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PERSISTENCE OF LOW PATHOGENIC AVIAN INFLUENZA VIRUSES IN FILTERED SURFACE WATER FROM WATERFOWL HABITATS IN GEORGIA, USA

Shamus P. Keeler,[1,2,4] Roy D. Berghaus,[3] and David E. Stallknecht[1]

[1] Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA
[2] Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA
[3] Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA
[4] Corresponding author (email: skeeler@uga.edu)

ABSTRACT: The natural reservoirs for avian influenza virus (AIV) are wild bird species of the orders Anseriformes and Charadriiformes. The primary route of transmission for wild birds is through fecally contaminated surface water on shared aquatic habitats. A distilled water model has shown that AIV remains infectious in water for weeks to months with pH, salinity, and temperature affecting stability. To evaluate the effect of pH, salinity, and temperature on AIV persistence in natural surface water, we measured the duration of infectivity for two common low pathogenic AIV subtypes in 15 filtered surface water samples collected from major waterfowl habitats in Georgia, USA. Trials were performed at three incubation temperatures 10, 17, and 28 C. Consistent with previous studies, pH and temperature had a significant effect on the stability of AIV in filtered surface water. Both viruses were less stable at warmer temperatures and in acidic water (pH<5.0). Due to the limited range of salinity of the field water samples, the role of salinity in AIV stability in surface water could not adequately be evaluated. Variations in persistence times between water samples with comparable pH and salinities indicated that other factors affect AIV stability in natural surface water. These results contribute to the current understanding of AIV persistence in aquatic habitats and may help in identifying areas with an increased likelihood of AIV persistence and potential transmission.

Key words: AIV, avian influenza virus, environment, persistence, low pathogenic, water.

INTRODUCTION

Species of wild aquatic birds in the orders Anseriformes and Charadriiformes are reservoirs for avian influenza virus (AIV; Stallknecht and Shane, 1988) and transmission of AIV between wild birds occurs through the fecal-oral route involving surface water in aquatic habitats (Hinshaw et al., 1979, 1980). Replication of low pathogenic (LP) AIVs occurs primarily in the intestinal tract of infected ducks and large amounts of infectious virus are shed in their feces (Webster et al., 1978). The duration of shedding can be as long as 28 days as reported in experimentally infected Pekin Ducks (*Anas platyrhynchos*) with large virus concentrations shed early in infection (Hinshaw et al., 1980). Experimentally infected Muscovy Ducks (*Cairina moschata*) may shed up to 1×10^{10} EID_{50} in a 24-hr period (Webster et al., 1978; Hinshaw et al., 1980). Contaminated aquatic habitats may serve as both a transmission medium for short-term transmission and potentially a reservoir for longer-term transmission. Avian influenza viruses have been isolated from the surface water of waterfowl habitats when infected ducks were present (Hinshaw et al., 1980; Markwell and Shortridge, 1982; Halvorson et al., 1985; Sivanandan et al., 1991; Ito et al., 1995) and from surface water and sediment of aquatic habitats following bird migration (Ito et al., 1995; Lang et al., 2008). Based on results from attempts to model AIV infection events in wild ducks, indirect fecal-oral transmission via contaminated water may be an important source of transmission and should be considered when attempting to understand AIV dynamics in wild birds (Breban et al., 2009; Roche et al., 2009; Rohani et al., 2009).

Most studies evaluating the persistence of AIV in water used distilled water as a

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model system (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009). The distilled water system allows for easy modification and control of pH, salinity, and temperature and adequately simulates the water column of aquatic systems. Laboratory trials using distilled water demonstrated that the virus could remain infectious in water for weeks to months and the duration of persistence showed an inverse relationship to temperature and salinity (Stallknecht et al., 1990a; Stallknecht et al., 1990b). Filtered surface water samples from waterfowl habitats in Louisiana, USA, were also tested, and the stability of AIV in these samples was consistent with the results of the distilled water trials (Stallknecht et al., 1990a). However, these trials included only four surface water samples with limited variation in pH and salinity. Work with surface water collected in Bulgaria supported earlier reports of the effects of pH, salinity, and temperature on AIV persistence and determined that AIV persistence was reduced in biologically intact water compared to filtered water samples (Zarkov, 2006). Brown et al. (2009) evaluated the effect of pH, salinity, and temperature on 12 LP AIV isolates representing hemagglutinin subtypes 1–12 using the distilled water laboratory model. Viral stability was greatest at a slightly basic pH (7.4–8.2), low temperatures (<17 C), and fresh to brackish water (0–20,000 parts per million (ppm); Brown et al., 2009). Direct comparisons of natural surface water and comparable distilled water support the inverse relationship between water temperature and AIV stability (Nazir et al., 2010).

