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Persistence of Avian Influenza Viruses in Water

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SUMMARY. Persistence of five avian influenza viruses (AIVs) derived from four waterfowl species in Louisiana and representing five hemagglutinin and neuraminidase subtypes was determined in distilled water at 17 C and 28 C. Infectivity was determined over 60 days by microtiter endpoint titration. One AIV was tested over 91 days at 4 C.

Linear regression models for these viruses predicted that an initial concentration of 1×10^6 $TCID_{50}$/ml water could remain infective for up to 207 days at 17 C and up to 102 days at 28 C. Significant differences in slopes for AIV persistence models were detected between treatment temperatures and among viruses.

Results suggest that these viruses are adapted to transmission on waterfowl wintering habitats. Results also suggest a potential risk associated with waterfowl and domestic poultry sharing a common water source.

RESUMEN. Persistencia de virus de Influenza aviar en el agua.

Se determinó la persistencia de cinco virus de influenza aviar provenientes de cuatro especies de aves acuáticas en el estado de Louisiana. Los virus representaron cinco subtipos de hemoaglutinina y neuroaminidasa y la persistencia fue determinada en agua destilada a temperaturas de 17 C y 28 C. La infectividad se determinó por más de 60 días mediante titulación en microplaca. Una de las cepas fue analizada durante más de 91 días a 4 C.

Los modelos de regresión linear para estos virus predijeron que una concentración inicial de 1×10^6 $DICC_{50}$/ml de agua podría permanecer infectante hasta por 207 días a 17 C y hasta por 102 días a 28 C. Se detectaron diferencias significantes en las curvas de persistencia entre las temperaturas de tratamiento y entre los virus.

Los resultados sugieren que estos virus están adaptados para la transmisión entre la población de aves acuáticas. Los resultados también sugieren un riesgo potencial asociado con las aves acuáticas y las aves comerciales que compartan la misma fuente de agua.

Although fecal-oral transmission of avian influenza virus (AIV) within wild waterfowl populations is thought to occur via contaminated water (8,16,18), little is known about viral persistence in this medium. Replication of AIV in ducks occurs primarily in the intestinal tract (20), with high concentrations of infectious virus shed in feces (7,21). Webster *et al.* (21) reported that experimentally infected Muscovy ducks (*Cairina moschata*) shed 6.4 g of fecal material per hour with an infectivity of $1 \times 10^{7.8}$ mean egg infective dose (EID_{50})/g. These birds shed an estimated 1×10^{10} EID_{50} of AIV within a 24-hr period.

Avian influenza virus has been isolated from unconcentrated surface water in Canada (9) and Minnesota (4), but virus has not been isolated from lake water in areas devoid of ducks (6). Information pertaining to persistence of AIV in water is limited to a single trial in which duck feces, naturally infected with A/duck/Memphis/546/74 (H3N6), was tested in Mississippi River water (21). The initial concentration of $1 \times 10^{8.1}$ EID_{50}/ml was reduced to $1 \times 10^{4.3}$ EID_{50}/ml after 32 days at 4 C and was undetectable after 4 days at 22 C.

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Persistence of AIV in water is pertinent to an understanding of the natural reservoir and in the prevention of poultry disease. Although it has been suggested that AIVs may overwinter in frozen lake water, the actual mechanisms of persistence of AIV in wild duck populations have not been defined (7). From the standpoint of poultry disease, contaminated surface and ground water both have been suggested as long- and short-term sources of AIV for domestic turkeys (5). Objectives of the present study were to quantify the persistence of waterfowl isolates of AIV in water and to determine the effects of water temperature and viral strain on AIV persistence.

MATERIALS AND METHODS

Avian influenza viruses. Five AIVs, isolated from hunter-killed ducks from Cameron Parish, Louisiana, were used to evaluate persistence. These included A/gadwall/LA/17G/87 (H3N8), A/blue-winged teal/LA/44B/87 (H4N6), A/mottled duck/LA/38M/87 (H6N2), A/blue-winged teal/LA/188B/87 (H12N5), and A/green-winged teal/LA/169GW/88 (H10N7).

Viruses were propagated in 9-day-old specific-pathogen-free (SPF) chicken eggs. Amnio-allantoic fluid (AAF) was harvested after 72 hr and frozen at −70 C until needed. All viral assays were performed on the third egg passage.

Experimental procedures. Infective AAF was thawed and diluted 1:50 in sterile glass-filtered distilled water (pH 7.3). Aliquots of inoculated water (1.5 ml) were transferred into 2.5-ml glass vials, which were evenly divided and placed in environmental chambers at 17 C and 28 C until tested. Selected temperatures represent mean winter and summer water temperatures for coastal Louisiana marsh habitats (13). One virus, A/green-winged teal/LA/169GW/88 (H10N7), was also tested at 4 C.

