated in our study. Similarly, the amount of infectious virus in air is likely an underestimation based on the collection efficiency and sampling processing issues that are known to make air sampling challenging in real-life settings. Lastly, all air samples from breath of individual cows were H5N1 negative, including from individual cows identified by a veterinarian or other trained professional as demonstrating obvious clinical signs suggestive of H5N1 infection. However, in these instances H5N1 infection was not always known at the time of sampling since rapid detection of H5N1 positive cows on a farm is not yet feasible. Remedies for these limitations would include more longitudinal sampling of individual cows on dairy farms during outbreaks, including assessment of individual quarters for H5N1 and mastitis, daily nasal swabs and respiratory emissions, as well as tracing of milking equipment. Individual cow sampling would allow for an assessment of the transmission efficiency and secondary attack rate of H5N1 in cows in a herd and a correlation between viral load and symptom onset. Additionally, a rapid diagnostic system that can identify H5N1 positive cows during milking is critical to isolating infected animals and facilitating field sampling [3].

Taken together, our data confirm the presence of infectious H5N1 virus in the air and reclaimed farm wastewater sites. In addition, we observed high viral loads in the milk of cows, including those without clinical signs, and heterogenous patterns of H5N1 positivity by quarter, suggesting that multiple modes of H5N1 transmission likely exist on farms. These transmission

routes could include contaminated milking equipment from an infected cow, aerosols generated within the milking parlor, and/or contact of teats with contaminated water used to clean housing pens. Multiple mitigation strategies should therefore be implemented to reduce the risk of H5N1 spread within a herd and to humans. Respiratory and ocular personal protective equipment (PPE) for farm workers to prevent deposition of virus-laden aerosols on these sites, especially in the milking parlor. Disinfection of milking equipment between each milking of each cow, such as with consistent use of backflush system, could also reduce spread of H5N1 between cows. Treatment of milk from sick cows to inactivate H5N1 prior to disposal as well as treatment of waste streams prior to their use on fields or on farms should also be considered. Finally, identification of infected cows, regardless of clinical signs, for isolation will help reduce the transmission of H5N1 on farms.

Methods (see separate document)

Data Availability

All data sets generated that support the findings of this study are available on FigShare (doi:10.6084/m9.figshare.29627840).

Code Availability

The custom Python code used for analysis of droplet size volumes can be found on figure are project associated with this manuscript (doi: 10.6084/m9.figshare.29627840).

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Author contributions
A.J.C. contributed to conceptualization, sample collection and processing, data analysis, and writing the manuscript; M.S. contributed to sample collection, processing, and data analysis; A.P.P. contributed to sample collection and processing, data analysis, and manuscript preparation; M.P. contributed to sampling processing and data analysis; M.V., W.J.F., and E.E.B., contributed to sampling processing and data analysis; C.S.S. contributed to sample collection, data analysis, and manuscript preparation; K.Bu. and K.Br. both contributed to sample processing; G.Q., N.S., and N.K. contributed to data analysis; M.W., L.C.M, and J.G contributed to data analysis and manuscript preparation; A.S.L. contributed to sample processing, data analysis, and manuscript preparation; J.L. contributed to study design and coordination, sample collection, data analysis, and manuscript preparation; S.S.L. contributed to conceptualization, data collection, analysis, sample processing, funding acquisition, and writing of the manuscript.

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Supplementary Data Tables

Supplemental Data Table 1. Details of 14 dairy farms that were sampled in California.

Dairy Farm	Location (region)	Date of H5 positivity	Approx. Number of Cows in Milk[a]	Primary Housing Pen Style	Milking Parlor Style
BB	Central Valley	11/23/24	800	Freestall	Parallel
BC	Central Valley	10/24/24	2900	Freestall	Rotary and Single Flat
BD	Central Valley	11/25/24	1000	Freestall	Parallel
BF	Central Valley	11/26/24	3000	Freestall	Rotary
BM	Central Valley	11/25/24	1400	Freestall	Herringbone
EA	Southern CA	3/3/25	300	Open Lot	Herringbone
EB	Southern CA	2/4/25	970	Open Lot	Unknown
EC	Southern CA	2/24/25	420	Open Lot	Herringbone
ED	Southern CA	12/2024*	500	Open Lot	Unknown
EE	Southern CA	12/2024*	1700	Open Lot	Herringbone
EF	Southern CA	12/2024*	1200	Open Lot	Herringbone
EG	Southern CA	2/26/24	500	Open Lot	Herringbone
FA	Central Valley	11/11/24	2,000	Freestall	Herringbone
FB	Central Valley	11/19/24	3,342	Freestall	Rotary

* Approximate date provided

a - Cows in milk refers to the number of cows currently lactating and being milked on a dairy farm, not the total number of animals on a farm.

Supplemental Data Table 2. Details of air sampling during the initial phase on farms in the Central Valley of California in late 2024.

