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.

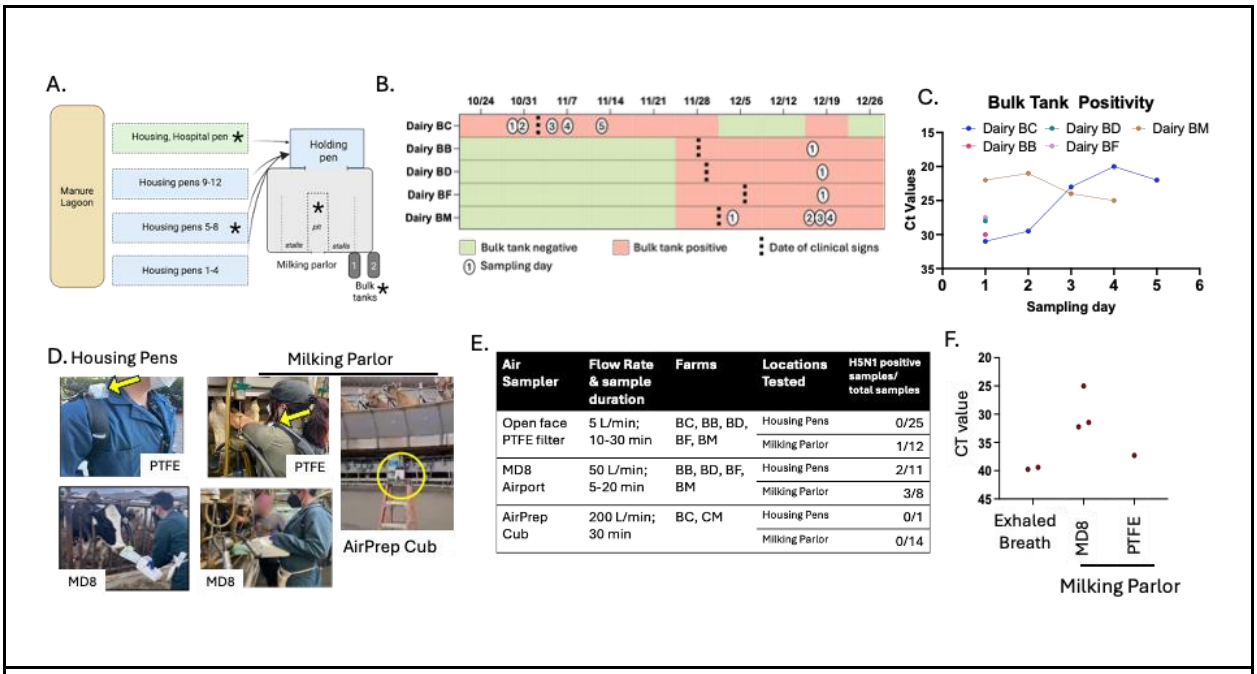
In this study, we describe the results of extensive sampling efforts at fourteen H5N1-positive dairy farms. We report detection of virus in the air during milking of suspected H5N1-infected cows on multiple farms and on multiple days, and in the exhaled breath of cows, suggesting that airborne transmission 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.

