Highly Pathogenic Avian Influenza A(H5N1) Virus Outbreak in New England Seals, United States

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We report the spillover of highly pathogenic avian influenza A(H5N1) into marine mammals in the northeastern United States, coincident with H5N1 in sympatric wild birds. Our data indicate monitoring both wild coastal birds and marine mammals will be critical to determine pandemic potential of influenza A viruses.

Highly pathogenic avian influenza (HPAI) viruses are of concern because of their pandemic potential, socioeconomic impact during agricultural outbreaks, and risks to wildlife conservation. Since October 2020, HPAI A(H5N1) virus, belonging to the goose/Guangdong H5 2.3.4.4b clade, has been responsible for >70 million poultry deaths and >100 discrete infections in many wild mesocarnivore species (*1*). As of January 2023, H5N1 infections in mammals have been primarily attributed to consuming infected prey, without evidence of further transmission among mammals.

We report an HPAI A(H5N1) virus outbreak among New England harbor and gray seals that was concurrent with a wave of avian infections in the region, resulting in a seal unusual mortality event (UME); evidence of mammal adaptation existed in a small subset of seals. Harbor (*Phoca vitulina*) and gray (*Halichoerus grypus*) seals in the North Atlantic are known to be affected by avian influenza A virus and have experienced previous outbreaks involving seal-to-seal transmission (*2*–*7*). Those seal species represent a pathway for adaptation of avian influenza A virus to mammal hosts that is a recurring event in nature and has implications for human health.

The Study

The first detections of HPAI clade 2.3.4.4b viruses in North America were in wild and domestic birds in November 2021 in Canada and late December 2021 in the United States (*8*–*11*). Starting on January 20, 2022, avian oropharyngeal or cloacal samples were collected from wild birds by personnel in 4 wildlife clinics in Massachusetts. Additional opportunistic samples were collected in Maine and Massachusetts in response to suspicious avian deaths in seabird breeding colonies. We screened samples from 1,079

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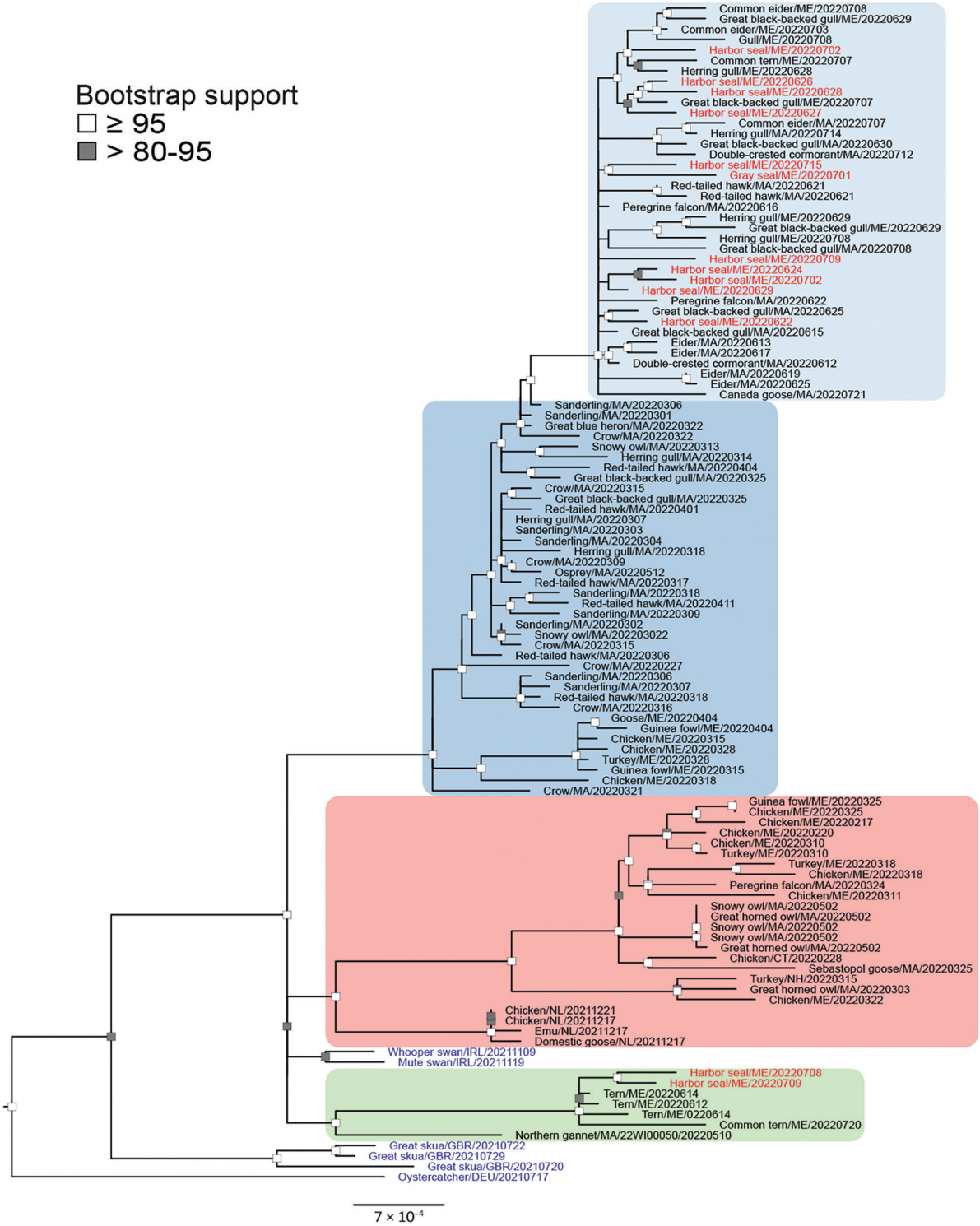

Figure 1. Phylogenetic analysis of highly pathogenic avian influenza A(H5N1) viruses from New England birds and seals, United States. Complete genomes of HPAI H5N1 viruses (GISAID database, https://www.gisaid.org) were compared by using IQ-TREE (https://www.iqtree.org) with the Ultrafast bootstrap (n = 10,000) option and A/chicken/NL/FAV-0033/2021 as a reference. Bootstrap support values >80 are shown at nodes. Red text indicates seal-derived sequences, black text avian-derived sequences from New England and Newfoundland, and blue text indicates avian-derived sequences from Europe . Branches are shaded on the basis of lineage groups: primary lineage from North America, pink; New England–specific lineage A, 1st wave blue, 2nd wave light blue; and New England–specific lineage B, green. All newly reported specimens were collected in the New England region during February–July 2022. Scale bar indicates nucleotide substitutions per site.

individual wild birds representing 78 avian species of concern for H5 influenza and identified 119 infected birds from 21 species (Appendix 1 Figure 1, panel A, https://wwwnc.cdc.gov/EID/article/29/4/22-1538-App1.pdf; Appendix 2, https://wwwnc.cdc.gov/EID/article/29/4/22-1538-App2.xlsx). Wild birds in New England experienced 2 waves of influenza infections during 2022. The first wave peaked in March and was largely represented by raptor deaths (39.1% influenza-positive birds). A second wave began in June; gull (38% influenza-positive) and eider (26.8% influenza-positive) deaths were most frequently reported during the second wave. Mortality events affected seabird breeding colonies throughout the coastal region during the second wave; 8 islands had ≥1 bird test positive for H5 (Appendix 1 Table).

