Title:

Unexpected Detection of Highly Pathogenic Avian Influenza (HPAI) H5N1 virus in bovine semen from a bull used for natural breeding on an affected dairy farm

Authors:

Ailam Lim[1,2†*], Keith Poulsen[1,3†], Leonardo C. Caserta[4], Lizheng Guan[5], Eryn Opgenorth[1], Maxwell P. Beal[6,7], Amie J. Eisfeld[5], Yoshihiro Kawaoka [5,8,9,10], Diego G. Diel[4]

Author Affiliations:

[1] University of Wisconsin-Madison Wisconsin Veterinary Diagnostic Laboratory, Madison, Wisconsin, USA (A. Lim, E. Opgenorth, K. Poulsen)

[2] University of Wisconsin-Madison Dept. of Pathobiological Sciences, Madison, Wisconsin, USA (A. Lim)

[3] University of Wisconsin-Madison Dept. of Medical Sciences, Madison, Wisconsin, USA (K. Poulsen)

[4] Cornell University Animal Health Diagnostic Center, Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Ithaca, New York, USA (L.C. Caserta, D.G. Diel)

[5] University of Wisconsin-Madison, Dept. of Pathobiological Sciences and Influenza Research Institute, Madison, Wisconsin, USA (L. Guan, A.J. Eisfeld, Y. Kawaoka)

[6] Mill Creek Veterinary Services, Visalia, California, USA (M. P. Beal)

[7] Dakota Dairy Health, Brookings, South Dakota, USA (M. P. Beal)

[8] University of Tokyo Department of Virology, Institute of Medical Science, Tokyo, Japan (Y. Kawaoka)

23 [9] University of Tokyo Pandemic Preparedness, Infection and Advanced Research Center
24 (UTOPIA), Tokyo, Japan (Y. Kawaoka)
25 [10] Japan Institute for Health Security, National Institute of Global Health and Medicine, Tokyo,
26 Japan (Y. Kawaoka)
27
28 † These authors contributed equally to this work
29
30 *Corresponding author. Ailam Lim, Wisconsin Veterinary Diagnostic Laboratory, 445 Easterday
31 Lane, Madison, WI 53706, USA. allim2@wisc.edu.
32 Ailam Lim is a Clinical Associate Professor in the Department of Pathobiological Sciences at the
33 University of Wisconsin–Madison, she serves as the Section Head of Virology and Molecular
34 Diagnostics at the Wisconsin Veterinary Diagnostic Laboratory, Madison. She specializes in
35 veterinary diagnostics and infectious disease, with primary focus on advancing molecular
36 diagnostic assays.
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38 **Running Title:** HPAI H5N1 detection in bovine Semen
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40 **Keywords:** Highly Pathogenic Avian Influenza, H5N1, Biosecurity
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42 **Abstract:**
43 Since March 2024, HPAI H5N1 virus has infected dairy cattle in the U.S., prompting concern
44 about novel transmission routes. During an outbreak in California, HPAI H5N1 RNA was

detected in an asymptomatic bull's semen. Although infectious virus was not isolated, questions remain about semen-associated transmission risks and biosecurity practices.

Main Text

Since March 2024, detection of clade 2.3.4.4b highly pathogenic avian influenza (HPAI) H5N1 in U.S. dairy cattle has raised concerns about the virus's ability for cross-species transmission, adaptation to mammals, and novel transmission routes, including milk (1,2). The knowledge that viruses are infectious in bovine semen and the finding of HPAI in turkey semen, has prompted questions about the potential role of HPAI transmission in bovine semen (3,4). The shedding of HPAI H5N1 in bovine semen could result in silent viral spread within herds and across geographic regions via artificial insemination (AI). While there are reports of clinical HPAI disease in female calves and pregnant animals, reports of diseased bulls in dairies or beef cattle are lacking. While many questions about the pathophysiology of HPAI H5N1 in U.S. dairy herds remain unanswered, movement of lactating cows is a recognized risk factor for interstate disease spread. This diagnostic study sought evidence of HPAI H5N1 shed via semen in natural breeding bulls on an HPAI H5N1-affected dairy farm in California.

The HPAI H5N1 genotype B3.13 outbreak in California began in August 2024, likely resulting from the interstate movement of infected cows, which led to the rapid spread of the virus within the state. In October 2024, infection in a 4,500-head Holstein dairy was detected by reverse transcription-polymerase chain reaction (RT-PCR) for H5N1 RNA in bulk tank milk samples. Clinical signs in lactating dairy cows included decreased milk production, mastitis, lethargy, dehydration, anorexia, and pyrexia (>104.0°F). The herd experienced a 60% morbidity rate over three weeks. About four weeks later, the herd veterinarian collected diagnostic samples

68 from three three-year-old Holstein bulls, including deep nasal swabs, preputial scrapings, pre-
69 ejaculate seminal fluid, semen and serum samples. These were sent to the Wisconsin Veterinary
70 Diagnostic Laboratory for testing. Subjectively, the semen appeared to have low sperm
71 concentration and volume, although total sperm count was not measured. The poor semen quality
72 was likely due to timing and suboptimal sampling conditions, as the bulls had been co-mingled
73 with cows the same day for breeding.