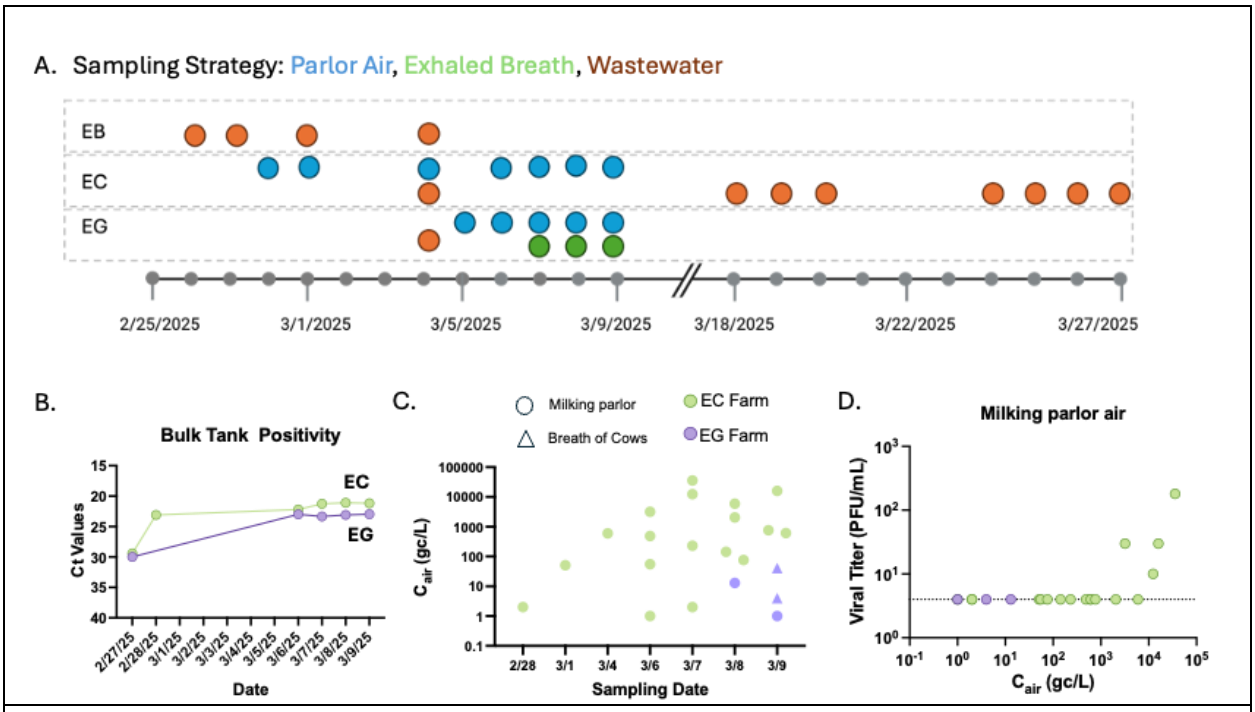
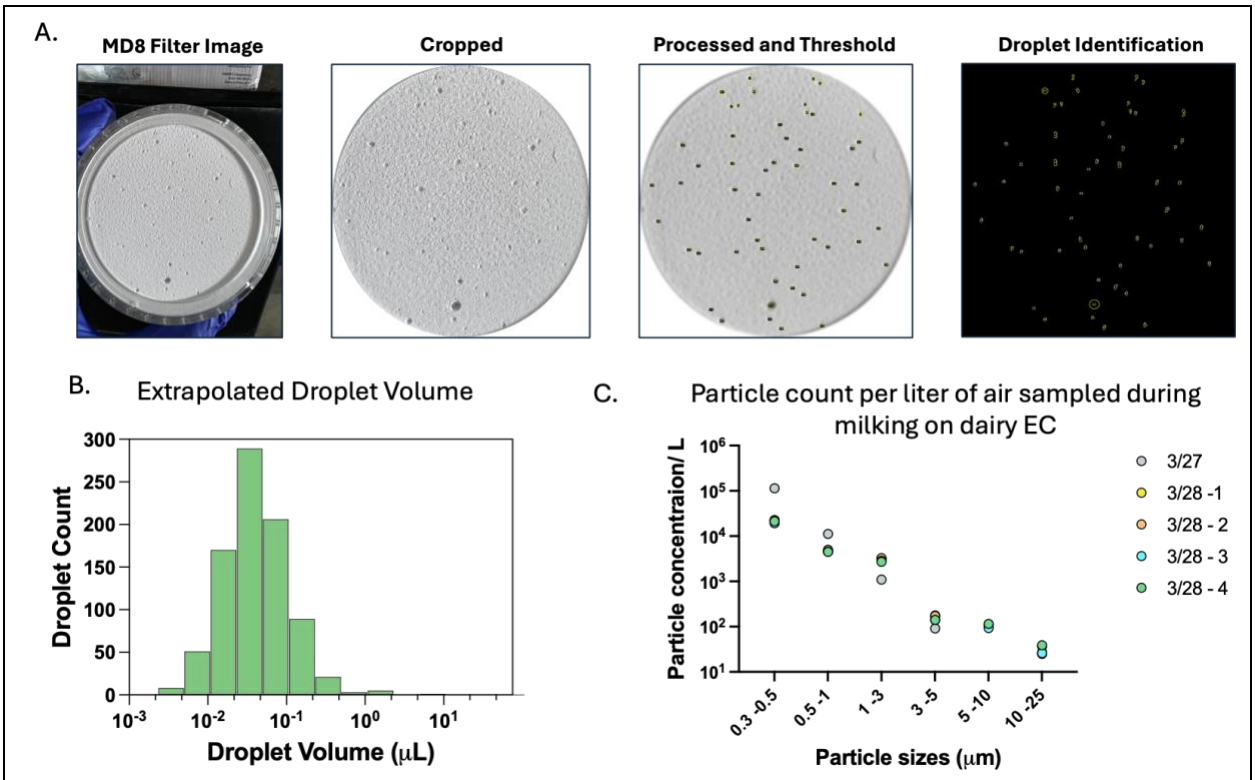