Air Sampler	Modification	Sample Location	Sample Descriptor	Farm	Date	Days post BTM+[a]	Positive/ Total
AirPrep Cub 210 (200 LPM)	None	Milking Parlor	Cows from multiple pens during milking. Sample duration 60 minutes	BC	10/29/24	5	0/4
				BC	10/30/24	6	0/3
				BC	11/4/24	11	0/2
				BC	11/7/24	14	0/1
				BC	11/11/24	18	0/2
				BM	12/4/24	9	0/2
		Housing Area[b]	Open-air pen of hospital cows[c] Sample duration 60 minutes	BM	12/4/24	9	0/1
Open Face PTFE (5 LPM)	Backpack[d]	Milking Parlor	Following milking process during milking of hospital pen[c] cows. Sample duration 20- 45 minutes	BC	10/29/24	5	0/3
				BC	10/30/24	6	0/3
				BC	11/4/24	11	0/1
				BB	12/16/24	23	0/1
			Following milking process during milking of cows recovered from clinical H5 signs	BD	12/18/24	23	0/1
			Following the parlor worker closely during milking of hospital pens of cows	BM	12/19/24	24	**1/1**
		Housing Area	Sampling of background air in housing pens. Sample duration 20- 45 minutes	BC	10/29/24	5	0/1
				BB	12/16/24	23	0/1
				BF	12/18/24	22	0/1
	Cone[e]	Milking Parlor	Cone directed at milking process, following worker during milking of healthy cows	BB	12/16/24	23	0/1
			Cone directed at milking process, following worker during milking of cows recovered from H5 clinical signs	BD	12/18/24	23	0/1
		Housing Area	Exhaled breath of 15 - 30 cows in the hospital pen; cone held close to muzzles. Sample duration 5- 15 seconds/cow	BM	12/16/24	21	0/1
				BF	12/18/24	22	0/1
				BM	12/19/24	24	0/2
			Exhaled breath of individual cow with 105F fever on 12/16/25 in hospital pen. Sample duration 2 minutes	BM	12/16/24	21	0/1
				BM	12/19/24	24	0/1
	No Cone	Housing Area	Exhaled breath of individual hospital pen cows. Sample duration 5- 15 minutes	BC	10/29/24	5	0/6
				BC	10/30/24	6	0/8
				BC	11/4/24	11	0/2
MD8 Airport (50 LPM)	Cone	Milking Parlor	Cone directed at milking process, following worker, during milking of a healthy pen of cows. Sample duration 10 minutes	BB	12/16/24	23	0/2
			Cone directed at milking process, following worker, during milking of the hospital pen. Sample duration 10 minutes	BB	12/16/24	23	0/2
			Cone directed at milking process, following worker during milking of cows recovered from H5 clinical signs. Sample duration 10 minutes	BD	12/18/24	23	0/1
			Following the milking process for hospital pens of cows; closely following parlor worker throughout. Sample duration 5- 15 minutes	BM	12/19/24	24	**3/3**
		Housing Area	Exhaled breath of 15-30 cows, cone held close to muzzles. Sample duration 5- 15 seconds/cow	BB	12/16/24	23	0/1
			Exhaled breath of 15 - 30 clinically recovered cows; cone held close to muzzles. Sample duration 5- 15 seconds/cow	BD	12/18/24	23	0/1
			Exhaled breath of 15-30 hospital pen cows; cone held close to muzzles. Sample duration 5- 15 seconds/cow	BF	12/18/24	22	0/1
				BM	12/17/24	22	0/2
				BM	12/19/24	24	**2/2**
			Exhaled breath of individual cow in hospital pen that had an H5+ nasal swab on 12/4/24. Sample duration 1- 2 minutes	BM	12/16/24	21	0/2
			Exhaled breath of individual cow with severe signs associated with H5, cow couldn't stand. Sample duration 2- 3 minutes	BF	12/18/24	22	0/1
			Exhaled breath of individual cow in hospital pen that had a 105F fever on 12/16/24. Sample duration 1-2 minutes	BM	12/19/24	24	0/1

a- Days post BTM+ - Days post first bulk tank milk positive

b - Housing Area refers to primary pens used to house cows; these can be open-air or freestall pens depending on individual dairy.

c - Hospital cows/pen refers to animals identified by farmers, according to internal criteria, that have clinical signs requiring their milk not go to the bulk tank. These animals are grouped into 'hospital pens' separated from healthy animals.

d - Backpack refers to wearing the PTFE filter as shown in Fig 1D; mimicking occupational exposure

e- Cone refers to using a plastic cone adapted to the front of the air sampler as shown in Fig 1D, to prevent aerosol dilution and to protect filters from direct splashes.

Supplemental Data Table 3. Air sampling details for dairies with 1 or less positive environmental samples, Feb-Apr 2025