During January 20–July 31, 2022, opportunistic nasal, oral, conjunctival or rectal swab samples were collected from 132 stranded seals along the North Atlantic coast from Maine to Virginia (Appendix 2). HPAI virus was not detected in any of the 82 seals that were sampled through May 31, 2022. Concurrent with the second wave of avian infections, increased seal strandings in Maine led to a National Oceanic and Atmospheric Administration declaration of a UME beginning on June 1, 2022, that included 164 harbor and 11 gray seals in Maine during June and July (*12*). Swab samples were collected from 41 of those animals; 17/35 harbor and 2/6 gray seals were HPAI-positive and were within coastal regions of known and suspected HPAI outbreaks among terns, eiders, cormorants, and gulls (Appendix Figure 1, panel B). Respiratory symptoms were observed with a subset of neurologic cases, although most stranded seals were deceased. The respiratory tract was the most consistent source of reverse transcription PCR–positive samples from affected seals (15/19 nasal, 16/19 oral, 6/19 conjunctiva, and 4/19 rectal samples).

We sequenced influenza A viruses from swab samples, resulting in 71 avian- and 13 seal-derived virus genomes from New England. We performed phylogenetic analysis of sequences from New England and the most closely related available virus sequences by using IQ-TREE (https://www.iqtree.org) (Figure 1; Appendix 1). We classified all but 1 virus as nonreassortant Eurasia 2.3.4.4b viruses and included those in further analyses (Appendix 1 Figures 2, 3). Sequences fell into 4 distinct clusters; 2 lineages were unique to New England. We found single-nucleotide polymorphisms (SNPs) (Appendix 1 Figures 4–7) and amino acid mutations (Figure 2) by using vSNP (https://github.com/USDA-VS/vSNP). Most sequences fell within a dominant New England–specific cluster that spanned the first and second waves (lineage A in this study). All second-wave viruses from lineage A exhibited the acquisition of new, shared mutations. That cluster spanned diverse species, including gulls, geese, eiders, raptors, and seals. A small number of raptor-derived sequences clustered with the primary lineage prevalent in North America at the time of sampling. All but 1 sample from the second wave of avian infections fell into either lineage A or a smaller, unique cluster primarily associated with terns (lineage B in this study).

We inferred that ≥2 spillover events occurred in the seal population during the second wave of avian infections. Of the sequences derived from seals, 11/13 clustered with second wave lineage A (Figure 1). We found 4 aa changes in specific proteins in both birds and seals that were distinct from the first wave of HPAI (polymerase acidic protein, A70V; polymerase acidic X protein, A62V; hemagglutinin protein, P152S; and nonstructural 1 protein, R67Q) (Figure 2). Within second wave lineage A, we found 37 aa changes in ≥1 seal sequence that were infrequent or absent from bird sequences. Most changes were unique; each occurred in only 1 animal. The polymerase basic 2 protein amino acid substitutions, E627K (in seal no. Pv/MME-22–131) and D701N (in seal no. Pv/MME-22–122), previously associated with mammalian adaptation were each present in 1 seal in second wave lineage A. An additional 2/13 seal-derived sequences clustered with lineage B; 10 aa mutations occurred in both bird and seal sequences (Figure 2). Another 10 aa changes occurred in at least 1 seal sequence that were infrequent or absent in the bird sequences. In contrast to lineage A, most amino acid changes were shared between the 2 seals in lineage B and were derived from animals stranded within the same town and sampled 1 day apart. The polymerase basic 2 protein substitution, D701N, was present in 1 seal from lineage B (Figure 2, seal no. PV12, MME-22–195).

Conclusions

Transmission from wild birds to seals was evident for ≥2 distinct HPAI H5N1 lineages in this investigation and likely occurred through environmental transmission of shed virus. Viruses were not likely acquired by seals through predation or scavenging of infected animals, because birds are not a typical food source for harbor or gray seals (*13*). Data do not support

		Lineage A													Lineage B		
Gene	aa change	A1	A2	Pv1	Pv2	Pv3	Pv4	Pv5	Pv6	Hg1	Pv7	Pv8	Pv9	Pv10	B	Pv11	Pv12
PB2	S12L	S					L										
	E627K	E						K									
	D701N	D					N								D		N
PB1	P13S	P			S				S			S					
	R211K	R											K				
	N213K	N														K	K
	N375T	N														T	
	R480K	R													K	K	K
	M523L	M												L			
	V527I	V												I			
	I728V	I									V						
PB1-F2	A56V	A				V		V									
	G70E	G													E	E	E
PA	I38V	I			V							V					
	A70V	A	V	V	V	V	V	V	V	V	V	V	V	V			
	E101G	E											G				
	E252G	E											G				
	T263K	T		K													
	V379M	V								M							
	L425F	L		F													
	T465I	T													I	I	I
	L672F	L														F	
PA-X	I38V	I			V							V					
	A70V	A	V	V	V	V	V	V	V	V	V	V	V	V			
	E101G	E											G				
	K227R	K													R	R	R
	K252E	K												E			
HA	T10I	T													I	I	I
	P152S	P	S	S	S	S	S	S	S	S	S	S	S	S			
	E201K	E												K			
	T226A	T													A	A	A
	S377N	S												N			
	A538S	A											S				
NP	S165P	S		P													
	A181S	A														S	
	V194I	V											I			I	I
	P318Q	P						Q									
	N377S	N											S			S	S
	Q409H	Q														H	
	Q415R	Q										R					
	P419S	P									S						
	M448V/1	M			I					V				V			
	D455N	D								N							
	G485R	G				R											
NA	M23I	M								I							
	A343T	A									T						
	I362T	I													T	T	T
	S439G	S													G	G	G
	K469R	K											R				
M1	F109S	F						S									
M2	I27V	I													V	V	V
NS1	R67Q	R	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q			
	S87T	S								T							
	M98I	M		I													
	N127T	N													T	T	T
	T197N	T														N	N
NS2	L40I	L														I	I
	H85R	H					R										
	I89V	I											V				

