

1 Characterization of highly pathogenic avian influenza virus in retail dairy products in the US

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14 Running head: HPAIV RNA detection in milk

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23 **Abstract**

24 In March 2024 Clade 2.3.4.4b H5N1 highly pathogenic avian influenza virus (HPAIV) was detected in  
25 dairy cattle in the US and it was discovered that the virus could be detected in raw milk. Although  
26 affected cow's milk is diverted from human consumption and current pasteurization requirements are  
27 expected to reduce or eliminate HPAIV from the milk supply, a study was conducted to characterize  
28 whether the virus could be detected by quantitative real-time RT-PCR (qrRT-PCR) in pasteurized retail  
29 dairy products and if detected, to determine whether the virus was viable. From April 18 to 22, 2024 a  
30 total of 297 samples of Grade A pasteurized retail milk products (23 product types) were collected from  
31 17 US states and represented products from 132 processors in 38 states. Viral RNA was detected in 60  
32 samples (20.2%) with titer equivalents of up to  $5.4 \log_{10}$  50% egg infectious doses (EID<sub>50</sub>) per ml, with a  
33 mean and median of  $3.0 \log_{10}/\text{ml}$  and  $2.9 \log_{10}/\text{ml}$  respectively. Samples that were positive for type A  
34 influenza by qrRT-PCR were confirmed to be clade 2.3.4.4 H5 HPAIV by qrRT-PCR. No infectious virus  
35 was detected in any of the qrRT-PCR positive samples in embryonating chicken eggs. Further studies are  
36 needed to monitor the milk supply but these results provide evidence that infectious virus did not enter the  
37 US pasteurized milk supply before control measures for HPAIV were implemented in dairy cattle.

38

39 **Importance**

40 Highly pathogenic avian influenza virus (HPAIV) infections in US dairy cattle were first confirmed in  
41 March 2024. Because the virus could be detected in raw milk a study was conducted to determine whether  
42 it had entered the retail food supply. Pasteurized dairy products were collected from 17 states in April  
43 2024. Viral RNA was detected in 1 in 5 samples but infectious virus was not detected. This provides a  
44 snap-shot of HPAIV in milk products early in the event and reinforces that with numerous safety  
45 measures, infectious virus in milk is unlikely to enter the food supply.

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47

48 **Introduction**

49 Cow's milk and milk products are an important source of nutrition for humans. In the US, "Grade A" milk  
50 is regulated by a federal-state partnership, the National Conference on Interstate Milk Shipments  
51 (NCIMS), and is administered through adopted regulations, the Pasteurized Milk Ordinance (PMO)  
52 (<https://www.fda.gov/media/140394/download>). The NCIMS helps the industry produce a safe and  
53 wholesome product for the consumer. This regulatory system has multiple layers to ensure food safety.  
54 Cows with mastitis and other disease conditions that could affect milk quality and safety are milked  
55 separately, and the abnormal milk is not included in the supply for human consumption. Milk is also  
56 typically picked up from the farm at regular intervals, and the bulk milk (milk pooled from 600-700 cows)  
57 is routinely tested for commonly used antibiotics and other substances before pasteurization  
58 (<https://www.fda.gov/food/food-compliance-programs/national-drug-residue-milk-monitoring-program>).  
59 Samples are also analyzed on a recurring basis for somatic cell and bacterial plate counts to monitor  
60 quality management practices.

61 Pasteurization is another pivotal layer of the federal-state milk safety system. The primary  
62 method for pasteurization of fluid milk is typically through a continuous flow pasteurizer by high  
63 temperature short time; 72°C for 15 seconds is the most used approved method by regulation in the US  
64 according to the PMO. Variations in pasteurization time and temperatures are allowed that achieve the  
65 same goal of killing pathogenic bacteria and to reduce spoilage bacteria that will in effect increase the  
66 shelf life of the milk. The milk is then packaged and sent to retail markets with strict temperature controls  
67 that further ensures the safety and quality of the product.