Sample Location	Sample Type	Sample Source	Sample Descriptor	Farm	Date	Days post BTM+[a]	Positives/ Total
Milking Parlor	Milk	Bulk Tank	Collection from bulk tank	EA	2/26/25	-5	0/1
				EA	2/27/25	-4	**1/1**
				EA	3/4/25	1	**1/1**
				EE	3/2/25	~ 90	0/1
				FA	4/1/25	141	0/1
				FA	4/2/25	142	0/1
				FA	4/3/25	143	0/1
				FB	4/1/25	133	0/1
				FB	4/2/25	134	0/1
				FB	4/3/25	135	0/1
		Milk from individual cows, 4 teats	Collected from cows sorted into the hospital pen[b] for signs such as mastitis and potential *E. coli* infection	EF	3/2/25	~90	0/3
			Collected from cows with abnormal milk consistent with descriptions of milk from H5+ cows such as yellowy color and thick consistency.	FB	4/2/25	134	0/2
		Bulk Sick Cow Milk	Bulk milk from cows whose milk didn't go to bulk tank. No specific clinical signs described	FA	4/1/25	141	0/1
			Bulk milk from cows whose milk didn't go to bulk tank. Abnormal milk only clinical sign described.	FB	4/2/25	134	0/1
	Air	Milking Process	MD8 Airport: cone directed at milking process; following worker. Sample duration 5- 15 minutes	EA	2/26/25	-5	0/1
				EF	3/2/25	~90	0/1
			Open Face PTFE worn on backpack following worker during milking. Sample suration 58 minutes	EE	3/2/25	~90	0/1
	Surface Swab	Milking Unit Inflations	Swab of the interior of all 4 inflations of one milking unit	EE	3/2/25	~90	0/2
Wastewater Stream	Air	Milk Line Cleanout	MD8 Airport held at close range to where wastewater from the milk lines exits the parlor to gravity-flow to the 'flush pump pit' below the manure lagoon. Sample duration 7 minutes	FB	4/2/25	134	0/1
		Sump pump	MD8 Airport: primary sump pump sampled while water flowing. Sample duration 7 minutes	EA	2/26/25	-5	0/1
			MD8 Airport lowered into large, deep sump pump while wastewater flowing. Sample duration 6 minutes	FA	4/1/25	141	0/1
				FA	4/2/25	142	0/1
			Open Face PTFE: primary sump pump sampled while water flowing. Sample duration 12 minutes	EA	2/27/25	-4	0/1
		Flush pump pit[c]	MD8 Airport held over area of most agitation of the flush pump pit. Sample duration 5 minutes	FB	4/1/25	133	0/2
				FB	4/2/25	134	0/1
				FB	4/3/25	135	0/1
		Manure Lagoon	MD8 Airport held, or suspended on a telescoping pole, over inlet for milking parlor wastewater into the lagoon. Sample duration 5- 9 minutes	EA	2/26/25	-5	0/1
				ED	3/2/25	~90	0/1
				EE	3/2/25	~90	0/1
				EF	3/2/25	~90	0/1
				FA	4/1/25	141	0/1
		Flush of Freestall Pens[d]	MD8 Airport held right in front of where water is pumped up into the freestall pens, vigorous water flow. Sample duration 5- 8 minutes	FB	4/1/25	133	0/2
				FB	4/2/25	134	0/1
				FB	4/3/25	135	0/1
	Wastewater	Milk Line Cleanout	Sample of residual milk and wastewater flushed out of lines as part of the cleaning process post- milking	ED	3/1/25	~90	0/1
				EF	3/2/25	~90	0/1
				EE	3/2/25	~90	0/1
				FB	4/2/25	134	0/1
		Sump pump	1L from primary pump while water flowing	EA	2/26/25	-5	0/1
				EA	2/27/25	-4	0/2
				EA	3/4/25	1	0/1
			1L from secondary pump while water flowing	EA	2/26/25	-5	0/1
				EA	2/27/25	-4	0/1
				EA	3/4/25	1	0/1
			1L sample from deep pump while wastewater flowing.	FA	4/1/25	141	0/2
				FA	4/2/25	142	0/1
				FA	4/3/25	143	0/1
		Flush pump pit	1L sample of wastewater, lots of agitation.	FB	4/1/25	133	0/1
				FB	4/2/25	134	0/1
				FB	4/3/25	135	0/1
		Manure Lagoon	1L sample from pipe inlet to lagoon	EA	3/4/25	1	0/1
				ED	3/2/25	~90	0/1
				EE	3/2/25	~90	0/1
				EF	3/2/25	~90	0/1
			1L sample from lagoon milking parlor wastewater was flowing into	FA	4/1/25	141	0/3
				FA	4/2/25	142	0/1
				FA	4/2/25	143	0/1
		Field	1L sample from pump adding a 1:1 mix of wastewater from the manure lagoon to well water onto in-use crop field	FA	4/1/25	141	**1/1**
		Flush of Freestall Pens	250mL sample of water collected as it flowed out into the pens.	FB	4/1/25	133	0/1
				FB	4/2/25	134	0/1
				FB	4/3/25	135	0/1
Housing Pens	Air	Exhaled Breath of Row of Cows	MD8 Airport held very close to ~ 12 cows' muzzles as they were headlocked into stanchions. Hospital pen sampled. Sample duration 10- 30 seconds/cow	EF	3/2/25	~90	0/1

a- Days post BTM+ - Days post first bulk tank milk positive

b - Hospital cows/pen refers to animals identified by farmers, according to internal criteria, that have clinical signs requiring their milk not go to the bulk tank.

c - The flush pump pit is a large basin that was used to mix wastewater from the manure lagoon with fresh water. Water from this pit is used to clean other areas of the dairy, such as freestall pens.

d - The freestall pens are flushed daily with water to clean them out. On the sampled farm this water came from the flush pump pit.