Application of the laboratory results to natural systems is difficult due to the complexity of aquatic habitats (Stallknecht et al., 2010). To increase the understanding of the environmental persistence of influenza viruses, it is necessary to perform laboratory-based trials using surface water from waterfowl habitats, but the complexity of natural water samples may hinder or distort our understanding. Studies have used surface water for persistence trials, but the number of surface water samples has been limited and the experimental approaches have varied, restricting comparability. Nazir et al. (2010) showed that bacterial colony-forming units increased significantly during the course of persistence trials with natural surface water, which could have a significant effect on viral stability and artificially alter the duration of persistence. To limit the number of confounding factors and allow comparisons between studies that have used distilled water as a model system and the current study, natural surface water was filtered to remove the majority of biologic material. Our objectives were to evaluate the effect of water temperature, pH, and salinity on the duration of LP AIV persistence in filtered surface water and to determine if these factors alone could account for the duration of AIV persistence in surface water samples in aquatic habitats that are naturally used by ducks.

MATERIALS AND METHODS

Water collection

We identified major waterfowl habitats within Georgia, USA, and selected 15 sites across the state for sampling. At each site, the body of water most likely to have at least a seasonal population of waterfowl was identified for sampling. In 2008, sites were sampled from 13 to 19 March. Water samples were taken within 1 m of the shoreline and about 3 cm below the surface; 1 L was collected in a low-density polyethylene wide-mouth bottle (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) and placed on ice for transport to the laboratory. At each site, pH and specific conductance readings were taken using a YSI 556 MPS handheld instrument (YSI, Inc., Yellow Springs, Ohio, USA), which had been calibrated before each use. In the laboratory, water samples were filtered using a bottle-top vacuum filter system with a 0.22-μm polyethersulfone membrane (Corning Inc., Corning, New York, USA) to remove most biologic material. The pH and specific conductance were determined for the filtered samples using a VWR sympHony SB80PC

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benchtop meter (VWR International, Radmor, Pennsylvania, USA). Filtered water samples were stored at 4 C until experimental trials.

Prescreening water for viruses

The RNA was extracted from two 50-μL aliquots of each filtered water sample using the MagMax 96 AI/ND Viral Isolation Kit (Ambion, Inc., Austin, Texas, USA) and a semiautomated nucleic acid purification system, KingFisher 24 (Thermo Scientific) following published protocols (Das et al., 2009). Real-time reverse transcriptase (rRT-) PCR was used to screen all samples for the matrix protein of influenza virus. Primers and cycling parameters were identical to those determined by Spackman et al. (2002) and were run on a SmartCycler (Cepheid, Inc., Sunnyvale, California, USA). Water samples were considered to be negative if the cycle threshold (Ct) value was 0 or >40.

Two 500-μL aliquots of each filtered water sample were diluted 1:1 in 2× serum-free Eagle's minimal essential medium. The diluted water samples were subjected to the same infectivity assay as the experimental water samples to screen for environmentally deposited cytopathic agents and ensure that cytopathicity observed during the experiment was due to the AIV added during the study.

Viruses and virus titrations

Two LP AIVs isolated from wild ducks were used: A/Mallard/MN/199036/99 (H3N2) and A/Mallard/MN/199057/99 (H4N6). The viruses were selected because these isolates were used for previous persistence trials in our laboratory, and they represent common subtypes from wild birds in North America (Brown et al., 2009; Wilcox et al., 2011). Stocks of both viruses were propagated in 9- to 11-day-old specific pathogen-free embryonating chicken eggs with viral-infected amnioallantoic fluid (AAF) harvested 4 days postinoculation (DPI). The median tissue culture infectious dose ($TCID_{50}$) for each stock was determined on Madin-Darby canine kidney (MDCK) cells (described below). The titers of the H3N2 (third passage in eggs) and H4N6 (second passage in eggs) were $10^{8.3}$ $TCID_{50}$/mL and $10^{7.6}$ $TCID_{50}$/mL, respectively. Viral stocks were stored at −80 C until experimental trials. All titrations were performed using MDCK cells as described by Brown et al. (2009).

Experimental design

For both viruses, infective AAF was diluted 1:100 in each of the 15 filtered surface water samples. Inoculated water samples were placed into 5.0-mL polystyrene tubes at a volume of 4.0 mL. Each virus and water sample combination was maintained at 10 and 17 C in low-temperature incubators and 28 C in a water bath. The three incubation temperatures were selected because they are temperatures that have been used extensively in the literature and they represent water temperatures that would be encountered in aquatic environments throughout the year in Georgia (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009). In addition, 15 mL of each water sample with no infective AAF was maintained at each temperature, and the pH of the water samples was evaluated using a VWR sympHony SB80PC benchtop meter (VWR) at all time points. Virus-inoculated water was sampled at the time of inoculation (0 DPI) and at least five times thereafter. The frequency of the additional time points was determined based on estimates of the time required for a viral titer reduction of 2 $\log_{10}$ $TCID_{50}$/mL (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2009). In cases in which the viral titer became undetectable before five samples were collected, the trial was rerun with a shorter sampling frequency. If a 2 $\log_{10}$ $TCID_{50}$/mL reduction of viral titer was not observed after five sampling times, additional samples were taken maintaining the same sampling frequency. The sampling frequency ranged from 2 hr for water samples with a low pH and high salinity at 28 C to five days for water samples with a neutral pH and low salinity at 10 C. All titrations were performed in duplicate. A $TCID_{50}$/mL was determined for each (Brown et al., 2009).