68 Infection of dairy cattle with clade 2.3.4.4b H5N1 highly pathogenic avian influenza virus  
69 (HPAIV) was first reported in the US on March 25, 2024 (1). Diagnostic testing of milk from the initial  
70 cases detected viral RNA by real-time RT-PCR. The potential for HPAIV to enter the food supply is  
71 believed to be mitigated because symptomatic cows have decreased milk quality and production thus  
72 preventing the milk from entering the food supply due to milk safety controls. Poor quality milk is

73 normally diverted from the milk supply for human consumption. However, because HPAIV has never  
74 been described in dairy cattle, milk has not been monitored for the virus.

75 Historically, documentation of influenza A virus infection in cattle has been sparse with only a  
76 few reports of clinical disease (2-4), and there has not been evidence of sustained transmission among  
77 cows (5). More recently, serologic studies on respiratory disease or drops in milk production were  
78 reported in Northern Ireland that were associated with a rise in convalescent antibody titers to influenza A  
79 subtypes that are consistent with human seasonal influenza but no virus was isolated to confirm the  
80 lineage present (3). Several experimental studies from the 1950s clearly show that the direct inoculation  
81 of the human PR8 influenza A virus strain or Newcastle disease virus into the udder of lactating dairy  
82 cows or goats could result in infection with measurable virus shedding, however, the studies did not  
83 describe clinical disease or mastitis in the challenged animals (6-9). Until the recent outbreak of clade  
84 2.3.4.4b HPAIV in dairy cattle with sustained transmission, infection of bovines with type A influenza  
85 was not previously reported and therefore was not considered to be an important pathogen of cattle which  
86 delayed initial recognition of the infection.

87 Because the clade 2.3.4.4b H5 HPAIVs belong to the goose/Guangdong/1996 H5 HPAIV lineage,  
88 which is known to have zoonotic potential (10), the objective of this study was to screen pasteurized retail  
89 dairy products for the presence of viral RNA. Positive samples were subsequently evaluated for the  
90 presence of live virus in embryonating chickens eggs. Importantly, human infections with clade 2.3.4.4  
91 H5 HPAIV are rare and numerous risk assessments have concluded that the risk to the general public is  
92 very low ([https://www.ecdc.europa.eu/en/infectious-disease-topics/z-disease-list/avian-influenza/threats-](https://www.ecdc.europa.eu/en/infectious-disease-topics/z-disease-list/avian-influenza/threats-and-outbreaks/risk-assessment-h5)  
93 [and-outbreaks/risk-assessment-h5](https://www.who.int/publications/m/item/assessment-of-risk-associated-with-recent-influenza-a%28h5n1%29-clade-2.3.4.4b-viruses), [https://www.who.int/publications/m/item/assessment-of-risk-](https://www.who.int/publications/m/item/assessment-of-risk-associated-with-recent-influenza-a%28h5n1%29-clade-2.3.4.4b-viruses)  
94 [associated-with-recent-influenza-a%28h5n1%29-clade-2.3.4.4b-viruses](https://www.fao.org/animal-health/situation-updates/global-aiv-with-zoonotic-potential/en), [https://www.fao.org/animal-](https://www.fao.org/animal-health/situation-updates/global-aiv-with-zoonotic-potential/en)  
95 [health/situation-updates/global-aiv-with-zoonotic-potential/en](https://www.fao.org/animal-health/situation-updates/global-aiv-with-zoonotic-potential/en)).

96

97 **Results**

98 **Virus detection.** A total of 297 samples representing 23 pasteurized dairy product types (Supplementary  
99 Table) were collected from 17 states which represent products produced at 132 processing locations in 38  
100 states. Of these, 20.2% (60/297) were positive for the detection of influenza A RNA by qrRT-PCR (Table  
101 1). Virus titer equivalents for positive samples ranged up to  $5.4\log_{10}$  50% egg infectious doses (EID<sub>50</sub>) per  
102 ml, with a mean and median of  $3.0\log_{10}/\text{ml}$  and  $2.9\log_{10}/\text{ml}$  respectively (Supplementary Table). Fluid  
103 milk with different fat contents represented 64.0% (n=190) of the products tested and 75% (n=60) of the  
104 samples in which influenza A was detected by qrRT-PCR.