Supplemental Data Table 4. Sampling details for dairy farm EB during spring 2025.

Sample Location	Sample Type	Sample Source	Sample Descriptor	Date	Days post BTM+[a]	Positives/ Total
Wastewater Stream	Air	Sump pump	MD8 Airport (50 LPM) sampling for 7 minutes while pump cycled	2/26/25	22	0/1
				2/27/25	23	0/1
		Field	MD8 Airport (50 LPM) sampling over the wastewater outlet in the field for 4- 6 minutes	2/26/25	22	0/2
				3/1/25	25	0/1
				3/4/25	28	0/1
	Wastewater	Sump pump	1L sample from sump pump	2/26/25	22	**1/1**
				2/27/25	23	**1/1**
		Field	1L sample from wastewater inlet to field, usually while pump running	2/26/25	22	**3/3**
				3/1/25	25	**1/1**
				3/4/25	28	**1/1**
		Manure Lagoon	1L sample; milkhouse wastewater not flowing here, lagoons only had run-off from housing pens and rainwater	2/26/25	22	**1/2**

a- Days post BTM+ - Days post first bulk tank milk positive

Supplemental Data Table 5. Sampling details for dairy farm EC during sampling spring 2025.

Sample Location	Sample Type	Sample Source	Sample Descriptor	Date	Days post BTM+[a]	Positives/ Total
Milking Parlor	Milk	Bulk Tank	Collection from bulk tank	2/27/25	3	**1/1**
				2/28/25	4	**1/1**
				3/1/25	5	**1/1**
				3/6/25	10	**1/1**
				3/7/25	11	**1/1**
				3/8/25	12	**1/1**
				3/9/25	13	**1/1**
				LS: 3/18 - 3/27[b]	22- 31	**10/10**
		Sick cow milk - 4 teats combined	Collected from cows with signs such as mastitis or a sudden stop in milk production.	3/1/25	5	**1/1**
				3/4/25	8	**1/2**
				3/6/25	10	**5/5**
				3/7/25	11	**5/6**
				3/9/25	13	**2/2**
		Dump bucket	Collection from communal bucket used for cows whose milk was discarded.	3/7/25	11	**1/1**
	Air	Milking process, following worker	MD8 Airport (50 LPM) with cone directed at milking process, closely following worker. Sample duration 3- 15 minutes.	2/28/25	4	**1/4**
				3/1/25	5	**1/1**
				3/4/25	8	**1/1**
				3/6/25	10	**4/5**
				3/7/25	11	**4/4**
				3/8/25	12	**4/4**
				3/9/25	13	**3/4**
				LS: 3/18 - 3/27[b]	22- 31	**5/14**
		Milking process, following worker	Open face PTFE (5 LPM) worn on backpack during parlor sampling while milking going on. Sampling duration from 3 hours 11 minutes to 4 hours 47 minutes.	2/28/25	4	0/1
				3/6/25	10	**1/1**
				LS: 3/18 - 3/27[b]	22- 31	0/4
	Surface Swab	Milking unit Inflations	Swab of the interior of all 4 inflations of one milking unit.	2/28/25	4	**1/4**
				3/1/25	5	**1/1**
				3/4/25	8	**1/1**
				3/6/25	10	**2/2**
				3/7/25	11	**1/1**
				3/8/25	12	**2/2**
				3/9/25	13	**1/1**
		Milking unit shells	Swab of the exterior of all 4 shells, or teatcups, of a milking unit.	3/1/25	5	**1/1**
				3/6/25	10	**1/1**
				3/7/25	11	**1/1**
				3/8/25	12	**1/2**
				3/9/25	13	0/1
Wastewater Stream	Air	Manure Lagoon	MD8 Airport (50 LPM) at close range to point of outlet for wastewater into the manure lagoon. Sampling from 3- 6 minutes.	2/27/25	3	0/1
				3/1/25	5	0/1
				3/4/25	8	0/1
				LS: 3/18 - 3/27[b]	22- 31	0/1
		Sump pump	MD8 (50 LPM) held over an open sump pump pit as wastewater from the milking parlor line cleaning flowed through. Sampling for 13 minutes.	3/4/25	8	**1/1**
	Wastewater	Milk line cleanout	Sample of bulk milk flushed out of lines as part of the cleaning process post-milking	3/4/25	8	0/1
				3/9/25	13	**1/1**[c]
				LS: 3/18 - 3/27[b]	22- 31	**7/7**
		Sump pump	1L sample of wastewater from sump pump pit	3/4/25	8	**1/1**
				LS: 3/18 - 3/27[b]	22- 31	**4/7**
		Manure Lagoon	1L sample sample from right next to wastewater inlet to lagoon	2/27/25	3	0/1
				3/1/25	5	**1/1**
				3/4/25	8	**1/1**
				LS: 3/18 - 3/27[b]	22- 31	**7/7**
Housing Pens	Air	Exhaled Breath of Row of On-Study Cows[e]	MD8 Airport (50 LPM) held very close to cows' muzzles. 10- 30 seconds per cow.	LS: 3/18 - 3/27[b]	22- 31	0/3[f]
	Nasal Swabs[d]	On-Study Cows[e]	A pre-assembled swab kit used to take nasal swabs from group of on-study cows. Swabs taken quickly, as far into the nasal cavity as possible.	LS: 3/18 - 3/27[b]	22- 31	0/48[f]

a- Days post BTM+ - Days post first bulk tank milk positive
b - LS, Longitudinal Study. Samples collected between 3/18/25 and 3/27/25 are aggregated.
c - This sample analyzed via qRT-PCR instead of ddPCR, thus it is not shown in Figure 3B.
d- On each day nasal swabs were taken, some on-study cows were not available.
e - On-study cows refers to the group of 14 cows that were selected for the longevity study wherein daily milk samples from each teat were collected.
f- Not all 14 on-study cows were available for each day sampling conducted