Statistical analysis

The titration data for all trials were $\log_{10}$ transformed. Simple linear regression was used to calculate the virus log reduction time (Rt), which is the time in days required for a decrease of viral titer by 1 $\log_{10}$ $TCID_{50}$/mL (i.e., a 90% reduction in infectivity; Minitab 15, Minitab Inc., State College, Pennsylvania, USA).

The variance of Rt values increased with the mean, so a log transformation was used for statistical analysis. Factorial repeated measures analysis of variance (ANOVA) was used to evaluate the effects of water temperature, pH category, and virus type on the log-transformed Rt values. Pairwise comparisons were performed using Tukey's procedure. A predictive model was obtained using multivariable linear regression with robust standard

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errors to account for repeated measurements on the same water samples. Multivariable model selection began with a maximum model containing main effect terms for all variables, with subsequent stepwise elimination of variables having $P>0.10$. After reaching a preliminary main effects model, all possible two-way interactions were evaluated. All testing assumed a two-sided alternative hypothesis, and $P<0.05$ was considered statistically significant. Statistical analysis was performed using commercially available software (Stata version 11.1, StataCorp LP, College Station, Texas, USA).

RESULTS

Study sites included most of the physiogeographic regions of Georgia including the ridge and valley, piedmont, and coastal plain (Fig. 1). All water samples tested negative for AIV by rRT-PCR with all samples having a Ct value of 0, and no cytopathic agents were detected in any of the filtered water samples. The pH and salinity of the collected water samples are summarized in Table 1 along with the calculated Rt values. The linear regression models (LRMs), adjusted R^2, P value, and estimated viral persistence for all experimental conditions are summarized in Appendix 1.

Water samples with a relatively low pH (4–5) had shorter Rts than did those with a near neutral pH (7); higher water temperatures also yielded shorter Rts (Fig. 2). Based on the repeated measures ANOVA of log-transformed Rt values, there was a significant effect of both pH ($P<0.001$) and temperature ($P<0.001$), but there was no difference between virus types ($P=0.49$). There were also no significant interactions between pH and virus ($P=0.38$); temperature and virus ($P=0.17$); temperature and pH ($P=0.21$); or pH, temperature, and virus ($P=0.14$). The geometric mean Rt values for temperature and pH categories are summarized in Table 2. The mean $\log_{10}$ Rt values for the pH 6.8–7.3 and 7.4–7.6 categories were not significantly different from each other, but both were significantly higher than for the pH 4.2–5.0 category. The mean $\log_{10}$ Rt values differed significantly between all three temperatures (10, 17, and 28 C), with the longest Rts at 10 C and the shortest at 28 C.