Figure 2: Presence of infectious H5N1 virus in air samples from dairy farms in southern California. (A) Timeline of sampling strategy for farms EB, EC, and EG. Blue dots indicate milking parlor air samples, green dots indicate exhaled breath samples from cows, red dots indicate water samples, and small 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.

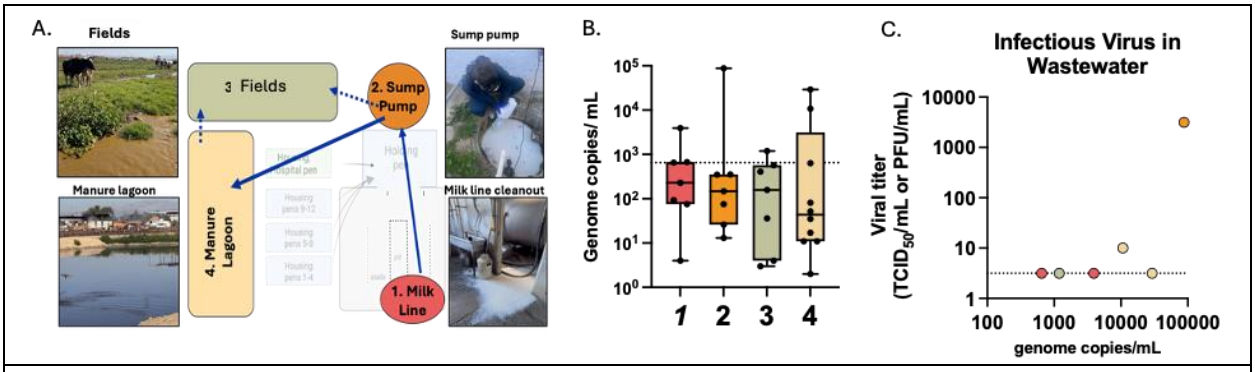
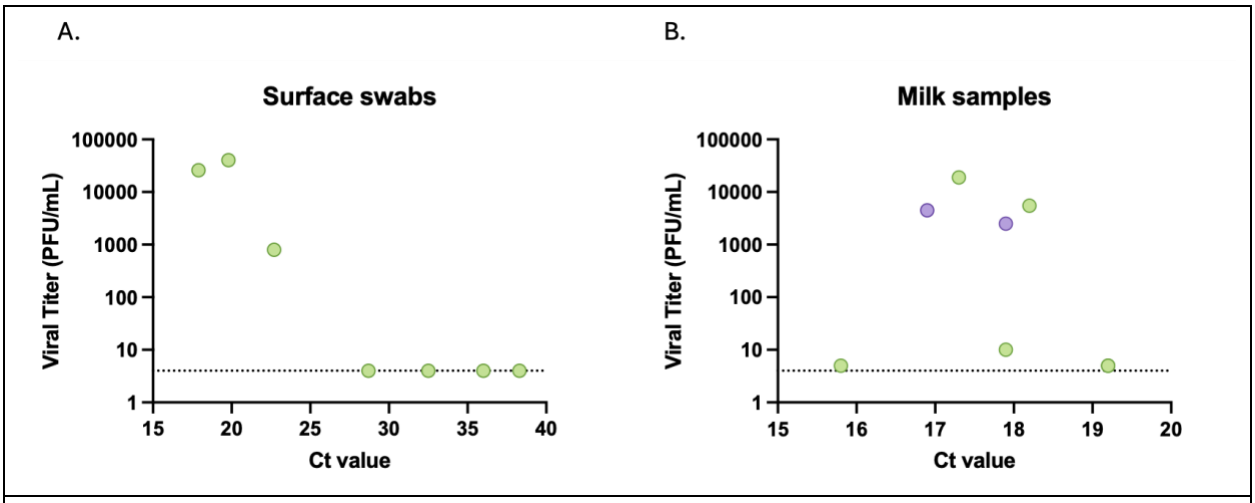