Supplemental Data Table 6. Sampling details for dairy farm EG during sampling in spring 2025.

Sample Location	Sample Type	Sample Source	Sample Descriptor	Date	Days post BTM+ [a]	Positives/ Total
Milking Parlor	Milk	Bulk Tank	Collection from bulk tank	3/6/25	8	**1/1**
				3/7/25	9	**1/1**
				3/8/25	10	**1/1**
				3/9/25	11	**1/1**
		Sick cow milk - 4 teats combined	Collected from cows sorted into the hospital pen that had mastitis at time of sampling.	3/5/25	7	**1/3**
		Bulk sick cow milk	Milk from sick cows that was diverted from the bulk tank into a smaller tank for a calf raising facility. Clinical signs of cows were decreased appetite and milk production, increased nasal discharge, and increased respiration rates.	3/6/25	8	**2/2**
	Air	Milking process, following worker	MD8 Airport (50 LPM) with cone directed at milking process, closely following worker during milking of the hospital pen cows. Sample duration was 10- 20 minutes.	3/5/25	7	0/1
				3/6/25	8	0/1
				3/7/25	9	0/1
				3/8/25	10	**1/1**
				3/9/25	11	**1/1**
Wastewater Stream	Wastewater	Field	1L sample from right next to pipe where wastewater from the parlor outlets into the field.	3/4/25	6	**1/1**
Housing Pens	Air	Exhaled breath of row of cows	MD8 Airport (50 LPM) held very close to cows' muzzles as they were headlocked into stanchions. Hospital pen sampled. ~15- 30 cows were sampled for 10- 30 seconds per cow.	3/7/25	9	0/2
				3/8/25	10	0/1
				3/9/25	11	**2/3**

a- Days post BTM+ - Days post first bulk tank milk positive

Supplemental Data Table 7. Consensus variants identified in environmental samples from dairy farm EC.

Sample	Date	Source	PB2	PB1	PA	HA	NP	NA	M	NS
EC22	3/4/25	Air, milking parlor, sick pen cows								
EC27	3/4/25	Wastewater, sump pump								G672A (NS1 syn; NEP G67E)
EC36	3/6/25	Air, milking parlor, pen 7 cows	A1231G (I411V), A1562C (K521T)	T1533C (syn)		A613G (N189D), A871G (N275D)		A834G (syn)		
EC38	3/6/25	Air, milking parlor, pen 8 cows	A1562C (K521T)							G538A (NS1 V180I; NEP syn)
EC47	3/7/25	Air, milking parlor, pen 7 cows		C191T (P64L), T1533C (syn)						
EC54	3/7/25	Air, milking parlor, sick pen cows			T1449C (syn)			A1352G (D451G)		
EC60	3/8/25	Air, milking parlor, pen 7 cows						A1352G (D451G)		G672A (NS1 syn; NEP G67E)
EC66	3/8/25	Air, milking parlor, sick pen cows		T1533C (syn)		A1271G (N408S)	T1005A (syn)	A233G (syn)		A427G (NS1 T143A)
EC70	3/9/25	Air, milking parlor, pen 8 cows			T1449C (syn)					
EC71	3/9/25	Air, milking parlor, sick pen cows			T1449C (syn)					

Differences in consensus nucelotide sequences from each sample are indicated relative to the consensus sequence of all environmental samples from farm EC.
Nucleotide positions are relative to the positive-sense gene coding portion of each segment.
Amino acid changes are indicated in parentheses; with numbering relative to the start codon position.
HA amino acid positions are based on the mature HA protein sequence, not the start codon position as suggested in Burke DF and Smith DJ (2014) *PLoS One*.
syn = synonymous mutation

Methods

Dairy farms

Initial sampling was conducted on five dairy farms in the Central Valley of California from October through December 2024; then subsequently on seven farms in southern California, and on two additional farms in the Central Valley from February to April 2025. Each of these dairy farms had confirmed H5N1 infections in cows as determined by screening of on-dairy bulk tank milk samples in accordance with the National Milk Testing Strategy Federal Order from December 2024. Bulk tank samples taken by our group were collected using either a clean metal dipper, a spigot in the tank, or aseptically with the needle-only QualiTru system into 50mL conical tubes, depending upon the setup of each dairy. The sizes and infrastructure of surveyed dairies varied and are detailed in Supplemental Data Table 1. Determinations of clinical signs and mastitis were made by dairy farm owners, workers, and/or veterinarians, depending on each dairy.