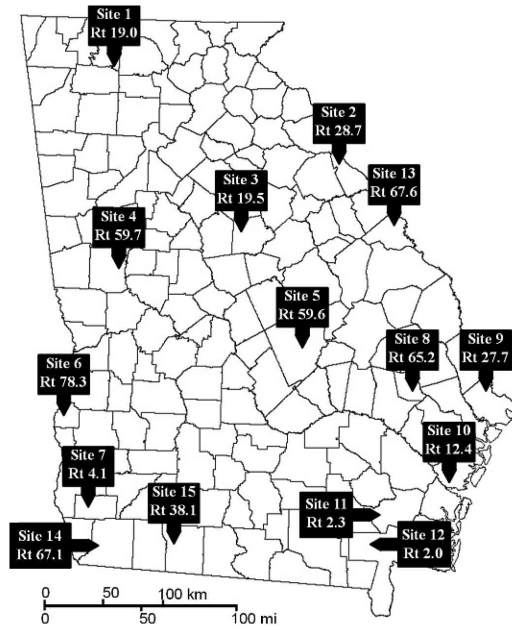

FIGURE 1. The 15 sampling sites in Georgia, USA, where water samples were collected in March 2008 for use in laboratory-based viral persistence trials. Water samples were filtered and persistence trials were performed for two low pathogenic avian influenza viruses. The experimentally determined average viral log reduction time for both viral isolates in each water sample at 10 C is listed under the site designation. The collection sites were 1) Carter's Lake in Coosawatte Wildlife Management Area (WMA; 34°35′51.11″N, 84°41′40.67″W); 2) Morris Creek in Fishing Creek WMA (33°52′23.66″N, 82°34′54.80″W); 3) B. F. Grant WMA (33°25′30.54″N, 83°29′06.61″W); 4) Joe Kurz WMA (33°07′31.62″N, 84°31′31.22″W); 5) Riverband WMA (32°27′33.12″N, 82°50′15.40″W); 6) Eufala National Wildlife Refuge (NWR; 31°59′31.74″N, 85°03′10.80″W); 7) Mayhaw WMA (31°12′13.07″N, 84°47′34.76″W); 8) Evans County public fishing area (32°07′33.49″N, 81°48′08.06″W); 9) Savannah NWR (32°09′48.47″N, 81°08′10.38″W); 10) Altamaha WMA (31°21′17.78″N, 81°27′06.44″W); 11) Laura Walker State Park–Dixon Memorial (31°08′26.45″N, 82°12′52.16″W); 12) Grand Bay WMA (30°56′03.59″N, 82°12′52.16″W); 13) Phinizy Swamp WMA (33°25′01.88″N, 81°59′05.68″W); 14) Lake Seminole (30°47′07.51″N, 84°44′35.66″W); and 15) Thomas Co. Plantation Ponds in Rivercreek WMA (30°54′39.42″N, 84°00′27.79″W).

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TABLE 1. Surface water samples were collected from 15 waterfowl habitats distributed throughout Georgia, USA, in March 2008. Water samples were filtered and laboratory-based viral persistence were performed with two low pathogenic avian influenza viruses at three incubation temperatures. This table summarizes the water sample characteristics and virus log reduction times (Rts) by virus type and temperature. The Rt values are the time (days) required for a decrease of viral titer by 1 log_{10} median tissue culture infectious dose per milliliter.

Site (reference No.)[a]	pH	Salinity (ppm)	A/Mallard/MN/199036/99 (H3N2) Rt values			A/Mallard/MN/199057/99 (H4N6) Rt values		
			10 C	17 C	28 C	10 C	17 C	28 C
Carter's Lake in Coosawatte WMA (1)	7.2	40	21.2	22.8	2.1	16.8	16.5	1.6
Morris Creek in Fishing Creek WMA (2)	7.3	40	24.9	20.6	6.7	32.4	25.7	6.9
B. F. Grant WMA (3)	7.4	110	15.8	11.9	7.9	23.2	9.6	8.1
Joe Kurz WMA (4)	6.8	60	58.1	44.8	6.5	61.4	37.2	9.7
Riverband WMA (5)	7.3	70	57.5	43.9	7.8	61.7	44.8	10.0
Eufala NWR (6)	7.4	60	88.5	47.4	9.5	68.0	73.0	12.7
Mayhaw WMA (7)	4.7	30	5.7	3.8	1.9	2.6	4.1	1.2
Evans County PFA (8)	6.9	60	51.0	49.8	7.0	79.4	66.7	11.6
Savannah NWR (9)	7.6	2,800	30.6	18.3	5.1	24.7	14.7	6.9
Altamaha WMA (10)	4.8	2,740	12.9	18.0	2.1	11.8	10.6	2.2
Laura Walker SP–Dixon Memorial (11)	4.3	50	0.7	0.8	0.3	3.8	0.7	0.2
Grand Bay WMA (12)	4.2	50	0.9	1.0	0.7	3.1	1.8	0.2
Phinizy Swamp WMA (13)	7.2	120	73.0	41.3	9.2	62.1	48.8	10.0
Lake Seminole Thomas County (14)	7.4	80	70.9	51.3	18.3	63.3	48.1	11.1
Plantation Ponds in Rivercreek WMA (15)	7.6	130	26.0	28.1	7.0	50.3	52.9	8.2

[a] WMA = Wildlife Management Area; NWR = National Wildlife Refuge; PFA = public fishing area; SP = state park.

Consistent with the results of the repeated measures ANOVA, only temperature and pH were significant predictors ($P<0.001$) in the final regression model (Table 3). Compared to water samples at 10 C, the mean log_{10} Rt value was 0.11 units lower at 17 C and 0.74 units lower at 28 C, corresponding to relative reductions in the untransformed Rt values of 22% and 82%, respectively. For every one unit increase in pH, the mean log_{10} Rt increased by 0.39 units, corresponding to a relative increase in the untransformed Rt values of 144%. The final regression model explained 79% of the variability in log_{10} Rt values.

The majority of the water samples collected during this study had salinities <130 ppm, well below the threshold for fresh water (<500 ppm). Two water samples, Savannah National Wildlife Refuge (NWR; Fig. 1: Site 9) and Altamaha NWR (Fig. 1: Site 10), could be considered brackish with salinities of 2,800 ppm and 2,740 ppm, respectively. Even these water samples were well below the salinity at which a significant reduction in the duration of viral persistence is observed in distilled water trials (Brown et al., 2009). Due to this limited range of salinity concentrations, the effect of salinity could not be adequately evaluated. Salinity was not statistically evaluated using the repeated measures ANOVA, but salinity was included in the multivariable linear regression and had no significant predictive value.