105 A subset of the samples positive for type A influenza by qrRT-PCR (n=30) were confirmed to be  
106 clade 2.3.4.4 HPAIV by a lineage specific qrRT-PCR test; 100% (30/30) were positive.

107 A total of 60 samples that were positive for type A influenza were tested for infectious virus by  
108 standard testing in ECE. Infectious virus was not detected in any samples (Supplementary Table).

109

## 110 **Discussion**

111 In March 2024 HPAIV was discovered in the milk of infected dairy cattle in the US. Samples were  
112 collected from retail markets in April 2024 to assess a variety of products to provide data for an initial  
113 safety risk assessment of the national milk supply. Samples were selected to be representative of dairy  
114 processors in states that have confirmed HPAIV infected dairy cattle, and states that have not reported  
115 infected herds. Of note, due to the complexity of the milk distribution system, the location of where milk  
116 was processed may not correlate with the location where the milk was produced. Commercial milk is  
117 typically pooled from several dairy farms and routed for bulk processing (i.e., pasteurization) and  
118 distribution to multiple states is a common industry practice. For example, a product could have been  
119 produced by cows in one state, then processed in a different state, and then sold commercially in a third  
120 state. □

121 Most importantly, although viral RNA was detected by qrRT-PCR in 20.2% of the samples, no  
122 infectious virus was detected by testing for replication in ECE, which is a highly sensitive bioassay for  
123 avian influenza virus detection (11, 12). Positive qrRT-PCR indicates that some viral RNA entered the

124 milk supply, however, it can't be determined at what stage, if any, the virus was infectious. First, cows  
125 rapidly develop antibodies after infection which are present in milk and will inactivate the virus. Second,  
126 virus is inactivated by pasteurization and possibly the high shear force of homogenization. Work with  
127 continuous flow pasteurization is in progress to confirm the conditions for virus inactivation.

128 This study has several limitations that make wider extrapolation of HPAIV RNA levels in  
129 pasteurized dairy products difficult. First, the sample size is small. The scope of this study was to obtain  
130 an initial snap-shot of whether dairy products had evidence of virus in retail milk samples after the  
131 detection of virus in raw milk from dairy cows. Further, some samples were intentionally collected from  
132 regions with known HPAIV infected dairy herds, therefore these data likely provide a higher positivity  
133 rate than would be expected from a random testing process. Since the recognition of dairy cattle infection  
134 with HPAIV, farmers are more aware of the disease, and diagnostic testing can occur in many of the  
135 USDA approved laboratories in the National Animal Health Laboratory network. Currently, dairy cattle  
136 must be tested before moving across state lines ([https://www.aphis.usda.gov/sites/default/files/dairy-  
137 federal-order.pdf](https://www.aphis.usda.gov/sites/default/files/dairy-federal-order.pdf)) helps mitigate contaminated milk from entering the human food supply. Finally,  
138 regardless of the specific detection of HPAIV infection, cows will develop mastitis which will also result  
139 in removing their milk from the food supply.

140 In general, numerous measures in the milk production process will greatly reduce, if not  
141 eliminate, the risk for infectious influenza A virus entering the retail milk supply. First, approximately  
142 99% of the US commercial milk supply ([https://downloads.usda.library.cornell.edu/usda-  
143 esmis/files/4b29b5974/hq37xb74r/s1786b07q/mlkpdi24.pdf](https://downloads.usda.library.cornell.edu/usda-esmis/files/4b29b5974/hq37xb74r/s1786b07q/mlkpdi24.pdf)) that is produced on dairy farms in the US  
144 comes from farms that participate in the Grade "A" milk program and follow the PMO  
145 (<https://www.fda.gov/media/140394/download>), which includes numerous layers of quality controls that  
146 help ensure the safety of dairy products. Second, the US federal-state milk safety system requires that  
147 milk from sick cows is diverted for further processing or is destroyed.

148 More studies are needed to characterize the risk of HPAIV entering the milk supply long term but  
149 this study provides initial evidence that infectious HPAIV has not reached the US retail milk supply. A

150 combination of the previously implemented sanitary control measures (e.g., PMO) and new HPAIV  
151 specific measures are expected to further ensure a safe milk supply.