Figure 2. Amino acid changes in highly pathogenic avian influenza A(H5N1) viruses from New England birds and seals, United States. Each single-nucleotide polymorphism (SNP) that resulted in an amino acid change within ≧1 seal-derived sequence is shown. SNPs were observed in 12 H5N1 virus genes resulting in amino acid changes in corresponding proteins: PB2, PB1, PB1-F2, PA, PA-X, HA, NP, NA, M1, M2, NS1, and NS2. All avian virus reference sequences are shaded gray. A/Sanderling/MA/CW_22–112 (H5N1) (GISAID database, https://www.gisaid.org) (labeled A1) was used as a reference for first wave lineage A sequences; A/common eider/MA/TW_22–1400 (H5N1) (labeled A2) was used as a reference for second wave lineage A sequences; and A/common tern/MA/20220612_1 (H5N1) (labeled B) was used as a reference for lineage B sequences. Four aa differ between first and second wave lineage A viruses and 10 aa differ between first wave lineage A and lineage B. Second wave lineage A and lineage B seal-derived virus sequences, sampling date, and sampling location in Maine, USA, are indicated for each seal as follows: Pv1, MME22-112, 2022 Jun 22, Wells; Pv2, MME22-117, 2022 Jun 24, Yarmouth; Pv3, MME22-121, 2022 Jun 26, Georgetown; Pv4, MME22-122, 2022 Jun 27, New Harbor; Pv5, MME22-131, 2022 Jun 28, Harpswell; Pv6, MME22-133, 2022 Jun 29, S. Portland; Hg1, MME22-144, 2022 Jul 1, Phippsburg; Pv7, MME22-150, 2022 Jul 2, Westport; Pv8, MME22-155, 2022 Jul 2, Falmouth; Pv9, MME22-198, 2022 Jul 9, Wells; Pv10, MME22-230, 2022 Jul 15, Kennebunkport; Pv11, MME22-191, 2022 Jul 8, Harpswell; Pv12, MME22-195, 2022 Jul 9, Harpswell. HA, hemagglutinin; Hg, gray seal; M, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic; PB, polymerase basic; Pv, harbor seal.

seal-to-seal transmission as a primary route of infection. If individual bird–seal spillover events represent the primary transmission route, the associated seal UME suggests that transmission occurred frequently and had a low seal species barrier. We observed novel amino acid changes throughout the virus genome in seals, including amino acid substitutions associated with mammal adaptation.

In contrast to outbreaks in agricultural settings, outbreaks of HPAI in wild populations can rarely be managed well through biosecurity measures or depopulation, which is particularly true for large, mobile marine species such as seals. Avian and mammalian colonial wildlife might be particularly affected by influenza A viruses, which could enable ongoing circulation between and within species, providing opportunities for reassortments of novel strains and study of mammalian virus adaptation. Migratory animals might further disseminate the viruses over broad geographic regions. Therefore, the interface of wild coastal birds and marine mammals is critical for monitoring the pandemic potential of influenza A viruses.

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All sequencing data are available in the GISAID database (https://www.gisaid.org) (EPI_ISL_14098915, EPI_ISL_14098917–24, EPI_ISL_16632466, EPI_ISL_16632487–8, EPI_ISL_16632494–6, EPI_ISL_16632498–524, EPI_ISL_16632536–42, EPI_ISL_16641764–94, EPI_ISL_16641796–9) or as a single file on GitHub (https://github.com/ksawatzki/H5N1_EID). All additional data are available in the appendices.

About the Author

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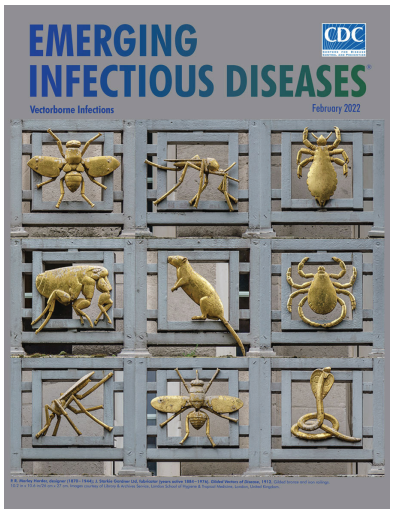

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Appendix 1

Materials and Methods

Sample Collection

Oropharyngeal or cloacal samples were collected from birds that were brought into wildlife clinics for care or were found deceased. Samples were collected from all live animals by experienced personnel within each facility (Tufts Wildlife Clinic, Cape Wildlife Clinic, New England Wildlife Centers, or Wild Care, Inc.) as part of diagnostic care. Deceased animals were either sampled in the field or brought to a wildlife center for sampling.

Oral, conjunctival, nasal, or rectal samples were collected from seals by staff at Marine Mammals of Maine, Seacoast Science Center, the National Marine Life Center, International Fund for Animal Welfare, Mystic Aquarium, and National Aquarium. Samples were obtained from both live and dead pinnipeds (gray, harbor, and harp seals) that were stranded along the Eastern Atlantic seaboard from Maine to Maryland during January–July 2022. Samples from stranded animals were collected as diagnostic samples under each organizations stranding agreement with the National Oceanic and Atmospheric Administration Fisheries Service.

Swab samples were collected by using polyester swabs (Puritan Medical Products, https://www.puritanmedproducts.com) that were placed into viral transport media (VTM) comprised of Medium 199, nystatin, gentamicin, benzylpenicillin, streptomycin, sulfamethoxazole, kanamycin sulfate (Sigma-Aldrich, https://www.sigmaaldrich.com) and bovine serum albumin (ThermoFisher Scientific, https://www.thermofisher.com). Samples were stored at −80°C or −20°C until processed.

