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Letter

Environmental Surveillance and Detection of Infectious Highly Pathogenic Avian Influenza Virus in Iowa Wetlands

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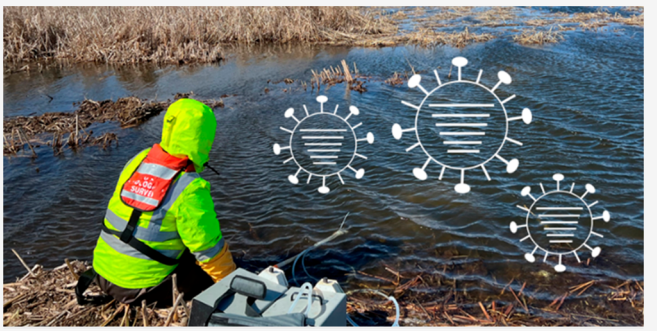
ABSTRACT: Avian influenza viruses (AIVs) infect both wild birds and domestic poultry, resulting in economically costly outbreaks that have the potential to impact public health. Currently, a knowledge gap exists regarding the detection of infectious AIVs in the aquatic environment. In response to the 2021−2022 Eurasian strain highly pathogenic avian influenza (HPAI) A/goose/Guangdong/1/1996 clade 2.3.4.4 lineage H5 outbreak, an AIV environmental outbreak response study was conducted using a One Health approach. An optimized method was used to temporally sample (April and May 2022) and analyze (culture and molecular methods) surface water from five water bodies (four wetlands and one lake used as a comparison location) in areas near confirmed HPAI detections in wild bird or poultry operations. Avian influenza viruses were isolated from water samples collected in April from all four wetlands (not from the comparison lake sample); HPAI H5N1 was isolated from one wetland. No virus was isolated from the May samples. Several factors, including increased water temperatures, precipitation, biotic and abiotic factors, and absence of AIV-contaminated fecal material due to fewer waterfowl present, may have contributed to the lack of virus isolation from May samples. Results demonstrate surface water as a plausible medium for transmission of AIVs, including the HPAI virus.



KEYWORDS: *avian influenza, highly pathogenic, surface water, wetlands, infectious*

INTRODUCTION

Eurasian strain highly pathogenic avian influenza (HPAI) virus A/goose/Guangdong/1/1996 (Gs/GD) clade 2.3.4.4 lineage H5 was first detected in wild waterfowl in the United States Atlantic flyway in January 2022. The virus continues to circulate in North America, causing the largest animal outbreak in U.S. history, with a record number of HPAI cases in wild birds, domestic poultry, and wild mammals. There have been pervasive wild bird HPAI detections in the U.S. and Canada. Avian influenza viruses (AIVs) infect wild birds and domestic poultry and can result in environmentally and economically devastating outbreaks.[1,2] Often AIVs are low pathogenicity, causing minimal clinical complications in poultry.[3] However, subtypes H5 and H7 can mutate into HPAI viruses and subsequently lead to high poultry mortality.[4] Recent outbreaks of clade 2.3.4.4b HPAI have had a significant impact on wild bird species globally.[2,5−8]

Wild birds can shed large amounts of AIV in feces, and fecal shedding of virus into surface water can lead to transmission among waterfowl via a fecal−oral route.[9−11] Concentrations of AIV in surface water are often too low to be detected by most methods, and past field studies focused on detection of viral RNA or were unsuccessful in isolating virus.[12−19] Select studies have been successful in isolating infectious AIV from surface water using small sample volumes (50 mL to 1 L); however, in these studies water samples were collected proximal to traps, nesting areas, or where large numbers of waterfowl gathered[11,20−22] and may not be representative of the potential wetland volume exposure to waterfowl. Dispersal and abundance of viruses in wetlands are not well understood; however, research has suggested virus dispersal is controlled by both water flow and agitation.[23−25] Laboratory studies have been successful in recovering both HPAI and low pathogenicity avian influenza (LPAI) virus isolates from artificial environmental conditions, for instance, experimental infections of mallards (*Anas platyrhynchos*) or seeded surface water in the laboratory.[12,26−33] These studies have suggested surface water as a means of transmission; however, to our knowledge, no studies have recovered infectious HPAI from surface waters in