Air sampling devices and methodology

Air samples were collected at sources including exhaled breath of individual cows and rows of cows in housing pens (15-30 cows per filter), within milking parlors during milking, and sites where wastewater is exposed to the environment such as open sump pumps, manure lagoons, and fields. The air samplers used in this study are described below. Details of sampling for each source on each farm can be found in Supplemental Data Tables 2-6.

PTFE open-face filter and GilAir 5 pump: The GilAir 5 Personal Air Sampler pump (Gilian) was set to an air flow rate of 5 L/min, the highest rate for this device, during all sampling periods. Aerosols were collected onto Fluoropore 37mm diameter, 3µm pore size PTFE membrane filters (Millipore Sigma Ref. FSLW03700) housed within open-faced plastic cassettes. Such filters have been shown to have a physical collection efficiency of ~95% for MS2, a bacteriophage that is smaller than influenza virus[1]. The GilAir 5 pump was worn in a backpack, with tubing connecting the pump to the filter cassette, which was either fastened onto the shoulder strap facing outward or was housed within a plastic cone to enable targeted sampling of cows or processes (Figure 1D).

MD8 Airport Sampler: The MD8 Airport (Sartorius) is a handheld air sampler that was set to an air flow rate of 50 L/min for all sampling periods. The sampler used 80mm diameter gelatin filters, which are designed to facilitate capture of pathogens while maintaining viability. The MD8 was always used with a plastic cone fitted onto the front to minimize dilution of aerosols from the source and to protect the gelatin cassette from direct contact with larger liquid droplets. When used around wastewater, the MD8 was held as close as possible to a source of aerosolization, where water was most agitated or the point of impact of source wastewater into its sink destination. To reach such points that could not be approached closely enough on foot, the MD8 was attached to the end of a 25-foot extendable metal pole for sampling.

AirPrep Cub 210 Air Sampler: The AirPrep Cub (InnovaPrep), with its higher air flow rate of 200 L/min, was used to sample ambient air in environments where groups of sick and potentially H5N1 positive cows gathered, such as the milking parlor and housing pens. The sampler was placed either on the ground or at a height of ~2 m and set to sample for 60-minute intervals.

Estimation of particle sizes and numbers in milking parlor air: An AeroTrak Handheld Airborne Particle Counter Model 9306 (TSI) was used to determine the sizes and number of particles in the milking parlor of dairy farm EC. Readings were collected for ~10 minutes while following the parlor worker in the pit during the milking process. Each interval corresponded to the total time it took to milk the cows on one side of the parlor, from the time the cows entered until they were released from the milking stalls. The AeroTrak reported particle counts in six size bins: 0.3-0.5µm, 0.5-1µm, 1-3µm, 3-5µm, 5-10µm, and 10- 25µm while sampling at a flow rate of 2.83 L/min. Sampling with the AeroTrak was done on two consecutive days on dairy farm EC.

Surface swabs

The interior surfaces of milking liners as well as their exterior shells were sampled with pre-assembled swab kits (Becton Dickinson Ref. 220526). For milking unit shells, the flocked swab was unwrapped, pre-moistened by briefly dipping into the included tube of viral transport media (VTM), pressed and dragged over the surface, sealed into the tubes, and bagged for subsequent processing (see below).

Wastewater sample collection

Wastewater samples were collected from multiple locations on each farm (see Supplemental Data Tables 3-6). Whenever possible, wastewater from the beginning of the milk line cleanout was collected at the end of each milking shift. Samples were also collected from sump pumps, manure lagoons, and/or fields whenever possible. Wastewater samples were collected aseptically. When waste flows were within reach, the external sides of 50mL falcon tubes or 250mL and 1L bottles were first sterilized with 10% bleach and then lowered into the water stream until tubes were filled. When waste flows were out of reach, a 25ft pole with a bottle adapter (VWR) and 1L bottles were used for sample collection. The base of the pole, bottle adapter, and a zip tie were sterilized by submerging them in 10% bleach for 5 minutes before sample collection. The outside of the bottle was also wiped with 10% bleach to sterilize and was then attached to the adapter with the zip tie. The sample was collected by dipping the bottle into the wastewater until full; full bottles were wiped with 10% bleach again and maintained at 4°C from time of collection until processing at Emory University.

Processing samples and quantifying H5N1 viral RNA and infectious virus

AirPrep Cub 210 electret filters: Used filters from the AirPrep Cub were put into 50mL conical tubes with 10 - 15 mL of Brain heart infusion (BHI) media (supplied by National Veterinary Services Laboratory, NVSL) at the end of each sampling day. Tubes were vortexed for 30 seconds and shipped overnight at 4°C to the National Animal Health Laboratory Network

(NAHLN) lab at Iowa State University, where they were assayed for H5N1 viral RNA per standard operating procedures and NAHLN protocols.