Reverse Transcription PCR

RNA was extracted from 50 µL of VTM containing the sample by using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek Inc., https://www.omegabiotek.com) on a semiautomated KingFisher Purification System robot (ThermoFisher Scientific) as previously described (*7*). RNA was screened for the influenza A virus matrix protein (MP) gene by reverse transcription PCR (RT-PCR) by using the StepOnePlus platform (ThermoFisher Scientific). Total RNA (5 µL) was added to qScript XLT One-Step RT-qPCR ToughMix ROX (VWR, https://www.vwr.com) containing forward M F25 (5′-AGATGAGTCTTCTAACCGAGGTCG-3′) and reverse 2002 M R124 (5′-TGCAAAAACATCTTCAAGTCTCTG-3′) primers and M P64 (FAM-TCAGGCCCCCTCAAAGCCGA-TAM) probe. One-step real time RT-PCR was performed as follows: 50°C, 10 min; 95°C, 1 min; and 45 cycles of 95°C, 3 s and 60°C, 30 s. All plates were run with multiple negative VTM controls and purified A/Puerto Rico/8/1934 H1N1 RNA as a positive control. Any sample with a cycle threshold <40 was screened for the H5 hemagglutinin gene by using the same protocol described but with forward H5 1456NA (5′-ACGTATGACTATCCACCATACTCA-3′), forward H5 1456EA (5′-ACGTATGACTACCCGCAGTATTCA-3′), and reverse H5 1685 (5′-ACCTCGATGGGCAATGTGTT-3′) primers and H5 1637 (FAM-CATGTCCCTCATATCAAAACCTTCGGAGG-TAM) oligonucleotide probe. Samples with a cycle threshold <35 were sent to the US Department of Agriculture, National Veterinary Services Laboratories (NVSL, https://www.aphis.usda.gov) for further confirmatory RT-PCR testing.

Sequencing

Whole genome sequencing was performed at the Icahn School of Medicine at Mount Sinai, New York, USA, and NVSL. At the Icahn School of Medicine, original sample material from 25 animals was provided. RNA was extracted by using QIAmp Viral RNA minikits (QIAGEN, https://www.qiagen.com) and amplified by using a modified 2-step RT-PCR. RT was performed by using the ProtoScript II kit (New England Biolabs, https://www.neb.com) and 7 µL RNA and 1 µL of the Opti1 primer set. Reactions were incubated at 65°C for 5 min to denature the RNA, after which 10 µL of ProtoScript II Reaction Buffer and 2 µL of ProtoScript II Reverse Transcriptase were added; the reactions were then incubated at 25°C for 5 min, 48°C for 30 min, and 80°C for 5 min (to inactivate the enzyme). PCR was performed on cDNA by using a Q5 High-Fidelity PCR kit (New England Biolabs) in accordance with the manufacturer's

instructions; reactions were adjusted to a 25 µL volume containing 5 µL cDNA and the Opti1 primer set, consisting of Opti1-F1 (5′-GTTACGCGCCAGCAAAAGCAGG-3′), Opti1-F2 (5′-GTTACGCGCCAGCGAAAGCAGG-3′), and Opti1-R1 (5′-GTTACGCGCCAGTAGAAACAAGG-3′) primers. DNA amplicons were purified by using an Agencourt AMPure XP 5 mL kit (Beckman Coulter, https://www.beckman.com) and prepared by using the Nextera XT DNA Library Preparation kit (Illumina, https://www. illumina.com) following the manufacturer's protocol; sequencing was performed on a MiSeq instrument (Illumina) with 2 × 150-bp end reads. At the NVSL, original sample material from 38 animals was PCR-amplified and cDNA libraries were prepared by using the Nextera XT DNA Sample Preparation Kit (New England Biolabs) following the manufacturer's instructions. Sequencing was performed by using Reagent Kit v2 (500-cycles) on the MiSeq platform.