Aliquots of inoculated water were sampled over a 60-day period, with a minimum of 16 assays per virus/temperature treatment. The single 4 C trial was performed over a 91-day period with eight assays.

Infectivity of AIV was quantified using a microtiter endpoint titration, as described below, and is expressed in units of mean tissue-culture infective dose ($TCID_{50}$)/ml of water. Primary cultures of chicken embryo fibroblasts (CEFs) from 11-day-old SPF chicken eggs (3) were used in these assays. Final cell suspensions of 3×10^6 CEF/ml were prepared in serum-free Eagle's minimum essential medium (MEM) supplemented with antibiotics (100 u penicillin G and 100 μg streptomycin sulfate/ml) and 25 mM HEPES buffer.

The identity of AIVs in all virus/temperature treatments was verified from water samples on days 27 and 47. Infective tissue-culture media from wells exhibiting cytopathic effect on endpoint titrations were tested by agar gel immunodiffusion (AGID) for the presence of type-A specific nucleoprotein (14). In addition, infected tissue-culture media from test plates were used to propagate all AIVs in 9-day-old SPF eggs. In all cases, AIV was isolated from test plates, and subtypes were verified by hemagglutination-inhibition and neuraminidase-inhibition tests by the National Veterinary Services Laboratories (NVSL), Science and Technology, Veterinary Services, APHIS, USDA, Ames, Iowa.

Endpoint titration procedure. A 0.5-ml sample of AIV-inoculated water was diluted 1:2 by addition of 0.5 ml 2× serum-free MEM. From this point, serial 10-fold dilutions were prepared in serum-free MEM.

Each well of a 96-well microtiter plate received 100 μl of cell suspension and 50 μl of the appropriate virus dilution. All wells were supplemented with 50 μl of MEM containing 2.8 μg/ml of highly purified trypsin (final concentration per well of 0.7 μg/ml).

Plates were covered and incubated at 37 C under 5% CO_2 for 96 hr. Examination for cytopathic effect was performed with light microscopy. To verify results, wells of culture plates were stained with 100 μl of 10% buffered formalin containing 1% crystal violet. Stain was removed after 2 hr, and plates were examined. Endpoints were recorded as 100% monolayer destruction with $TCID_{50}$ calculated as described (15).

Statistical analysis. All data were $\log_{10}$ transformed and analyzed using linear regression analysis. Differences in slope between linear models were evaluated using a test for heterogeneity of slope. All analyses were performed using the Statistical Analysis System (SAS) General Linear Models Procedure (17).

RESULTS

At 17 C, infectivity was detected throughout the 60-day sample period. At 28 C, infectivity for three of five AIVs reached an undetectable level by day 60. Table 1 and Fig. 1 present linear regression models and estimates of half-life for the five AIVs tested at 17 C and 28 C. All models and all parameters (y-intercept and slope) were significant ($P < 0.05$). Coefficients of determination (R^2) values for these linear models ranged from 0.66 to 0.97.

Differences in slope were detected among temperature treatments for all AIVs (Table 1). Differences in slope were also apparent among test AIVs at both 17 C ($P = 0.045$) and 28 C ($P < 0.0001$).

The R^2 value for the linear model for A/green-winged teal/LA/169GW/88 (H10N7) at 4 C was

Table 1. Linear regression models for persistence of five avian influenza viruses in water.

Virus strain	Temperature (C)	Linear regression model[A]	R^2	Half-life (days)
A/gadwall/LA/17G/87 (H3N8)	17	$y = 5.11 - 0.031x$	0.83	9.71
	28	$y = 5.38 - 0.092x$*	0.85	3.27
A/blue-winged teal/LA/44B/87 (H4N6)	17	$y = 4.55 - 0.028x$	0.70	6.27
	28	$y = 4.72 - 0.075x$*	0.97	4.01
A/mottled duck/LA/38M/87 (H6N2)	17	$y = 4.55 - 0.028x$	0.78	8.85
	28	$y = 4.78 - 0.065x$**	0.89	4.89
A/blue-winged teal/188B/87 (H12N5)	17	$y = 4.54 - 0.048x$	0.66	6.27
	28	$y = 5.80 - 0.197x$***	0.83	1.52
A/green-winged teal/LA/169GW/88 (H10N7)	17	$y = 5.60 - 0.41x$	0.89	7.34
	28	$y = 5.32 - 0.59x$*	0.83	5.10
	4	$y = 4.84 - 0.004x$	0.15	75.26

[A] $y = \log_{10}$ TCID$_{50}$; x = persistence in days; asterisks indicate significant differences in slope between models for 28 C and 17 C at: * $P < 0.0001$; ** $P = 0.0002$; *** $P = 0.0152$.