DISCUSSION

While it is known that AIV is stable in distilled water for months, our understanding of the factors that affect the persistence of the virus in natural surface water are limited. The importance of the abiotic factors pH, salinity, and temperature have been evaluated in previous studies, but these studies mainly used distilled water as a laboratory model with a small number of surface water samples. Wild birds use a wide range of habitats, and it is reasonable to presume that individual habitats will vary widely in their

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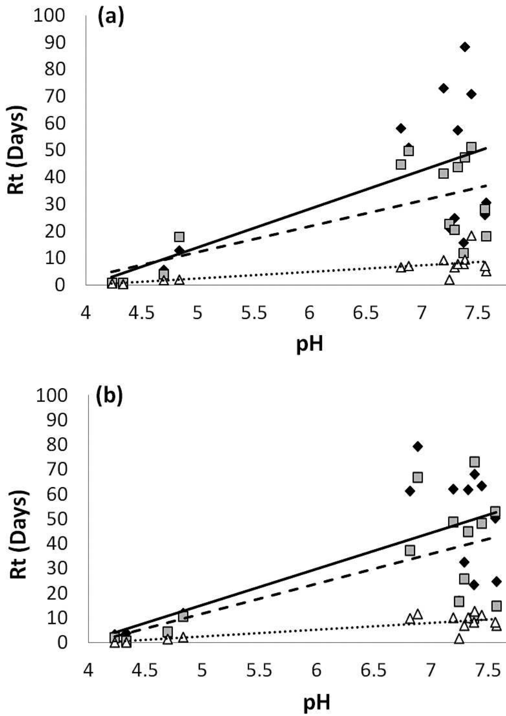

FIGURE 2. The virus log reduction times (Rt) for two low pathogenic avian influenza virus isolates in 15 filtered surface water samples with varying pH from 4.23 to 7.57 at three temperatures. (A) The Rt values for A/Mall/MN/199036/99 (H3N2) with general trend line for all Rt values at each temperature; black diamonds and solid line: 10 C, grey squares and dashed line: 17 C, white triangles and dotted line: 28 C. (B) The Rt values for A/Mall/MN/199057/99 (H4N6) with general trend line for all Rt values at each temperature; black diamonds and solid line: 10 C, grey squares and dashed line: 17 C, white triangles and dotted line: 28 C.

abiotic constituents. Evaluating the role of pH, salinity, and temperature in filtered surface water from waterfowl habitats enhances our understanding of the role of these abiotic factors in determining AIV stability and allows us to compare the laboratory-determined persistence trends to natural water without the confounding effect of biologic material.

The results of the trials with filtered surface water are consistent with some of the general trends of AIV persistence previously established (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Zarkov, 2006; Brown et al., 2007; Brown et al., 2009). The pH and temperature of surface water have a significant effect on the stability of AIV in filtered surface water. Both viruses persisted longer at the two lower temperatures (10 and 17 C) compared to the highest temperature (28 C) evaluated in this study, which is consistent with the inverse relationship between AIV persistence and temperature reported previously (Stallknecht et al., 1990a; Brown et al., 2009; Nazir et al., 2010). The pH of the surface water samples in our study was 4.23 to 7.57 with most samples having a pH close to neutral (6.81–7.57). Studies using the distilled water model established that AIV is most stable at a slightly basic pH (7.4–8.2; Stallknecht et al., 1990a; Brown et al., 2009). Six of our water samples had neutral pHs (6.8–7.4), which were slightly outside the laboratory-established ideal

TABLE 2. Surface water samples were collected from 15 waterfowl habitats distributed throughout Georgia, USA, in March 2008. Water samples were filtered and laboratory-based viral persistence were performed with two low pathogenic avian influenza viruses at three incubation temperatures. This table summarizes the experimentally determined geometric mean (min–max) virus log reduction times (days) in the filtered water samples by temperature and pH category for both virus isolates (H3N2 and H4N6) combined.

pH range	n[a]	10 C	17 C	28 C	Total
4.2–5.0	4	3.3 (0.7, 13)	2.8 (0.8, 18)	0.7 (0.3, 2.1)	1.9[a] (0.3, 18)
6.8–7.3	6	45 (21, 73)	36 (21, 50)	6.6 (2.1, 9.2)	22[b] (2.1, 73)
7.4–7.6	5	42 (16, 89)	29 (12, 51)	8.9 (5.1, 18)	22[b] (5.1, 89)
Total[b]	15	22(A) (0.7, 89)	17(B) (0.8, 51)	4.0(C) (0.3, 18)	11 (0.3, 89)

[a] Number of water samples in each pH category.