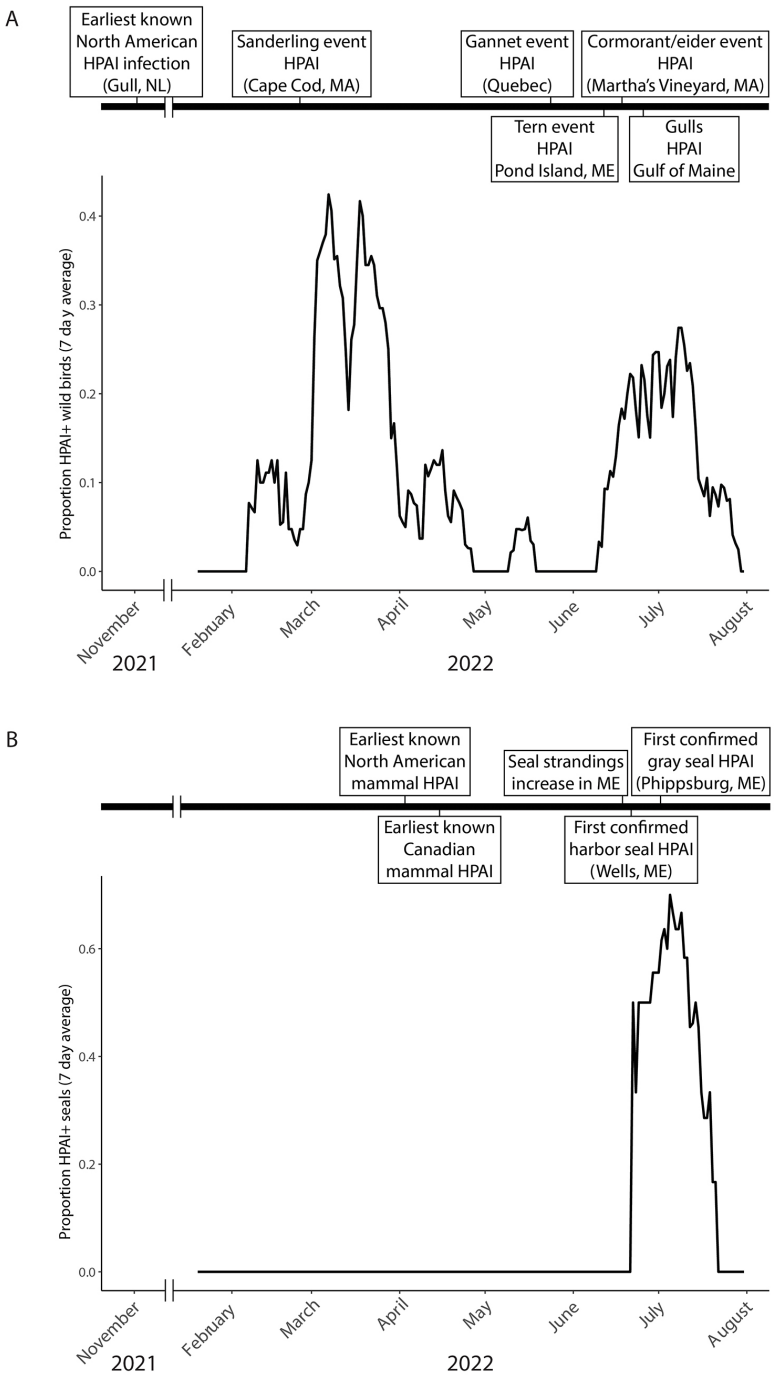
Phylogenetic analysis

Representative viruses obtained from a BLAST (https://www.ncbi.nlm.nih.gov/BLAST/) search were most closely related to viruses originating in Europe and North America but not to viruses from Africa or Asian. Therefore, we downloaded all available H5 influenza genome sequences from Europe (July 1, 2021–September 1, 2022) and North America (December 17, 2021–June 29, 2022) from the GISAID database (https://www.gisaid.org, accessed September 1, 2022). All virus segments were aligned with Clustal Omega v1.2.4, trimmed to the coding sequence, then concatenated to single supergenes. Low quality sequences were removed. Maximum-likelihood phylogenetic trees were estimated by using IQ-TREE v.2.1.3 with model selection on the total viral sample (n = 1,311) (Appendix Figure 2), a subset of all viruses from North America and the most closely related viruses from Europe (n = 407) (Appendix Figure 3), and a subset of only viruses from New England and Maritimes and the most closely related viruses from Europe (n = 107). The smallest subset was further analyzed by using the ultrafast bootstrap (n = 10,000) branch support and re-rooted by using TreeTime v0.9.4. One New England-derived virus had evidence of recombination (great black-backed gull/MA/22HP00488/20220723) and was excluded from the final subset. Comparative single nucleotide polymorphism (SNP) analysis was performed by using the NVSL vSNP pipeline (https://github.com/USDA-VS/vSNP) and Chicken/NL/FAV-0033/20221221 as a reference.

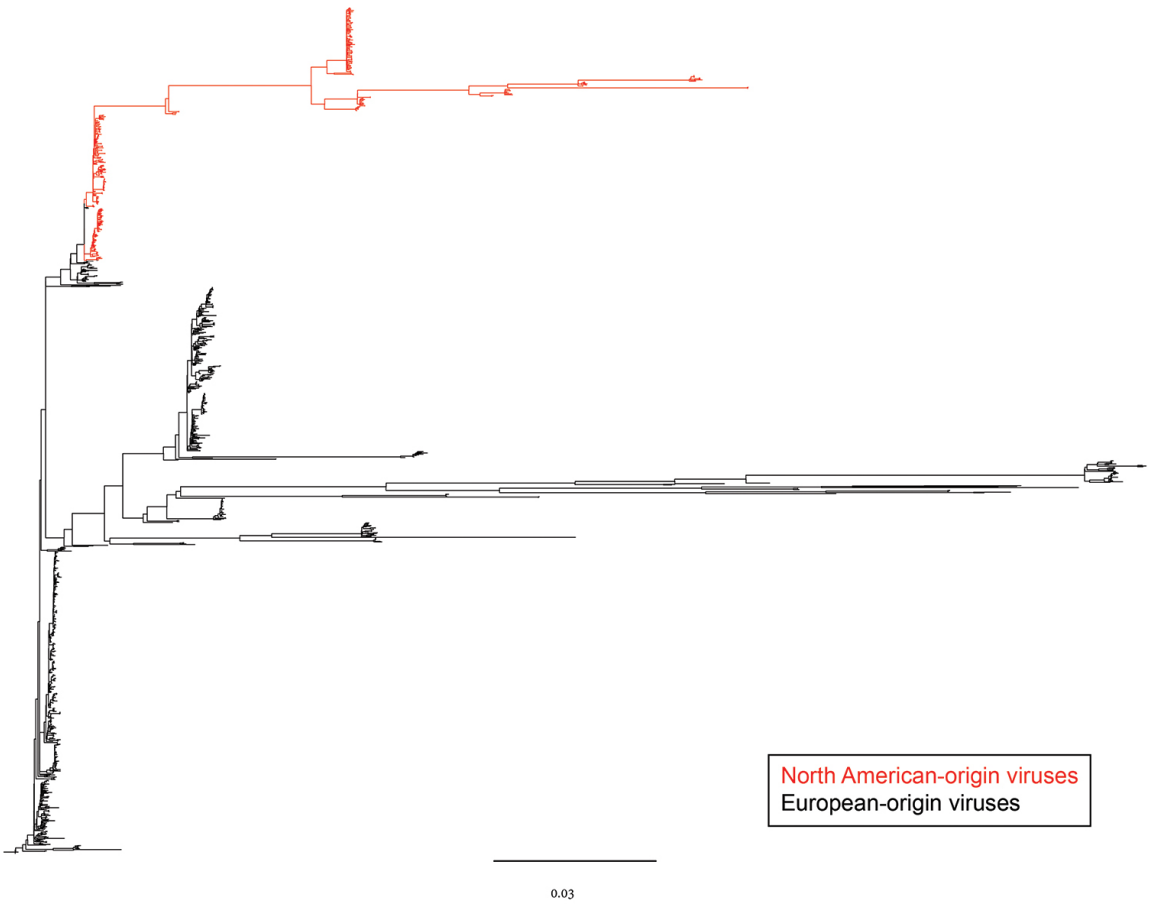
Appendix Table. New England seabird breeding colonies where suspicious avian deaths were observed*

Breeding colony locations	Town, state	GPS coordinates	Deaths	HPAI
Ship Island	Steuben, Maine	44.4334, −67.89743	Eiders	Confirmed
Green Island	Steuben, Maine	44.37341, −67.87305	Gulls, eiders, cormorants	Confirmed
Petit Manan Island	Steuben, Maine	44.3673, −67.86527	Gulls, eiders	Confirmed
Swans Island	Swans Island, Maine	44.18795, −68.44547	Gulls	Confirmed
Great Duck Island	Frenchboro, Maine	, 44.155, −68.24985	Gulls	Suspected
Mount Desert Rock	Mt Desert Rock, Maine	43.96869, −68.12778	Gulls	Suspected
Metinic Island	Vinalhaven, Maine	43.88708, −69.12581	Gulls, terns	Confirmed
Pond Island NWR	Phippsburg, Maine	43.73952, −69.77064	Terns	Confirmed
Appledore Island	Appledore Island, Maine	42.98678, −70.61424	Gulls	Suspected
Thacher Island NWR	Rockport, Massachusetts	42.63904, −70.57491	Gulls	Confirmed
Straitsmouth Island	Rockport, Massachusetts	42.65981, −70.59115	Eiders	Confirmed