152

## 153 **Materials and Methods**

### 154 **Retail dairy product sample collection.**

155 The US Food and Drug Administration (FDA) collected 297 samples at retail locations in 17 states  
156 between April 18 and 22, 2024. Sample sites were selected by local FDA Milk Specialists and field staff,  
157 in the Office of Regulatory Affairs. Samples were shipped directly by overnight courier to the US  
158 National Poultry Research Center, USDA- Agricultural Research Service where testing was conducted.  
159 Sample collection was designed to include both products processed in states where HPAIV infections in  
160 dairy herds had been confirmed by the National Veterinary Services Laboratories, APHIS-USDA, at the  
161 time of collection, as well as samples from states with no confirmed infections in dairy herds. Within  
162 these bounds, sample collection was random and based on retail availability. Samples represented  
163 pasteurized retail dairy products produced at 132 processors in 38 states (AR, AZ, CA, CO, CT, FL, GA,  
164 IA, ID, IL, IN, KS, KY, MA, ME, MI, MN, MO, NC, ND, NE, NH, NJ, NV, NY, OH, OK, OR, PA, SC,  
165 TN, TX, UT, VA, VT, WA, WI, WV). Samples included fluid milk (whole, 1%, 2%, skim), cream (heavy  
166 cream, light cream, and similar), half & half, cottage cheese (and similar), sour cream, and yogurt  
167 (Supplementary Table). All samples were Grade A pasteurized dairy products regulated under the PMO.  
168 (<https://www.fda.gov/media/140394/download>, [https://www.fda.gov/food/guidance-documents-](https://www.fda.gov/food/guidance-documents-regulatory-information-topic-food-and-dietary-supplements/milk-guidance-documents-regulatory-information)  
169 [regulatory-information-topic-food-and-dietary-supplements/milk-guidance-documents-regulatory-](https://www.fda.gov/food/guidance-documents-regulatory-information-topic-food-and-dietary-supplements/milk-guidance-documents-regulatory-information)  
170 [information](https://www.fda.gov/food/guidance-documents-regulatory-information-topic-food-and-dietary-supplements/milk-guidance-documents-regulatory-information)) by FDA and its state milk regulatory partners.

171 **Sample processing.** Samples were immediately processed after receipt. Product with temperatures  $>7^{\circ}\text{C}$   
172 were discarded and are not included in the sample numbers of this study. Samples were assigned a unique  
173 accession number and the original packaging was labeled and stored at  $4^{\circ}\text{C}$ . Product origin (US state) and  
174 product type were recorded.

175           Approximately 50ml of each product was portioned into sterile containers. Each sample was  
176 processed for RNA extraction and quantitative real-time RT-PCR (qrRT-PCR) as described below.  
177 Positive samples with titer equivalents of  $\geq 3.9\log_{10}$  50% egg infectious doses (EID<sub>50</sub>)/ml based on qrRT-  
178 PCR were quantified in embryonating chicken eggs (ECE) and samples with titers  $\leq 3.8\log_{10}$  EID<sub>50</sub>/ml  
179 were tested for viable virus in ECE as described below. The cut-off for quantification was selected  
180 because it was expected that, if present, the quantity of infectious virus would be lower than the quantity  
181 detected by qrRT-PCR and quantification of low levels would not be informative.

182 **RNA extraction.** RNA was extracted from fluid homogenized dairy products using the MagMax  
183 magnetic bead extraction kit (Thermo Fisher Scientific, Waltham, MA) in accordance with manufacturer's  
184 instruction. Semi-solid products (e.g., sour cream, yogurt, cottage cheese) were extracted using a hybrid  
185 procedure with Trizol LS (Thermo Fisher Scientific) and the MagMax magnetic bead kit. Semi-solid  
186 products were portioned by spatula based on weight (approximately 0.25g). Briefly, VetMAX Xeno  
187 (Thermo Fisher Scientific) was used as an extraction and internal positive control was added to the Trizol  
188 LS for each reaction prior to sample addition. Then 0.25ml or 0.25g of product was added to 0.75ml of  
189 Trizol LS and mixed. The mixture was incubated at room temperature for 7-10minutes and 0.2ml of  
190 chloroform was added and mixed, incubated at room temperature for an additional 7-10minutes and  
191 centrifuged for 10minutes at 15,000xg at 4°C. RNA was recovered from 0.05ml of the aqueous phase by  
192 the MagMax magnetic bead kit in accordance with the kit instructions.