[b] Within rows and columns, marginal means followed by the same letter are not significantly different with a level of significance of 5% over all comparisons.

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TABLE 3. Surface water samples were collected from 15 waterfowl habitats distributed throughout Georgia, USA, in March 2008. Water samples were filtered and laboratory-based viral persistence were performed with two low pathogenic avian influenza viruses at three incubation temperatures. Multivariable linear regression with robust standard errors was used to develop a predictive model. This table summarizes the multivariable linear regression model for the prediction of $\log_{10}$ virus reduction time in the water samples collected from 15 locations (R^2=78.6%).

Variable	Coefficient (robust SE)	95% CI	P[a]
Temperature (C)			
10	0.74 (0.05)	0.64, 0.83	<0.001
17	0.63 (0.06)	0.51, 0.75	
28	Referent	Referent	
pH	0.39 (0.07)	0.24, 0.53	<0.001
Constant	−1.93 (0.46)	−2.92, −0.94	0.001

[a] Based on Wald statistics.

range. There was no statistical difference between AIV stability in these six water samples compared to the five water samples with pH in the ideal range. This suggests that the ideal pH range for the stability of AIV in surface water is wider (neutral to slightly basic) than previously established. Four of our field water samples had acidic pHs (4.23–4.83), and AIV persistence was significantly reduced in these samples. The pHs observed in these samples were much lower than those used in previous experiments (Stallknecht et al., 1990a; Brown et al., 2009).

Overall, a multivariable LRM including only temperature and pH explained 79% of the variability in $\log_{10}$ Rt values. Despite the satisfactory predictive ability of the model, other factors may also be important for determining the duration of AIV persistence. Under experimental conditions, salinity could not be evaluated in our study, but it can affect AIV stability (Stallknecht et al., 1990a; Brown et al., 2007; Brown et al., 2009). In Georgia, saline habitats are not available to dabbling ducks outside of coastal regions, and for this reason, most of the water samples were from fresh water with salinities <500 ppm. Such habitats may be important in other areas of North America. A large range of variability of persistence was observed between the field water samples, particularly between water samples with neutral pHs and at lower temperatures, which are the more favorable conditions for AIV persistence. While other investigators have reported variability of persistence between viral subtypes and isolates (Brown et al., 2007; Brown et al., 2009), virus subtype was not found to be a significant determinant of AIV stability in this study. The surface water samples used in this study were filtered with a 0.2-μm filter to remove the majority of biologic material, but filtering does not entirely eliminate the influence of biologic material. The filter would not have removed small molecules secreted or released by organisms, and the filtering process itself may have lysed organisms, releasing cellular contents into the water. Previous studies have shown that biologic constituents of surface water reduce AIV persistence, but it is unclear whether microorganisms or their byproducts cause the reduction in infectivity (Zarkov, 2006; Nazir et al., 2010). While the observed variability could be related to these or other biologic factors, these results may also indicate that other abiotic factors beyond pH, salinity, and temperature affect AIV persistence in surface water. Additionally, in natural aquatic systems, other factors not represented in the laboratory trials, such as ultraviolet light and water current, could reduce the duration of AIV infectivity by inactivating the virus or dispersing the virus and reducing the localized viral titer.

As we gain a better understanding of AIV stability in surface water, we may be better able to predict how long AIV will persist in a particular aquatic habitat. With this type of information, we could target the areas with the greatest capacity for AIV persistence for environmental sampling, which could be used to enhance current surveillance programs or for studies of AIV ecology and transmission. Based on the laboratory persistence trials, several of the sites sampled in this study

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may be habitats where AIV could persist longer term (i.e., weeks to months) and be sites with an increased likelihood of environmental transmission risk between migratory waterfowl populations, but further trials and environmental sampling are required before particular sites could be identified as habitats that facilitate long term persistence. In addition to identifying areas of high environmental persistence, we could also identify areas with limited capacity for AIV persistence. In this study, the four sample sites with the lowest pHs had a much lower capacity for surface water persistence and could be considered of low concern for long-term stability and transmission of AIV. Waterfowl habitats are made up of many different types of aquatic systems, each with their own abiotic and biotic constituents, and all undergo changes over time. Unfortunately, our current understanding of the factors affecting AIV persistence is limited, and any conclusions about the persistence capacity of an area based on laboratory persistence trials are limited to the water sample collected for the study. For example, the water sample from Altamaha Wildlife Management Area (WMA) evaluated in this study indicated that this area has a lower capacity for surface water persistence of AIV. Altamaha WMA is considered to be one of the premier waterfowl migrating and wintering areas in Georgia and encompasses an area of about 109 km^2 with about 20 impoundments ranging from tidal freshwater to brackish water. The surface water persistence capacity of Altamaha WMA cannot be judged on a single water sample taken from one impoundment, but the persistence trials give an indication of suitability of the immediate area surrounding the sampling site. In time, it may be possible to estimate the persistence capacity of an aquatic system without performing laboratory persistence trials.