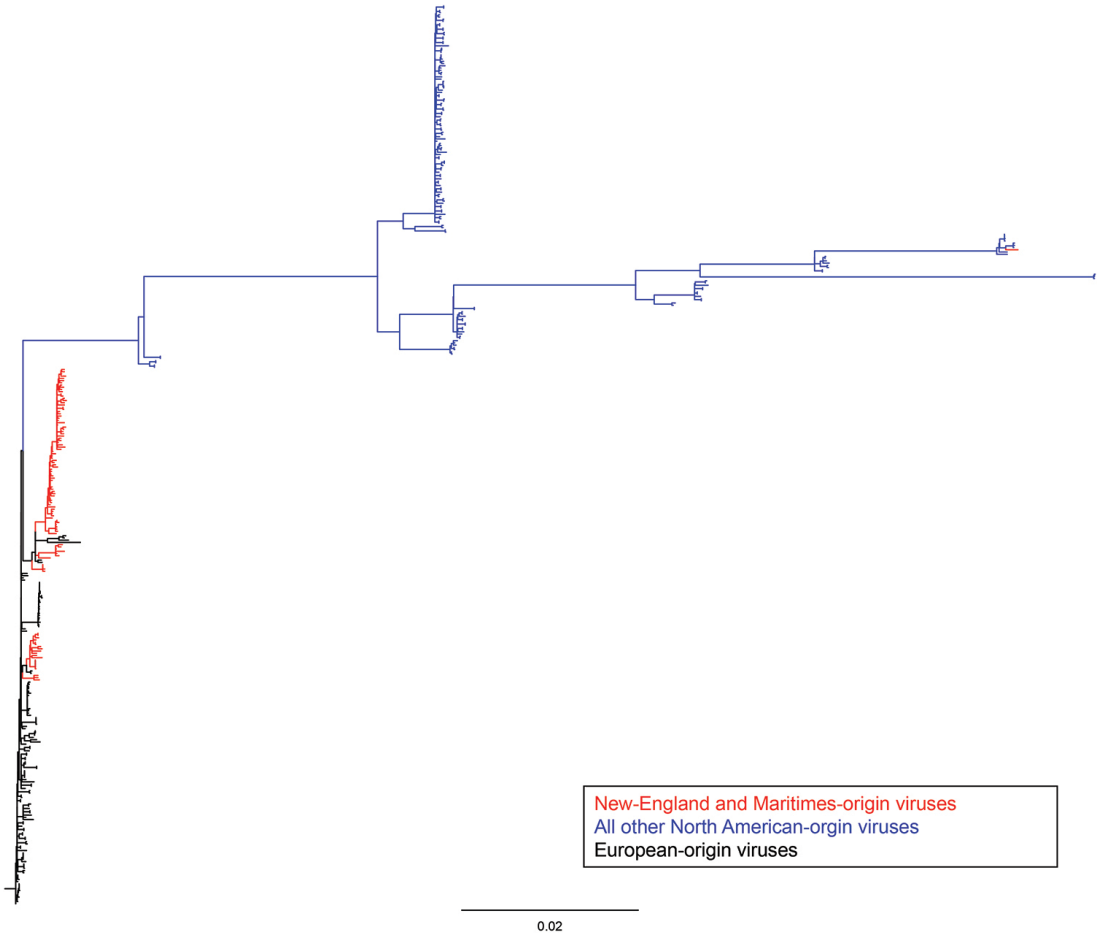
*GPS, global positioning system; HPAI, highly pathogenic avian influenza.



Appendix Figure 1. Opportunistic surveillance of New England birds and seals for highly pathogenic avian influenza (HPAI) H5N1 virus. Detection of HPAI in New England began in February 2022. A) Rolling 7-day average of H5-positive birds detected by reverse transcription PCR from New England wildlife clinics and opportunistic field collection (n = 1,079 unique birds). Timeline shows concurrent avian mortality events. Two waves of HPAI were observed in the region between February 2021 and August 2022. B) Rolling 7-day average of H5-positive seals stranded along the US Atlantic coast detected by reverse transcription PCR (n = 132 unique seals).



Appendix Figure 2. Phylogenetic analysis of H5N1 influenza viruses originating in North America and Europe. All available high-quality H5 influenza genome sequences from North America and Europe were collected, aligned, and trimmed to the coding sequence (n = 1,311). Gene segments were concatenated and maximum-likelihood phylogenetic trees were estimated by using IQ-TREE v.2.1.3 (https://www.iqtree.org) with model selection. Branches for Europe are shown in black and in red for North America. Scale bar indicates nucleotide substitutions per site.



Appendix Figure 3. Phylogenetic analysis of H5N1 influenza viruses from New England and North America. All available high-quality H5 influenza genome sequences from North America and closely related Europe-derived sequences (as inferred in Appendix Figure 1) were aligned and trimmed to coding sequence (n = 407). Gene segments were concatenated and maximum-likelihood phylogenetic trees were estimated using IQ-TREE v.2.1.3 (https://www.iqtree.org) with model selection. Branches for viruses from New England and Maritimes are shown in red, all other viruses from North America are shown in blue, and branches for Europe are shown in black. Only 1 virus from New England had evidence of reassortment (great black-backed gull/MA/22HP00488/20220723, top right in tree) and was excluded from the final subset. Scale bar indicates nucleotide substitutions per site.

	PB2:39	PB2:321	PB2:555	PB2:1788	PB1:507	PB1:750	PB1:1362	PB1:1524	PB1:1836	PB1:2091	PB1:2100	PB1:2221	PA:666	PA:752	PA:1184	HA:300	HA:897	HA:969	HA:1032	HA:1194	HA:1263	HA:1405	NP:138	NP:465	NP:570	NP:816	NA:720	NS:592	NS:717	PB2:1490	PB2:1578	PB1:201	PB1:366	PA:147	NA:1097	MP:963	NS:835
Chicken/NL/FAV-0033/2021 reference	G	T	T	A	T	A	G	G	G	G	C	G	C	A	G	T	A	G	A	C	G	C	G	G	G	T	T	C	A	G	G	C	A	G	G	T	T
Peregrine falcon/MA/22MM00242/20220324	A	C	C	G	C	T	T	A	A	A	T	T	T	G	A	C	T	A	G	T	A	T	A	A	A	C	C	T	G	·	·	·	·	·	·	·	·
Great horned owl/MA/22MM00468/20220420	A	C	C	G	C	T	T	A	A	A	T	T	T	G	A	C	T	A	G	T	A	T	A	A	A	C	C	T	G	A	A	T	G	A	A	C	C
Gray seal/ME/20220701	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220709	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220715	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Peregrine falcon/MA/20220616	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Common eider/MA/20220707	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/MA/20220625	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220622	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220629	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220624	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Peregrine falcon/MA/20220622	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor_seal/ME/20220702	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220627	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/ME/20220707	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220628	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220626	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220702	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Herring gull/ME/20220628	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Common eider/ME/20220703	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/ME/20220629	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Herring gull/ME/20220629	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/MA/20220708	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Double-crested cormorant/MA/20220612	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Canada goose/MA/20220721	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sanderling/MA/20220306	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sanderling/MA/20220301	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Snowy owl/MA/20220313	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/MA/20220325	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Red-tailed hawk/MA/20220411	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sanderling/MA/20220302	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Red-tailed hawk/MA/20220317	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Red-tailed hawk/MA/20220306	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Crow/MA/20220227	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Crow/MA/20220321	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Northern gannet/MA/20220510	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Common tern/ME/20220720	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Tern/ME/20220612	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220708	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220709	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