Figure 3: Detection of H5N1 virus in multiple reclaimed wastewater sites on southern California and Central Valley farms. (A) Top-down schematic of a representative dairy farm layout with farm wastewater sources depicted and on-site representative pictures included. Sources tested in the schematic match 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.

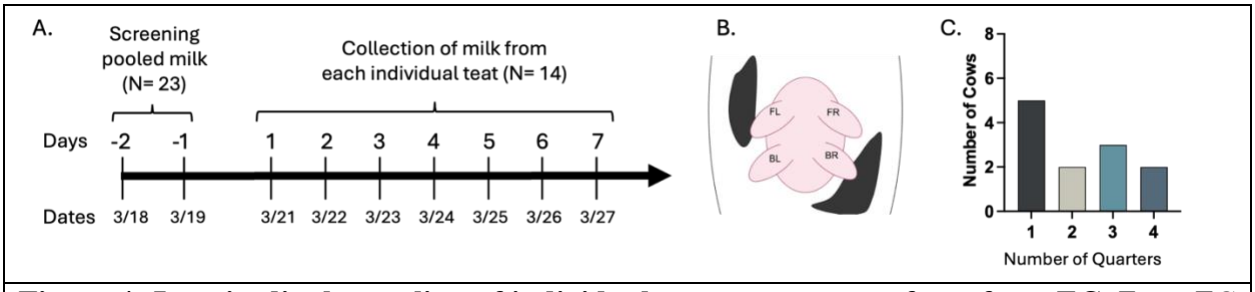


Figure 4: Longitudinal sampling of individual quarters on cows from farm EC. Farm EC tested positive on 2/24/2025. (A) Timeline of screening and longitudinal sampling (for 7 days, starting 3/20 or 3/21) of individual cows. (B) A worm-view schematic identifying the location of each quarter on the udder: 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.