74 Initially, the samples were examined for influenza A virus (IAV) using various methods
75 (Table 1 and Supplementary File 1). Deep nasal swabs, preputial scrapes, pre-ejaculate seminal
76 fluids, and semen were tested using multiple IAV Matrix RT-PCR assays (5). The IAV RNA
77 was detected in the semen from Bull 1 (147816-25) by three different PCR assays, but Bulls 2
78 and 3 were tested negative. No detection was reported in any other samples. The IAV RNA
79 detection in Bull 1 was confirmed with an additional RNA extraction to rule out laboratory
80 contamination. The IAV strain was further identified as HPAI using the H5N1 2.3.4.4b lineage
81 subtyping RT-PCR assay.

82 Targeted IAV sequencing at the Cornell Animal Health Diagnostic Center confirmed the
83 presence and yielded a partial H5N1 genome (Supplementary Table S1, GISAID accession
84 number EPI_ISL_20206713). Attempts to assemble the full genome were unsuccessful due to
85 the low viral load in the semen. Phylogenetic analysis of the partial concatenated genome
86 sequences indicated that the viral RNA in Bull 1 (147816-25) clustered within the B3.13
87 genotype and was closely related to a sample collected from a Californian dairy farm worker
88 during the same period (Figure 1) (6).

89 All Bull 1 samples were processed for virus isolation at the University of Wisconsin—
90 Madison Influenza Research Institute to attempt recovery of an isolate. Serially diluted samples

91 inoculated into 10-day-old specific-pathogen-free embryonated chicken eggs or Madin–Darby
92 canine kidney cells showed no embryo death or cytopathic effect, respectively. All samples were
93 confirmed negative by hemagglutination assay.

94 The sera from all bulls tested negative by antigen-based influenza A ELISA (7).
95 However, the S/P ratio for Bull 1 was 0.620, which is close to the validated 0.5 assay cutoff,
96 potentially indicating seroconversion.

97 Because of the limited semen volume for analysis, further confirmation testing at the
98 national reference laboratory was not performed. Additional samples were requested several
99 months later for convalescent testing, but Bull 1 had been culled from the herd. The importance
100 of identifying HPAI H5N1 in bovine semen remains uncertain, as the virus could have been
101 actively shed in semen or the ejaculate could have been contaminated by the environment.
102 Although detecting RNA does not confirm the presence of infectious viruses, this finding
103 warrants further investigation into whether HPAI H5N1 can be shed in semen and raises
104 questions about farm biosecurity amid the ongoing outbreak. Good biosecurity measures are
105 essential to preventing infections and, if infection occurs, slowing disease spread on the farm.

106 If these findings were to be repeated and confirmed, it would have significant
107 implications for natural breeding and biosecurity for AI collection centers. Further research and
108 risk assessments are needed to determine whether naturally infected HPAI H5N1 bulls shed virus
109 in semen, and, if so, evaluate the risk of disease spread on dairy farms and with AI programs.

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141 Table 1: Influenza A virus testing results for the samples collected from the three bulls in an

142 H5N1 infected farm in Central Valley of California.

Bull	Specimen	IAV ELISA result (S/P ratio)	IAV RT-PCR result (CT)	H5 RT-PCR result (CT)	2.3.4.4b RT-PCR result (CT)	Virus Isolation
Bull 1 (147816-25)	serum	Negative (0.620)	-	-	-	Negative
	deep nasal swabs	-	Negative	-	-	Negative
	preputial scrape	-	Negative	-	-	Negative
	pre-ejaculate	-	Negative	-	-	Negative
	semen	-	Detected * (38.2, 39.5) ‡ (Negative, 39.1) § (37.9, 38.9) £	Negative	Detected (39.6)	Negative
Bull 2	serum	Negative (1.000)	-	-	-	-
	deep nasal swabs	-	Negative	-	-	-
	preputial scrape	-	Negative	-	-	-
	pre-ejaculate	-	Negative	-	-	-
	semen	-	Negative	-	-	-
Bull 3	serum	Negative (0.880)	-	-	-	-
	deep nasal swabs	-	Negative	-	-	-
	preputial scrape	-	Negative	-	-	-
	pre-ejaculate & semen	-	Negative	-	-	-