Primary North American lineage

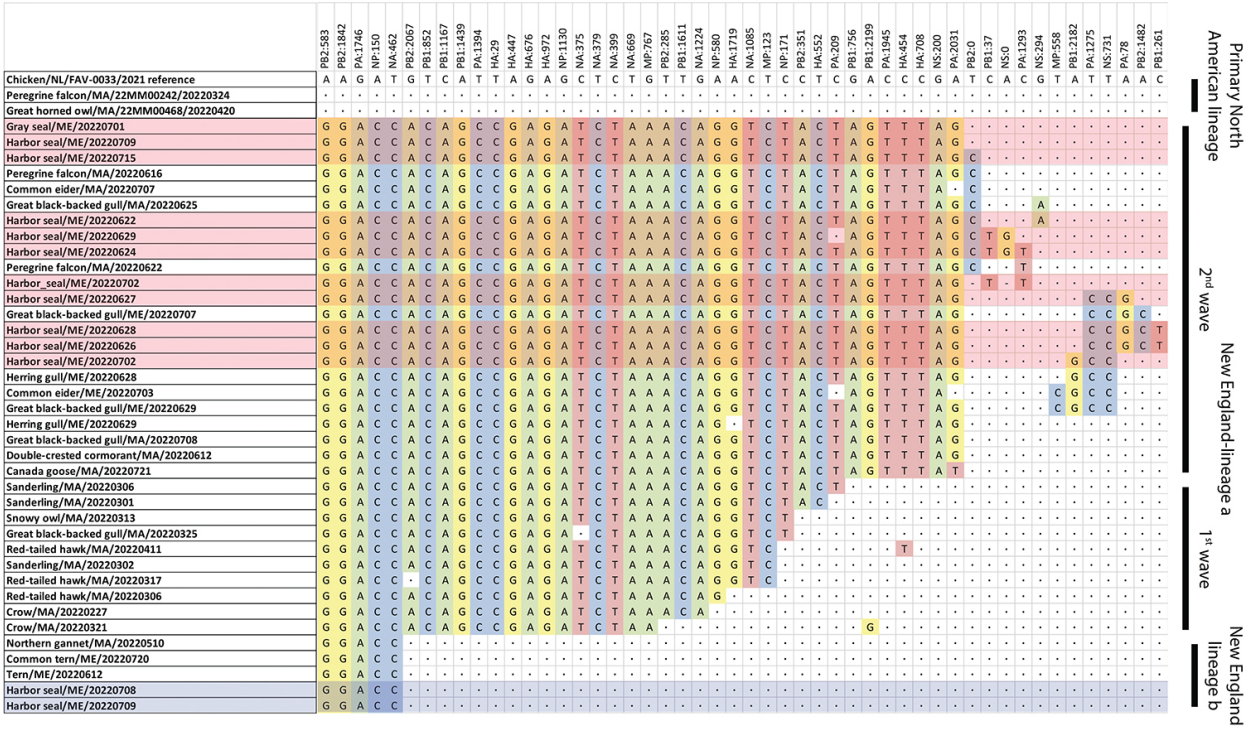
New England-lineage a

2nd wave

1st wave

New England lineage b

Appendix Figure 4. Single nucleotide polymorphism pattern for primary H5N1 lineage from North America. Eighty-four H5N1 sequences collected from birds and seals in New England were processed by using the vSNP pipeline (https://github.com/USDA-VS/vSNP) and Chicken/NL/FAV-0033/2021 (GISAID database, https://www.gisaid.org) as a reference. Avian samples with redundant single nucleotide polymorphism (SNP) motifs were removed, and the earliest observed member was retained. All SNPs observed in a single bird were removed. All seal SNPs were retained. Seal viruses in lineage A are highlighted in red, and seal viruses in lineage B are highlighted in blue. The SNP motif associated with the primary North American lineage is shown. Complete SNP data are available in Appendix 2 (https://wwwnc.cdc.gov/EID/article/29/4/22-1538-App2.xlsx).



Appendix Figure 5. Single nucleotide polymorphism pattern for New England H5N1 lineage A. Eighty-four H5N1 sequences collected from birds and seals in New England were processed by using the vSNP pipeline (https://github.com/USDA-VS/vSNP) and Chicken/NL/FAV-0033/2021 (GISAID database, https://www.gisaid.org) as a reference. Avian samples with redundant SNP motifs were removed, and the earliest observed member was retained. All single nucleotide polymorphisms (SNPs) observed in a single bird were removed. All seal SNPs were retained. Seal viruses in lineage A are highlighted in red, and seal viruses in lineage B are highlighted in blue. The SNP motif associated with New England lineage A is shown. Complete SNP data are available in Appendix 2 (https://wwwnc.cdc.gov/EID/article/29/4/22-1538-App2.xlsx).