We evaluated the role of pH and temperature in determining AIV stability in natural surface water by limiting the influence of biotic components through filtration. The study was designed to be complementary to previous studies that had used sterile distilled water as a model system. The results indicated that many of the previously established persistence trends are applicable to natural surface water; in particular the duration of viral persistence increases as water temperature decreases and as the pH approaches neutral to slightly basic. We also stressed the necessity to develop research on brackish to salt water to evaluate precisely the effects of salinity in AIV persistence in such habitats. Further studies using surface water are necessary to differentiate the effect of biotic and abiotic constituents and to identify the other factors affecting AIV persistence in water and predict sites with high potential for virus transmission in wild waterfowl populations

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APPENDIX 1

Linear regression models (LRMs), adjusted R^2, *P* value, and estimated viral persistence for two low pathogenic avian influenza virus isolates, A/Mall/MN/199036/99(H3N2) and A/Mall/MN/199057/99(H4N6) at three temperatures in 15 filtered surface water samples collected in Georgia, USA.

Site[a]	Virus	Temperature (C)	LRM[b]	R^2 (Adj)	*P* value	Estimated persistence[c] (Rt value)[d]
1	H3N2	10	y=5.30–0.0472x	61.3	0.02	127.1 (21.2)
		17	y=4.71–0.0439x	47.1	0.05	136.7 (22.8)
		28	y=4.61–0.476x	97.7	<0.01	12.6 (2.1)
	H4N6	10	y=4.55–0.0594x	77.0	0.03	101.0 (16.8)
		17	y=4.51–0.0607x	95.1	<0.01	98.9 (16.5)
		28	y=4.80–0.612x	33.0	0.03	9.8 (1.6)
2	H3N2	10	y=6.56–0.0401x	97.2	<0.01	149.6 (24.9)
		17	y=6.32–0.0486x	99.3	<0.01	123.5 (20.6)
		28	y=6.08–0.149x	91.8	<0.01	40.3 (6.7)
	H4N6	10	y=5.01–0.0309x	82.3	<0.01	194.2 (32.4)
		17	y=4.37–0.0389x	72.7	0.01	154.2 (25.7)
		28	y=5.25–0.144x	75.3	<0.01	41.7 (6.9)
3	H3N2	10	y=6.65–0.0633x	96.1	<0.01	94.8 (15.8)
		17	y=6.10–0.0840x	91.4	0.01	71.4 (11.9)
		28	y=5.67–0.127x	76.7	<0.01	47.2 (7.9)
	H4N6	10	y=4.67–0.0431x	57.0	0.05	139.2 (23.2)
		17	y=4.85–0.104x	86.0	0.02	57.7 (9.6)
		28	y=3.10–0.123x	55.1	0.06	48.8 (8.1)
4	H3N2	10	y=5.71–0.0172x	51.7	0.02	348.8 (58.1)
		17	y=5.44–0.0223x	75.3	<0.01	269.1 (44.8)
		28	y=6.09–0.153x	99.7	<0.01	39.2 (6.5)
	H4N6	10	y=4.72–0.0163x	35.1	0.05	368.1 (61.4)
		17	y=4.99–0.0269x	67.9	<0.01	223.1 (37.2)
		28	y=4.96–0.103x	88.7	<0.01	58.3 (9.7)
5	H3N2	10	y=5.63–0.0174x	59.3	0.01	344.8 (57.5)
		17	y=5.46–0.0228x	70.9	<0.01	263.2 (43.9)
		28	y=5.84–0.129x	97.0	<0.01	46.5 (7.8)
	H4N6	10	y=4.74–0.0162x	55.2	0.01	370.4 (61.7)
		17	y=4.59–0.0223x	77.9	<0.01	269.1 (44.8)
		28	y=4.85–0.0998x	82.3	0.01	60.1 (10.0)
6	H3N2	10	y=5.77–0.0113x	66.7	0.01	531.0 (88.5)
		17	y=5.89–0.0211x	86.3	<0.01	284.4 (47.4)
		28	y=5.69–0.105x	79.3	0.03	57.1 (9.5)
	H4N6	10	y=4.52–0.0147x	62.9	0.01	408.2 (68.0)
		17	y=4.69–0.0137x	60.6	0.01	438.0 (73.0)
		28	y=4.51–0.0789x	98.7	<0.01	76.0 (12.7)
7	H3N2	10	y=2.94–0.177x	64.6	0.06	33.9 (5.7)
		17	y=2.88–0.257x	52.2	0.06	23.4 (3.9)
		28	y=4.55–0.525x	82.9	<0.01	11.4 (1.9)
	H4N6	10	y=3.65–0.383x	81.6	<0.01	15.7 (2.6)
		17	y=3.54–0.242x	49.3	0.03	24.8 (4.1)
		28	y=3.52–0.805x	83.5	<0.01	7.5 (1.2)
8	H3N2	10	y=5.55–0.0196x	58.4	0.01	306.1 (51.0)
		17	y=5.52–0.0201x	53.5	0.02	298.5 (49.8)
		28	y=5.78–0.142x	89.4	<0.01	42.3 (7.0)
	H4N6	10	y=4.64–0.0126x	74.8	<0.01	476.2 (79.4)
		17	y=4.58–0.0150x	65.8	0.01	400.0 (66.7)
		28	y=4.86–0.0865x	100.0	<0.01	69.5 (11.6)
9	H3N2	10	y=4.90–0.0327x	69.3	<0.01	183.5 (30.6)
		17	y=5.27–0.0548x	83.7	<0.01	109.4 (18.3)
		28	y=5.63–0.197x	99.8	0.02	30.5 (5.1)