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the United States; however, Zhang et al.[11] isolated HPAI from 40 mL of frozen, archived surface water samples using a method incorporating formalin-fixed erythrocytes. Previous research suggests AIV, including Gs/GD HPAI viruses, may remain infectious for prolonged periods in surface water; however, persistence of Gs/GD HPAI viruses in the environment is poorly understood.[2,30,32,33]

Currently, there is a research gap regarding the extent the aquatic environment plays in the transmission of AIVs among wild birds and domestic poultry. HPAI detections steadily increased in the United States and Canada starting in January 2022, in both wild birds and commercial poultry and backyard poultry flocks. Avian influenza virus in the environment (environmental health) has the potential to influence the health of humans and wildlife. Wildlife health, including fecal shedding and virus transport, can influence environmental health, as waterbodies can act as a reservoir and source of virus. Thus, an AIV environmental outbreak response study was conducted using a One Health approach considering the intersection of environmental, human, and wildlife health. Surface waters in Iowa (United States) with known cases of HPAI in either wild birds or domestic poultry were sampled by using an optimized method to recover infectious AIV and viral RNA from surface waters.

MATERIALS AND METHODS

Site Information. Four wetlands and one lake used for wildlife habitat and human recreation (e.g., birding, hunting, swimming, and boating) were sampled in Iowa (Figure 1). Sampling locations were (1) wetlands that experienced a wild bird die-off related to HPAI (n = 1), (2) wetlands in counties near confirmed HPAI detections in commercial poultry (n = 2), or (3) wetlands in counties with several (n = 3−9) HPAI detections in wild birds (n = 2). One recreational lake with minimal waterfowl habitat was selected as a comparison location (Figure 1). Sampling locations represent areas with expected use of the waterbodies by waterfowl. No baiting areas or nesting areas were selected for this study. Federal, state, and county refuge, wildlife, or park managers assisted with wetland selection and coordinated access and permission to the wetlands. Wetlands were sampled on April 12−14 (T1) and resampled May 16−18 (T2) 2022 to examine infectious AIV in surface waters.

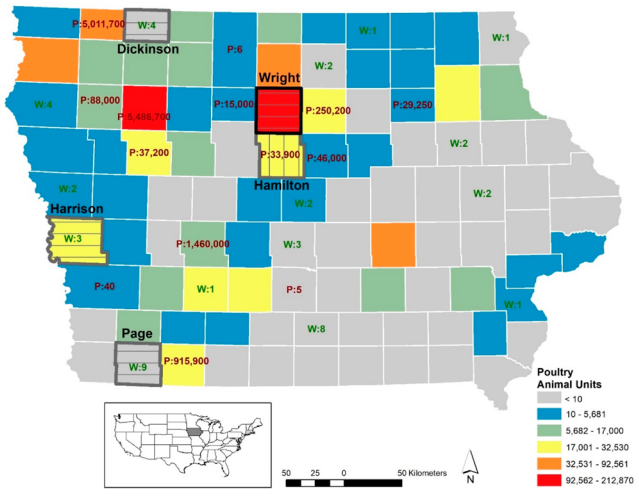

Figure 1. Map of Iowa Counties with sampled wetlands for the infectious avian influenza virus. Counties where wetland water was sampled outlined in gray. County where a sample from a wetland was confirmed as HPAI H5 outlined in black. County names are written above or below these respective counties. Horizontal cross hatch indicates one wetland sampled per county. Vertical cross hatch indicates one lake sampled per county. Numbers indicate total birds affected from confirmed commercial and backyard flock premises (red numbers, proceeded by "P:") and confirmed detections of HPAI in wild birds (green numbers, proceeded by "W:") within each county as of 5/18/2022.[34] Poultry animal unit data (animal unit = animal feeding operation number of head × factor) obtained from Iowa Geospatial data.[35]

Sample Collection and Processing. Sample collection and processing included the following steps: (1) filtration (primary concentration), (2) filter elution, (3) centrifugation (secondary concentration), (4) centrifugation (tertiary concentration), (5a) egg inoculation (virus isolation), and (5b) extraction (RNA isolation) (Figure S1).