	PB2:1824	HA:1392	NA:705	MP:681	PB1:1992	PB2:1254	PB2:1734	PB2:2097	PB1:303	PB1:1278	PB1:1497	PB1:2178	PA:681	PA:1281	PA:1971	HA:72	HA:111	HA:519	HA:1017	HA:1146	NP:66	NP:237	NA:234	NA:408	NA:663	NA:1315	NA:1317	MP:-7	NS:267	PB2:1596	PB1:639	PA:330	NS:590
Chicken/NL/FAV-0033/2021 reference	A	C	T	C	C	A	G	A	G	T	T	A	A	G	G	T	A	C	A	C	C	G	G	G	C	A	C	A	C	A	C	T	C
Peregrine falcon/MA/22MM00242/20220324	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great horned owl/MA/22MM00468/20220420	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Gray seal/ME/20220701	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220709	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220715	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Peregrine falcon/MA/20220616	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Common eider/MA/20220707	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/MA/20220625	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220622	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220629	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220624	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Peregrine falcon/MA/20220622	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor_seal/ME/20220702	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	-	·	·	·	·	·
Harbor seal/ME/20220627	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/ME/20220707	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220628	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220626	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Harbor seal/ME/20220702	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Herring gull/ME/20220628	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Common eider/ME/20220703	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/ME/20220629	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Herring gull/ME/20220629	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/MA/20220708	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Double-crested cormorant/MA/20220612	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Canada goose/MA/20220721	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·
Sanderling/MA/20220306	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sanderling/MA/20220301	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Snowy owl/MA/20220313	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Great black-backed gull/MA/20220325	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Red-tailed hawk/MA/20220411	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sanderling/MA/20220302	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Red-tailed hawk/MA/20220317	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Red-tailed hawk/MA/20220306	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Crow/MA/20220227	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Crow/MA/20220321	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Northern gannet/MA/20220510	G	T	C	T	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Common tern/ME/20220720	G	T	C	T	·	G	A	G	A	C	C	C	G	A	A	C	G	T	G	T	A	A	A	A	T	G	G	G	T	·	·	·	·
Tern/ME/20220612	G	T	C	T	T	G	A	G	A	C	C	C	G	A	A	C	G	T	G	T	A	A	A	A	T	G	G	G	T	·	·	·	·
Harbor seal/ME/20220708	G	T	C	T	T	G	A	G	A	C	C	C	G	A	A	C	G	T	G	T	A	A	A	A	T	G	G	G	T	G	A	C	A
Harbor seal/ME/20220709	G	T	C	T	T	G	A	G	A	C	C	C	G	A	A	C	G	T	G	T	A	A	A	A	T	G	G	G	T	G	A	C	A

Primary North American lineage

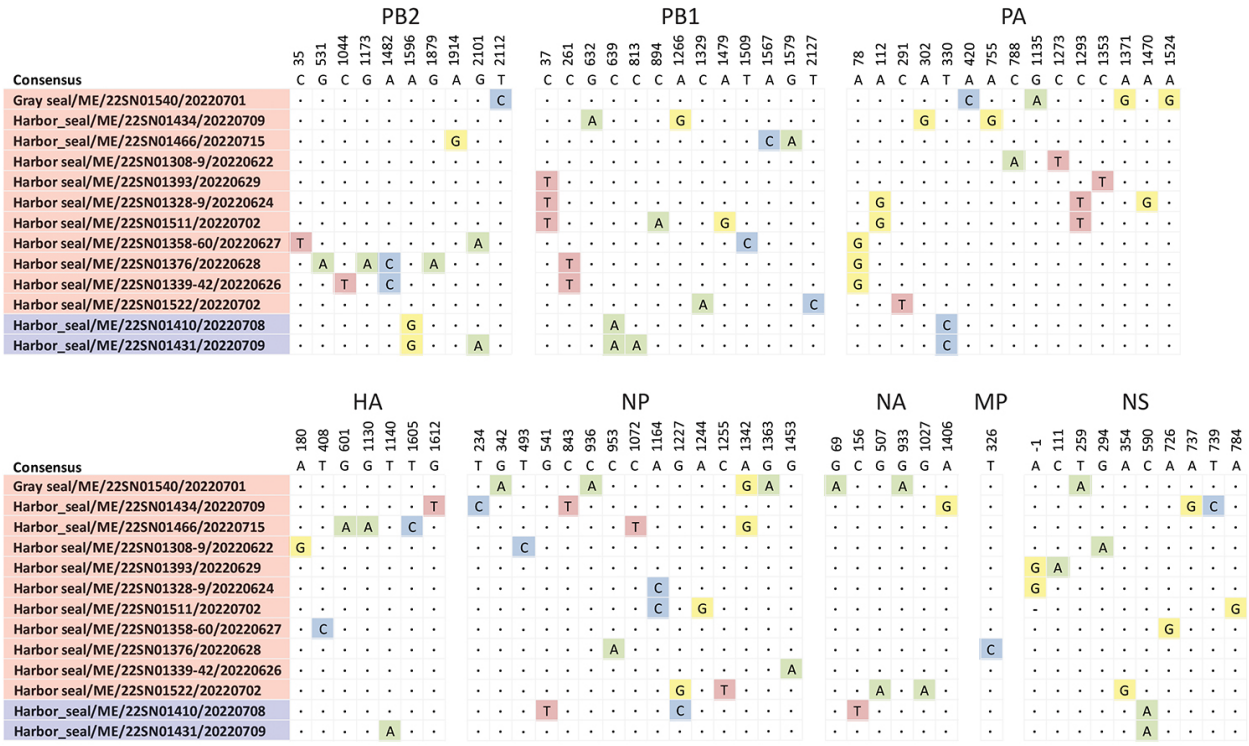
New England-lineage a

2nd wave

1st wave

New England lineage b

Appendix Figure 6. Single nucleotide polymorphism pattern for New England H5N1 lineage B. Eighty-four H5N1 sequences collected from birds and seals in New England were processed by using the vSNP pipeline (https://github.com/USDA-VS/vSNP) and Chicken/NL/FAV-0033/2021 (GISAID database, https://www.gisaid.org) as a reference. Avian samples with redundant SNP motifs were removed, and the earliest observed member was retained. All SNPs observed in a single bird were removed. All seal SNPs were retained. Seal viruses in lineage A are highlighted in red, and seal viruses in lineage B are highlighted in blue. The SNP motif associated with New England lineage B is shown. Complete SNP data are available in Appendix 2 (https://wwwnc.cdc.gov/EID/article/29/4/22-1538-App2.xlsx).



Appendix Figure 7. Single nucleotide polymorphisms observed in seal-derived H5N1 viruses. Thirteen H5N1 sequences collected from seals in New England were processed by using the vSNP pipeline (https://github.com/USDA-VS/vSNP) and Chicken/NL/FAV-0033/2021 (GISAID database, https://www.gisaid.org) as a reference. All SNPs observed in each seal are shown. Seal viruses in lineage A are highlighted in red, and seal viruses falling into lineage B are highlighted in blue. Complete SNP data are available in Appendix 2 (https://wwwnc.cdc.gov/EID/article/29/4/22-1538-App2.xlsx).