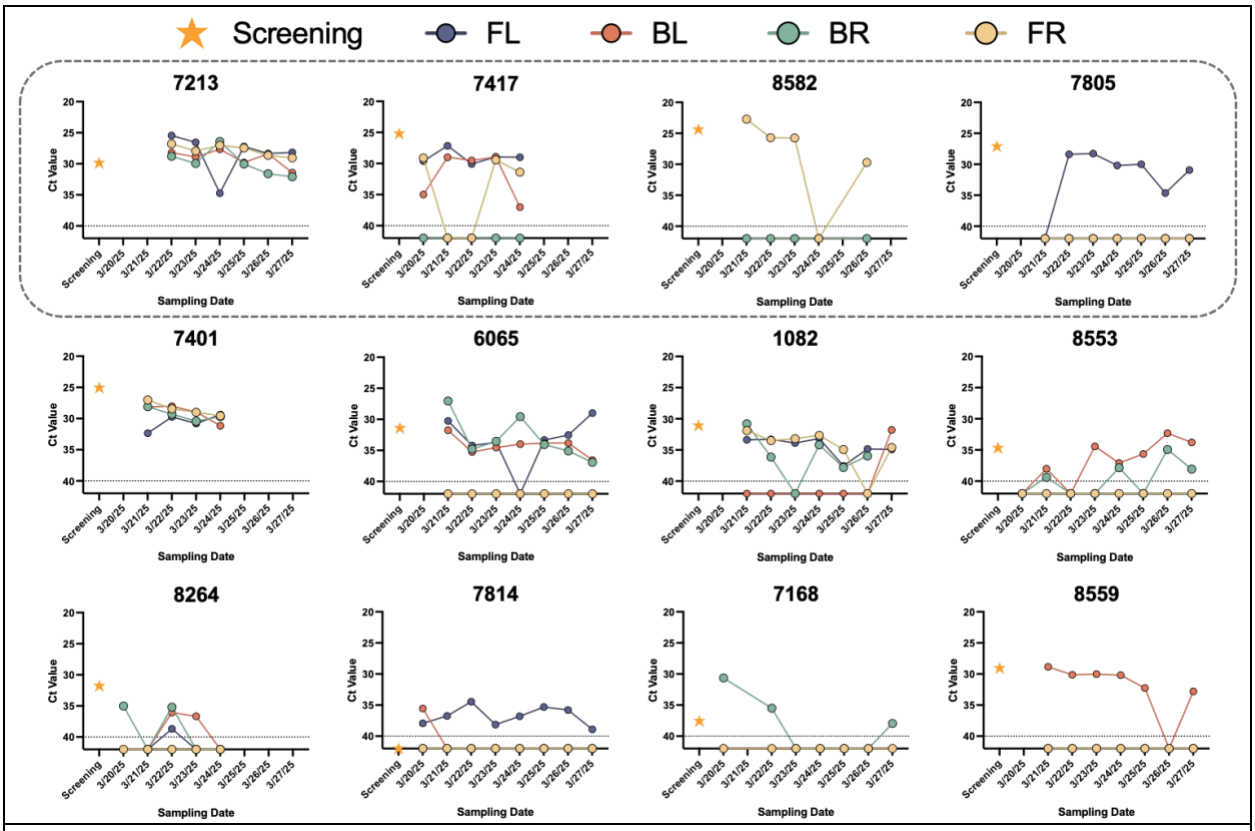


Figure 5: Heterogeneity in H5N1 positive quarters among naturally infected cows. H5N1 viral RNA was measured in milk from individual quarters of 14 cows; 12 PCR-positive cows are shown here, including one cow that had originally screened negative (#7814). Ct values averaged from technical duplicates 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.

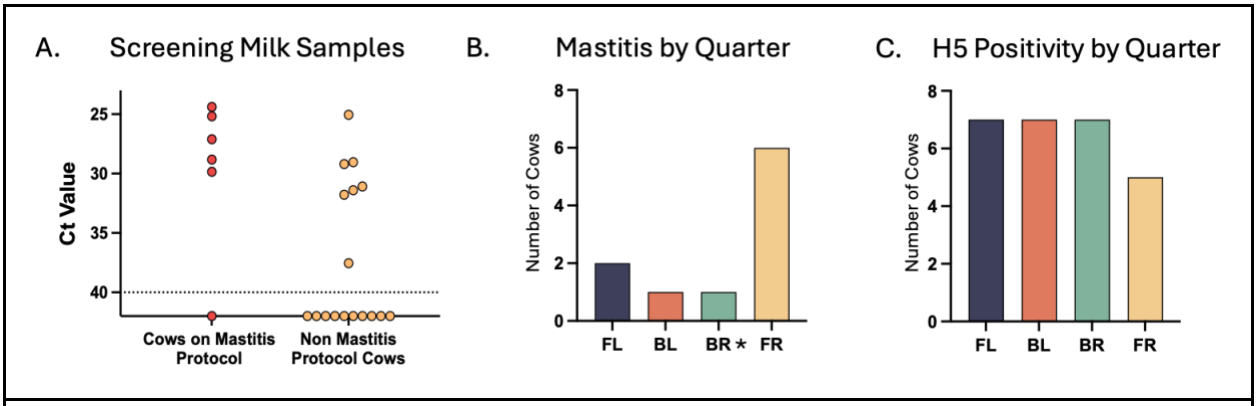


Figure 6. H5N1 infection does not always correlate with clinical mastitis. (A) Cows

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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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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