The wetland (n = 4) and lake (n = 1) samples were sampled in 1−3 near-shore transects with partial to whole coverage of the wetland perimeter (10.8−100%, median 18.7%, mean 43.0%) or multiple stationary locations (Figure S2, Table S1).[36] Wetland or lake water was concentrated continuously during the transect at ambient outdoor air temperatures by dead-end ultrafiltration (DEUF)[37] at a pump speed of 1,000 mL/min.[38,39] Wetland DEUF sample volumes were approximately 40 L. Quality control included 10 LPAI H3N8 AIV spikes (5 at T1, 5 at T2) and three negative controls (1 field, 2 laboratory). No replicate samples were collected. For the LPAI H3N8 spikes, 30 L of wetland or lake water was concentrated in the field using DEUF (duplicate ultrafilter), and a 10 L LDPE cubitainer of wetland water was collected and shipped with the ultrafilters for subsequent virus spike of LPAI H3N8 in the laboratory[40] (for a total of 40 L; Figure S1). A field negative control was collected by filtering 20 L of deionized water (DI) from a sterile container in the field at ambient outdoor air temperature. Additionally, wetland water was characterized in situ using a multiparameter water quality sonde (Yellow Springs Instruments; Yellow Springs, OH, USA), and standard water chemistry measurements (pH, temperature, specific conductance, dissolved oxygen, and turbidity) were recorded at T1 and T2 (Table S2).[36]

Following filtration, ultrafilters and 10 L cubitainers of wetland water were shipped overnight on wet ice to the processing laboratory (U.S. Geological Survey Michigan Bacteriological Research Laboratory, MI-BaRL; Lansing, MI). To field validate recovery, the 10 L cubitainers of wetland water were spiked with 5,000 EID_{50}/L A/mallard/Minnesota/UGAI14-2812/2014(H3N8), DEUF through the duplicate ultrafilter and processed as described below. Spike mean embryonated chicken eggs (ECE) infectious dose was quantified at Southeastern Cooperative Wildlife Disease Study (SCWDS) using Spackman et al.[41] Two laboratory negative controls were conducted by filtering 20 L of DI through ultrafilters in the laboratory at the beginning and end of environmental sample processing and processed as described below. Ultrafilters were eluted using a solution modified from Smith and Hill[37] (Table S3). Eluate was centrifuged, and the pellet discarded. The eluate was further concentrated (median concentration factor = 12,700, standard deviation = 7,000) using a Centricon Plus-70 centrifugal filter (EMD Millipore; Billerica, MA, USA; Table S3).[40]

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Table 1. Environmental Results[a]

Wetland ID	Treatment	Transect	T1 Collection date	T1 AIVM rRT-PCR (POS/NEG)	AIV Virus isolation result[c] (subtype)	$10^{X.XX}$ EID_{50}/mL	T2 Collection date (Days since T1)	T2 AIVM rRT-PCR (POS/NEG)	AIV Virus isolation result[c]	$10^{X.XX}$ EID_{50}/mL	GenBank accession #
Page	wetland	A	4/12/2022	n/d	POS (H3N2)	1.40	5/16/2022 (34)	n/d	NEG	n/t	OQ968375-OQ968382
Page	wetland	B	4/12/2022	n/d	POS (H3N2)	1.40	5/16/2022 (34)	n/d	NEG	n/t	OQ968383-OQ968390
Page	wetland	C	4/12/2022	n/d	POS (H3N2)	2.63	5/16/2022 (34)	n/d	NEG	n/t	OQ968391-OQ968398
Harrison	wetland	A	4/12/2022	n/d	POS (Mixed H3,N2,N3)	n/d	5/17/2022 (35)	n/d	NEG	n/t	
Dickinson	wetland	A	4/13/2022	n/d	POS (H10N7)	1.40	5/17/2022 (34)	n/d	NEG	n/t	OQ968367-OQ968374
Dickinson	wetland	B	4/13/2022	n/d	NEG	n/t	5/17/2022 (34)	n/d	NEG	n/t	
Hamilton	lake comparison	A	4/14/2022	n/d	NEG	n/t	5/18/2022 (34)	n/d	NEG	n/t	
Hamilton	lake comparison	B	4/14/2022	n/d	NEG	n/t	5/18/2022 (34)	n/d	NEG	n/t	
Wright	wetland	A	4/14/2022	POS	POS (HP H5N1)	n/t[b]	5/18/2022 (34)	n/d	NEG	n/t	OQ843962-OQ843969