143 *Sample was subjected to further sequencing analysis

144 ‡ Wisconsin Veterinary Diagnostic Laboratory IAV assay

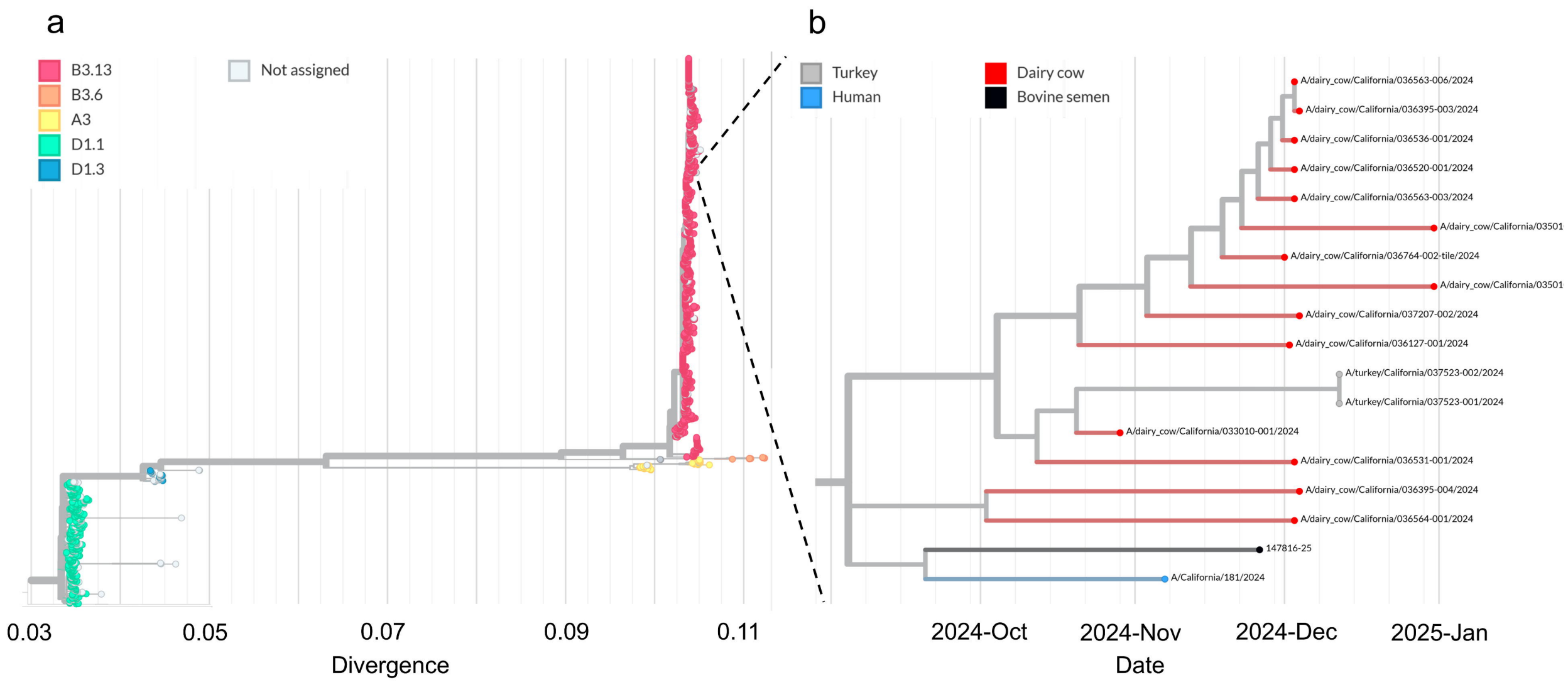
145 § National Animal Health Laboratory Network IAV Matrix assay

146 £ Modified National Animal Health Laboratory Network IAV Matrix assay

147 - Not performed

148

149 **Figure 1.** Phylogenetic analysis of partial concatenated genome sequences confirming detection
150 of highly pathogenic avian influenza A(H5N1) virus genotype B3.13 from bovine semen. A)
151 Tree showing broader phylogeny of H5N1 virus genotypes; B) timescale tree showing closer
152 examination of the virus from the Bull 1 semen (sample no. 147816-25) and closely related virus
153 sequences.


a
B3.13
B3.6
A3
D1.1
D1.3
Not assigned
0.03
0.05
0.07
0.09
0.11
Divergence
b
Turkey
Human
Dairy cow
Bovine semen
A/dairy_cow/California/036563-006/2024
A/dairy_cow/California/036395-003/2024
A/dairy_cow/California/036536-001/2024
A/dairy_cow/California/036520-001/2024
A/dairy_cow/California/036563-003/2024
A/dairy_cow/California/03501
A/dairy_cow/California/036764-002-tile/2024
A/dairy_cow/California/03501
A/dairy_cow/California/037207-002/2024
A/dairy_cow/California/036127-001/2024
A/turkey/California/037523-002/2024
A/turkey/California/037523-001/2024
A/dairy_cow/California/033010-001/2024
A/dairy_cow/California/036531-001/2024
A/dairy_cow/California/036395-004/2024
A/dairy_cow/California/036564-001/2024
147816-25
A/California/181/2024
2024-Oct
2024-Nov
2024-Dec
2025-Jan
Date