PTFE filters: PTFE filters from farm BC were put into 50 mL conical tubes with 10-15 mL BHI at the end of each sampling day, vortexed for 30 seconds, and shipped at 4°C overnight to the Iowa State Veterinary Diagnostic Laboratory, where they were assayed for H5N1 viral RNA per standard operating procedures and NAHLN protocols. PTFE filters from farms BB, BD, BF, and BM were processed at the end of each sampling day at the Lander Veterinary Clinic (LVC). Filters were placed into 50 mL conical tubes with 750 µl of VTM (BD Ref. 220526) and vortexed for 60 seconds, ensuring the entirety of the filter was coated by VTM. Tubes were centrifuged for 10 minutes at 1,000 rpm, and samples were stored at 4°C until RNA extraction and RT-qPCR at LVC as described below. PTFE filters from 2/26/25 – 4/3/25 were placed into 50mL conical tubes with 1mL of VTM (BD Ref. 220526), ensuring VTM coated the filter, and then stored at 4°C until shipment and processing at Emory University. The conical tubes containing the filters were centrifuged for 10 minutes at 1,000 rpm and media removed for RNA extraction; the remaining sample was stored at -20°C.

MD8 Airport gelatin filters: MD8 gelatin filters from BB, BD, BF, and BM were processed at the LVC at the end of each sampling day. Filters from all other farms were shipped to and processed at Emory University. All filters were processed using the same protocol. Briefly, filters were carefully broken up using sterile forceps and placed into 50mL conical tubes with either 3 mL of VTM or 1.5 mL VTM and 1.5mL phosphate buffered saline (PBS). Tubes were vortexed for 60 seconds to ensure all pieces of the filter were in contact with the liquid media. To fully dissolve the gelatin filter, tubes were placed in a warm water bath for 10 minutes. After, an aliquot was removed for RNA extraction while the remaining sample was stored at 4°C (LVC) or -20°C (Emory).

Surface Swabs: Pre-assembled swab tubes (BD Ref. 220526) containing surface samples were processed using the same protocol at LVC and Emory University. Tubes were vortexed for 10 seconds with the flocked swab inside, then centrifuged for roughly 10 seconds at 1,000 rpm to collect all liquid at the bottom of the tube. An aliquot was then removed for RNA extraction, and the remaining sample was stored at 4°C (LVC) or -20°C (Emory).

RNA extraction and RT-qPCR: For samples processed at LVC, RNA extraction was carried out using 200 – 400 µl of sample, using the MagMax CORE Nucleic Acid Purification Kit (Applied Biosystems, Cat. A32700) paired with the Swine Influenza Virus RNA Test Kit (Applied Biosystems, Cat. 4415200) on the KingFisher Flex 96 system (ThermoFisher). 8ul of extracted RNA samples were used in RT-qPCR reactions with the QuantStudio 5 Real-Time PCR System (ThermoFisher, Cat. A28569) according to manufacturer's instructions, with the modification of increased cycling from 40 to 45 to better visualize late-cycle amplification. Samples processed at Emory University used the iTaq Universal Probes One-Step kit (Bio-Rad, Cat. No. 1725141) on

a CFX Connect real-time PCR system (Bio-Rad) using published primers and cycling conditions.[2,3]

Wastewater sample processing and ddPCR: Bottles with wastewater samples were wiped down with 70% ethanol prior to opening, and then approximately 45 mL of raw sample was centrifuged at 16,500 x g for 5 minutes to remove large solids and debris. 10 mL of the supernatant was input into a concentration step using NanoTrap Microbiome Particles A & B (Ceres Nanosciences) in a KingFisher Apex (ThermoFisher); an endogenous control bovine respiratory syncytial virus (BRSV) was added to each sample prior to concentration. Total nucleic acids were extracted using the standard KingFisher MagMax Viral Pathogen kit (ThermoFisher) and eluted in 100 µl. The ddPCR assays targeting the influenza A M gene (total influenza A) and hemagglutinin H5 were run in duplex on all wastewater samples according to previously published protocols.[4] Briefly, droplets were generated using an AutoDG (Bio-Rad), targets amplified using a thermal cycler (Bio-Rad) and quantified using the QX600 (Bio-Rad). Three ddPCR replicates were performed per sample, and the three wells were merged for analysis. A sample was considered positive for a target when three or more droplets were positive. To ensure extraction efficiency, BRSV was quantified in all samples using a singleplex ddPCR assay on a single well.[3]

Infectious virus titration: Samples were tested for infectious virus by titering on confluent (MDCK) epithelial cells (ATCC) by plaque assay as previously described[5] or tissue culture infectious dose 50 ($TCID_{50}$) assay using the Reed and Muench method[6]. Air samples that tested positive for H5N1 viral RNA as determined by a cycle threshold (Ct) value below 40 by RT-qPCR were assayed for presence of infectious virus as described above. All positive milk samples from three of the cows in the longitudinal study (7213, 1082, 8582) were tested for infectivity, as well as milk samples collected from all four teats of individual cows with Ct values below 20. All four positive surface swabs of milking unit shells as well as three swabs of milking inflation liners were assayed for infectivity. Six positive wastewater samples were selected for infectivity testing with both $TCID_{50}$ and plaque assay based on having genome copy per milliliter concentrations above 650 and location of sample collection.