[a]T1, wetlands sampled April 12−14, 2022; T2, wetlands resampled May 16−18, 2022; AIV, avian influenza virus; AIVM, avian influenza virus matrix gene; rRT-PCR, real-time reverse-transcription polymerase chain reaction; $10^{X.XX}$ EID_{50}/mL, value (scientific notation) 50% egg infectious dose per milliliter; n/d, not detected; n/t, not tested; n/c, no collection; NEG, negative; POS, positive. [b]Virus isolate was unable to be quantified as it was identified as presumptive H5 and became a select agent. Intact material was required to be inactivated/destroyed. [c]Viral isolate considered positive if both hemagglutination assay and rRT-PCR positive.

Sample Screening. Concentrated samples were shipped on ice overnight to SCWDS, and antibiotics/antimycotics (penicillin G 200 units/mL, Streptomycin 0.2 mg/mL, and Amphotericin B 0.5 μg/mL; Sigma-Aldrich, St. Louis, MO) were added to each to reduce bacterial and fungal overgrowth in the eggs and stored at 4 °C. Nucleic acids from water samples were extracted using the KingFisher magnetic particle processer using the MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion/Applied Biosystems, Foster City, CA) as previously described[42] 2−4 days after sample collection and screened by real-time reverse transcriptase polymerase chain reaction (rRT-PCR) targeting the matrix gene where a cycle threshold (Ct) value <40 was considered "positive" (Table S3).[42,43] Virus isolation (VI) was performed in ECE[44] 2−4 days postcollection. After 48 h of incubation of ECE, amnioallantoic fluids were tested by a hemagglutination assay (HA) using 0.5% chicken red blood cells (RBC).[45] All samples that did not hemagglutinate RBC after one passage were subjected to a second passage in ECE, and procedures for the HA assay followed, as noted above. Resultant viral isolates were extracted using QIAgen Viral RNA Mini Kit as per the manufacturer's instructions and further screened using rRT-PCR assays targeting AIV matrix genes as well as subtypes H5 and H7, including an assay specific for Gs/GD lineage 2.3.4.4b H5. Viral isolates were considered positive if both the HA assay and rRT-PCR were positive. A subset of primary samples that yielded an isolate (n = 4) were titrated in ECE through 10-fold serial dilution series until an end point was reached.[3] Samples screened and identified as H5 by rRT-PCR are presumed positive HPAI virus, became select agents regulated under 9 CFR §121.3(c), and required proper inactivation/disposal of all intact material.[46] All presumptive AIV isolates were submitted to the U.S. Department of Agriculture National Veterinary Services Laboratories for confirmation and typing/sequencing. To confirm results of VI, viral isolates were characterized by whole genome sequencing as previously described.[7] Briefly, nucleic acids were amplified using universal primers. Sequencing libraries were prepared from cDNA using the Nextera XT Sample Preparation Kit (Illumina), and libraries were sequenced using a 500 cycle MiSeq Reagent Kit v2 (Illumina). Sequence reads were assembled and summary statistics were generated using IRMA v0.6.7 (Table S3).[47] A single consensus sequence was generated for each segment (8 segments per virus) with 100% genome coverage (Table S4). Consensus sequences were analyzed in Geneious Prime 2023.1.2. Sequences were aligned using MAFFT,[48] and phylogenetic trees were generated using RAxML.[49] Output trees were annotated using FigTree v1.4.[50]

RESULTS AND DISCUSSION

Environmental Samples. Avian influenza viruses were isolated from all four wetlands (T1, April 2022, n = 6; Table 1). No virus was isolated from the T1 lake comparison samples (n = 2). Overall, AIV RNA was identified in one of nine T1 samples (11.1%) via rRT-PCR, and infectious AIV was identified in six of nine T1 samples (66.7%) by VI (Table 1). One T1 wetland isolate was confirmed as clade 2.3.4.4b HPAI A(H5N1) (Figure 1, Table 1, Table S4). Sequence analysis showed the virus was genotype B2.1, which was the predominant genotype circulating in wildlife in the upper Midwest United States in Spring 2022 (Figure 2).[51] This is the first report of infectious HPAI isolated from a waterbody in the United States and has implications for human and wildlife health, virus surveillance, and outbreak management. The sequence was 99.8−99.9% similar to other wild bird and poultry viruses detected in the region at the time of collection (Figure S3; Table S5).[36] Sequences are available from GenBank (OQ843962-OQ843969; Table 1).