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APPENDIX 1. Continued.

Site[a]	Virus	Temperature (C)	LRM[b]	R^2 (Adj)	P value	Estimated persistence[c] (Rt value)[d]
	H4N6	10	y=3.51–0.0415x	58.0	<0.01	144.6 (24.1)
		17	y=4.23–0.0679x	85.4	0.01	88.4 (14.7)
		28	y=3.93–0.144x	75.2	<0.01	41.7 (6.9)
10	H3N2	10	y=4.91–0.0773x	78.9	0.01	77.6 (12.9)
		17	y=4.83–0.0557x	85.3	0.01	107.7 (18.0)
		28	y=4.68–0.478x	89.3	0.01	12.6 (2.1)
	H4N6	10	y=4.00–0.0846x	92.4	<0.01	70.9 (11.8)
		17	y=4.04+0.0943x	15.5	0.11	63.6 (10.6)
		28	y=3.98–0.458x	91.7	0.01	13.1 (2.2)
11	H3N2	10	y=4.03–1.39x	86.4	<0.01	4.3 (0.7)
		17	y=3.82–1.25x	81.4	<0.01	4.8 (0.8)
		28	y=3.90–3.16x	93.8	0.02	1.9 (0.3)
	H4N6	10	y=2.15–0.263x	40.7	0.03	22.8 (3.8)
		17	y=3.21–1.53x	93.9	0.02	3.9 (0.7)
		28	y=3.50–6.32x	68.3	0.05	1.0 (0.2)
12	H3N2	10	y=4.09–1.14x	50.9	0.07	5.3 (0.9)
		17	y=3.80–1.04x	90.3	<0.01	5.8 (1.0)
		28	y=3.56–1.48x	64.8	0.06	4.1 (0.7)
	H4N6	10	y=2.66–0.276x	58.3	0.01	21.7 (3.6)
		17	y=3.24–0.555x	96.4	0.09	10.8 (1.8)
		28	y=3.97–6.44x	96.0	<0.01	0.9 (0.2)
13	H3N2	10	y=5.91–0.0137x	51.5	0.01	438.0 (73.0)
		17	y=5.94–0.0242x	93.0	<0.01	247.9 (41.3)
		28	y=5.72–0.109x	94.2	<0.01	55.1 (9.2)
	H4N6	10	y=5.07–0.0161x	77.1	<0.01	372.7 (62.1)
		17	y=4.55–0.0205x	57.7	0.01	292.7 (48.8)
		28	y=5.18–0.0998x	88.6	<0.01	60.1 (10.0)
14	H3N2	10	y=5.87–0.0141x	77.4	<0.01	425.5 (70.9)
		17	y=5.72–0.0195x	78.7	<0.01	307.7 (51.3)
		28	y=4.90–0.0546x	60.8	0.04	109.9 (18.3)
	H4N6	10	y=4.87–0.0158x	42.6	0.05	379.8 (63.3)
		17	y=4.94–0.0208x	89.0	<0.01	288.5 (48.1)
		28	y=4.99–0.0901x	99.5	<0.01	66.6 (11.1)
15	H3N2	10	y=5.97–0.0385x	81.6	<0.01	155.8 (26.0)
		17	y=5.49–0.0356x	64.6	0.01	168.5 (28.1)
		28	y=5.68–0.143x	78.4	0.03	42.0 (7.0)
	H4N6	10	y=4.83–0.0199x	75.8	0.01	301.5 (50.3)
		17	y=4.66–0.0189x	50.8	0.03	317.5 (52.9)
		28	y=5.09–0.122x	84.4	0.05	49.2 (8.2)

[a] The site numbers corresponding to sampling sites defined in Fig. 1.

[b] y = log10 median tissue culture infectious dose per milliliter; x = persistence in days.

[c] Estimated persistence in days for 1×10^6 $TCID_{50}$/mL water.

[d] Time in days required for a decrease of viral titer by 1 log_{10} $TCID_{50}$/mL.

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