Calculation of virus concentration per liter of air: All RT-qPCR plates processed at Emory University contained a dilution series of an *in vitro* transcribed RNA product of known copy number, allowing generation of a standard curve. This was used for absolute quantification of the genome copy numbers present in each reaction and per milliliter. The genome copy numbers per milliliter were then used to estimate the number of genome copies present per liter of air; the volume of air sampled, in liters: V_{air} = *air flow rate (LPM) × sample duration (minutes).* The virus concentration per liter of air was calculated as:

$$C_{air} = \frac{RT\text{-}qPCR\ concentration\ (\frac{gc}{mL}) \times RNA\ elution\ volume\ (mL) \times \left(\frac{Total\ volume\ used\ to\ process\ sample\ (mL)}{Volume\ of\ sample\ used\ in\ RNA\ extraction\ (mL)}\right)}{V_{air}}$$

Estimation of aerosolized droplet sizes deposited on MD8 Airport filters
Photos were taken of 14 MD8 Airport gelatin filters immediately after air sampling. These photos were analyzed to determine the size of aerosolized droplets deposited on the filters using ImageJ. Photos were cropped to include only the filter surface area, then rescaled to 800 x 800 pixels to standardize and allow size conversion from pixels to millimeters since MD8 gelatin filters have 80mm diameter. To detect spots, a Gaussian blur (σ=2 pixels) was applied to reduce noise with the "Find Maxima" function to generate a mask from the detected maxima. The Regions of Interest (ROIs) were selected with a circularity filter applied (> 0.5) to select only spherical spots and the ROIs were restored onto the original unmasked image for accurate pixel measurement. All detected ROIs were manually inspected to remove false positives and artifacts from maxima detection. The ROI measurement function was used to calculate the number of spots and their area (pixel^2), and the data was exported as a .csv file for processing through a custom Python script (available at FigShare, DOI: 10.6084/m9.figshare.29627840) to convert spots from pixels^2 to mm^2 and calculate spot volumes.

Whole genome sequencing analysis
Whole genome sequencing was performed on nine air samples and one wastewater sample collected from farm EC (Supplemental Data Table 7). For five of the air samples, a cell passaged viral population was sequenced in addition to the original sample. For the wastewater sample, only a cell passaged viral population was sequenced. Nucleic acids were extracted using the NucleoMag RNA kit (Machery-Nagel) with an epMotion 5075 (Eppendorf). The 8 genomic segments of IAV were amplified in a one-step multiplex RT-PCR.[7] For each specimen, extracted RNA, was amplified with each of three different primer sets: Uni12/Inf1, Uni12/Inf3, Uni13/Inf1[7]; MBTuni-12, MBTuni-12.4, MBTuni-13[8]; OptiF1, OptiF2, OptiR[9]. cDNA samples were prepared for sequencing using the Oxford Nanopore Technologies (ONT) platform. Unique barcodes were ligated to RT-PCR products from each sample using end repair and ligation kits (New England Biolabs) and then pooled. These pools were ligated to pore adaptors and sequenced with a GridION instrument. Sequence reads were aligned to the nearest IAV reference and sample consensus sequences were generated using IRMA[10]. Influenza virus clades were assigned based on hemagglutinin sequences using Nextclade[11]. The consensus sequences from each sample and primer set were aligned using MUSCLE in MegAlign Pro (DNA Star) and mutations were identified relative to the overall consensus sequence of all the samples. Due to variable sequencing coverage across the genome, mutations were only identified if they were found in each of a sample's sequences from the three primer set amplifications that had sequencing read coverage at that genetic locus. All reported mutations were identified in at least one amplification reaction with 24-fold or greater coverage depth.

Longitudinal study of infected cows
Longitudinal sampling was conducted on dairy farm EC from 3/18/25 – 3/27/25. Cows were screened for H5N1 virus infection on 3/18 – 3/19 based on being in treatment for mastitis (as determined by dairy farm parlor workers) or were randomly selected during milking. Milk from cows on mastitis treatment doesn't go into the bulk tank. A composite sample of milk was collected from all four quarters and shipped overnight at 4°C to Emory University for RNA extraction and RT-qPCR as described above. Screening results informed the selection of cows for inclusion in the study group; selected cows were marked on right and left flanks to enable identification in pens and the milking parlor, and identifications were confirmed with cows' ear

tag numbers. Eleven H5 positive cows and three H5 negative cows were selected for the study group. One cow (7814) was selected with a negative sample at screening, but on every subsequent study day tested positive for H5N1. Daily, from 3/20/25 – 3/27/25, a sample of milk from each teat of the selected cows was collected into separate 50mL conical tubes and stored at 4°C until shipping and processing at Emory University for viral RNA extraction, RT-qPCR, and/or virus titering as detailed above. Some cows from this study were not available on all testing days. Nasal swabs were performed on cows in the study group on 3/19, 3/22, 3/24, and 3/26 while the cows were in headlocks prior to milking. Preassembled swab kits (BD Ref. 220526) were used and the flocked swab was inserted as far into the cows' nasal cavity as possible before placement in the included tube of VTM. Sampling of the exhaled breath of all available cows on study on a single MD8 Airport gelatin filter was done immediately post nasal swab sampling. All swab and air filter samples were stored at 4°C until they could be processed at Emory University and stored at -20°C prior to RNA extraction and RT-qPCR as described above.

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