The remainder of the T1 isolates from wetlands (n = 5) were identified as North American wild bird lineage (AM) AIV subtype H3N2, H10N7, and a mixture of H3N2 and H3N3 (Table 1). No AIVs were detected in wetland samples (n = 9) from T2 (May 2022, 34−35 days later) via rRT-PCR or VI (Table 1). Four concentrated T1 water samples (held at 4 °C with 2× antibiotic/antimycotic solution and titrated in July

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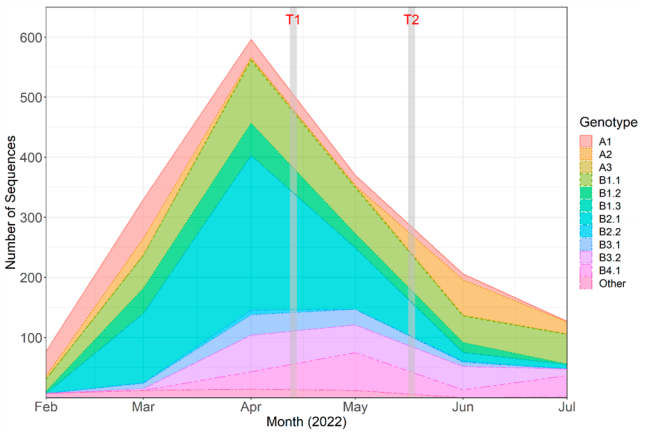

Figure 2. Chart with distribution of HPAI H5N1 genotypes detected from U.S. wildlife between February and July 2022.[51] Genotype B2.1 (identified as the Wright A water sample genotype) is shown in cyan. T1 and T2 are shown in gray lines. *Y*-axis is the total number of whole genome sequences (proportion of each genotype) generated from individual samples during the course of the outbreak.

2022) had a concentration range of $10^{1.4}$–$10^{2.63}$ EID_{50}/mL ($10^{4.4}$–$10^{5.63}$ EID_{50}/L), 1–2 orders of magnitude larger than the spike concentration of 5,000 EID_{50}/L (Table 1). Low pathogenicity isolates from wetland samples were identified as single subtypes in four of five samples (80.0%) from T1 (Table 1). Sequences are available from GenBank (Table 1).

Quality Assurance/Quality Control Samples. All three negative controls (one field, two laboratory) were negative for AIV via rRT-PCR and VI. Viral RNA from LPAI H3N8 AIV spikes was identified in three of five samples (T1, 60.0%) and four of five samples (T2, 80.0%; Table S6).[36] rRT-PCR Ct values ranged from 30.57 to 38.25 (T1-T2; Table S6).[36] An inhibition assessment was not included when analyzing these samples as inhibition assessment is not included in the published molecular methods frequently used in diagnostics of avian influenza virus.[42,43] Recovery efficiencies were not calculated for the LPAI H3N8 AIV spikes, as the rRT-PCR method was run as presence/absence and did not include a standard curve. In future studies, standard curves and inhibition assessment will be incorporated into the rRT-PCR. Virus was isolated from in 9 of 10 spikes and positive via rRT-PCR in 7 of 10 spikes (Table S6). Virus was not isolated from the lake comparison T2 spike; either laboratory error occurred during spiking, or conditions during T2 affected our ability to isolate virus at this location.