193 **Quantitative real-time RT-PCR.** A qrRT-PCR test targeting the influenza A M gene was run on a  
194 QuantStudio5 (Thermo Fisher Scientific) as described (13). The primers and probe for the internal control  
195 were used as directed by the kit instructions. Titer equivalents were determined by including a standard  
196 curve derived from RNA extracted from a 10-fold dilutions series of quantified avian influenza virus  
197 stocks (14). A subset of the influenza A qrRT-PCR positive samples were tested with an additional qrRT-  
198 PCR test that is specific for the 2.3.4.4b H5 lineage with a highly pathogenic cleavage site (15).

199 **Virus detection and quantification in embryonating chickens eggs.** All samples (1ml) were treated for  
200 1hr at ambient temperature (approximately 21°C) with antibiotics (final concentration: penicillin G 1000



201 IU/ml, streptomycin 200 µg/ml, gentamicin 100 µg/ml, kanamycin 65 µg/ml, amphotericin B 2 µg/ml).  
202 Then dilutions were made in brain heart infusion (BHI) broth with antibiotics for samples that were  
203 quantified. Semisolid samples were mixed 1:1 (0.5g:0.5ml) with brain heart infusion broth prior to  
204 inoculation into ECE or dilution. Samples were inoculated for virus detection (undiluted for 2 passages)  
205 or quantified using standard methods (16, 17). Hemagglutination assay was used to confirm the presence  
206 of avian influenza virus (18).

207

## 208 **Acknowledgements**

209 The authors gratefully thank Nathan Anderson, Frankie Beacorn, Tarrah Bigler, Nick Chaplinski, Suzanne  
210 DeBlois, Edna Espinoza, Jesse Gallagher, Javier Garcia, Jessica Gladney, David Haley, Anne Hurley-  
211 Bacon, Lindsay Killmaster, Scott Lee, Stephen Norris, David Pearce, Timothy Roddy, Melinda  
212 Vonkunghong, Stephen Walker, Robin Woodruff, and Ricky Zoller for technical assistance with this  
213 work.

214 Any use of trade, firm, or product names is for descriptive purposes only and does not imply  
215 endorsement by the US Government. This research was supported by US Department of Agriculture  
216 (USDA)-Agricultural Research Service Project No. 6040-32000-081-00D and the US Food and Drug  
217 Administration by IAA contract number pending. All opinions expressed in this paper are the authors' and  
218 do not necessarily reflect the policies and views of the USDA or FDA. The USDA is an equal opportunity  
219 provider and employer.

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268

**Table 1.** Detection of influenza A in pasteurized retail dairy products by quantitative real-time RT-PCR.

Titer values are expressed as  $\log_{10}$  50% egg infectious doses determined by a standard curve using quantified virus. No infectious virus was detected in any of the qrRT-PCR positive samples.

<b>Product</b>	<b># positive / total tested (% positive)</b>	<b>Mean titer equivalents (<math>\pm</math> standard deviation)</b>
Whole milk	16/68 (23.5)	3.0 $\pm$ 1.1
2% reduced fat milk	16/58 (27.6)	3.1 $\pm$ 1.2
1% low fat milk	9/28 (32.1)	3.1 $\pm$ 1.2
Skim milk	4/36 (11.1)	3.3 $\pm$ 0.7
Half and half	6/25 (24.0)	2.3 $\pm$ 1.0
Yogurt	0/14 (0)	Not applicable
Cream	3/17 (17.6)	2.3 $\pm$ 0.9
Cottage cheese	1/21 (4.8)	2.6 $\pm$ 0.0
Sour cream	5/30 (16.7)	3.4 $\pm$ 1.2
<b>Total</b>	<b>60/297 (20.2)</b>	<b>3.1 <math>\pm</math> 1.1</b>