Contributing Environmental Factors. Virus isolated in samples did not always coincide with positive rRT-PCR results, indicating possible PCR extraction or amplification inhibition in several of the samples. Low detection by PCR was evident in both the LPAI H3N8 AIV spikes (60.0–80.0%) and the environmental (11.1%) sample results. The extraction technique used for these samples was optimized to remove inhibitors from fecal samples;[42] however, the surface water samples may introduce additional or different inhibitors such as humic and fulvic acids, organic matter, metals, and toxins with potential antiviral properties.[52−56] In addition, the extraction procedure, which utilizes 50 μL of primary material, may not adequately capture a homogeneous portion of the concentrated water sample (970–5000 μL). The molecular results demonstrate that the current commercially available molecular methodology used for diagnostic AIV detection may not detect AIV in large volume wetland water sample concentrates. Following the current diagnostic molecular procedures, our environmental positive detections would have been missed without the addition of VI to determine infectivity. These results support the need for virus isolation in addition to molecular techniques when assessing AIV presence in surface water as many of the VI positive samples would have been categorized as negative if only rRT-PCR was used to assess AIV presence. Our VI and sequencing results confirm the presence and recovery of infectious AIV, including HPAI, from wetlands. The results also underscore the need for (1) improved extraction techniques for reduction or removal of PCR inhibitors in concentrated water samples and (2) incorporating an internal positive control in rRT-PCR.

Sampling at multiple locations in the wetland improved the ability to isolate AIV and captured a more representative water sample (Table 1; Table S1). As with all water sampling, inherent variability in the dispersion of biological contaminants and infectious AIV likely contributed to the variability in VI success and subtype in these wetlands. For instance, Dickinson A and B were adjacent transects; however, AIV was isolated from one sample (A) and not the other (B; Table 1; Table S1). Further research, including sampling multiple points or transects in a wetland, will help assess variability in virus isolate subtypes and occurrence.

Results for this study documented that while AIV was isolated from the four wetlands during T1, AIV was not isolated upon repeat sampling of the same wetlands 5 weeks later (i.e., T2) even though previous research has documented persistence for extended periods (i.e., longer than six months) during cool water conditions.[30,31,57,58] Multiple factors may have contributed to the lack of T2 detections, including temperature, dilution, changes to contribution of new fecal material, UV penetration, biotic effects, viral aggregation,[59,60] sequestration, deposition, etc. First, sustained above-normal air temperatures approximately 7 days prior to T2 (Figure S4, Table S7)[36] caused dramatic increases in water temperatures from a median of 10.2 °C (1.6–17.8 °C) during T1 to a median of 19.0 °C (16.2–27.1 °C) during T2. Research has shown a general inverse relation between viral persistence and water temperature.[30,61,62] Second, above normal precipitation occurred between T1 and T2 in four of the five counties (Figure S4 and Table S7) that could have diluted any viable AIV still remaining in the sampled wetlands. Third, the sampling period was at the end of wild bird migration;[63] thus, little new fecal material (and AIV) was likely being deposited in the wetlands in the period between T1 and T2.

Environmental Implications. This field research demonstrated the applicability of our laboratory method to recover infectious AIV from surface water. In addition, these results provide further evidence that surface water can serve as a plausible medium for the environmental transmission of AIVs among birds and mammals, including HPAI H5N1. Clade 2.3.4.4b HPAI A(H5N1) was isolated in an Iowa wetland and was 99.8–99.9% similar to other wild bird and poultry viruses detected in the region at the time of collection, suggesting the virus was deposited in the water by wild birds and remained infectious and potentially transmissible. In addition, the LPAI H3 and H10 AIVs isolated in the samples were of relevant and potentially infectious concentrations compared to previous studies of LPAI H7 mean infectious doses in chickens, turkeys, and ducks.[64−66] Considering One Health, the study results indicate that surface water (environment) can be a reservoir

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and a source of infectious AIV capable of infecting wildlife and humans.

Further research is needed to improve the collective understanding of AIV in the environment, including persistence and potential for transmission via water to birds and mammals, supporting early detection of HPAI viruses and other AIVs, and mitigation to reduce the spread of disease into domestic poultry and potentially to other wildlife. This information also has potential human health implications, especially in public use waterways, where people may have direct exposure to AIV with primary or secondary contact. Methods to detect AIV using rRT-PCR more consistently from these concentrated surface waters would help improve environmental detection.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.3c00668.

Additional figures and tables; including experimental procedure details; virus sequence information; quality assurance data; and air temperature, precipitation, and field parameters (XLSX)

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Notes

The authors declare no competing financial interest.

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