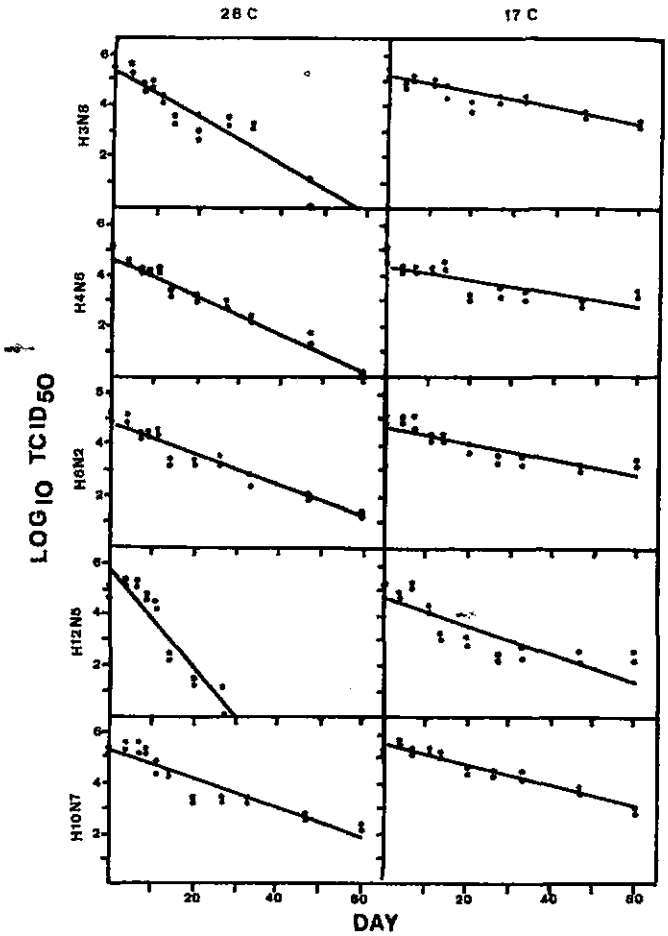

Fig. 1. Linear regression models for persistence of five AIVs in water at 17 C and 28 C.

Table 2. Estimated duration of infectivity in days for 1×10^6 $TCID_{50}$ of five avian influenza viruses in water at 17 C and 28 C.

Virus	17 C	28 C	% Reduction[A]
A/gadwall/LA/17G/87 (H3N8)	194	66	66%
A/blue-winged teal/LA/44B/87 (H4N6)	207	80	61%
A/mottled duck/LA/38M/87 (H6N2)	176	98	43%
A/blue-winged teal/LA/188B/87 (H12N5)	126	30	76%
A/green-winged teal/LA/169GW/88 (H10N7)	146	102	30%

[A][100 − (persistence in days at 28 C/persistence in days at 17 C)] × 100.

0.15 (Table 1). The slope was not different from zero ($P = 0.338$).

For comparison, duration of infectivity for an initial concentration of 1×10^6 $TCID_{50}$/ml water was estimated for each AIV at 17 C and 28 C (Table 2). Persistence of infectivity varied from 126 to 207 days at 17 C and from 30 to 102 days at 28 C. Considerable variation was apparent in the extent of the temperature effect among AIVs. Persistence of infectivity for virus strains at 28 C compared with 17 C was reduced from 30% to 76%. At 4 C, it is estimated that 1×10^6 $TCID_{50}$/ml water of A/green-winged teal/LA/169GW/88 (H10N7) would remain infective for 1333 days.

DISCUSSION

Linear regression models adequately explained results in all cases with coefficients of determination, indicating that individual models account for 66% to 97% of observed variation in data. All models and all parameters in these models were significant ($P < 0.05$), suggesting that they can be used to realistically predict persistence of AIV under experimental conditions.

Although data indicate long-term persistence, the linear regression model for A/green-winged teal/LA/169GW/88 (H10N7) at 4 C was not significant. This failure to fit a linear model to these data probably resulted from a relatively small sample size ($n = 8$) coupled with a slight change in infectivity (slope) over the 91-day test period.

Differences in slope between viruses were detected at both 17 C and 28 C. Variations in temperature tolerance have been reported for different AIVs isolated from domestic poultry (12). These differences may help explain persistence of specific AIV subtype combinations within wild waterfowl populations.

Persistence estimates for test AIVs at both temperature treatments exceed the duration reported for A/duck/Memphis/546/74 by Webster *et al.* (21). Infectivity for $1 \times 10^{8.1}$ EID_{50}/ml of this virus at 22 C in Mississippi River water was undetectable after 4 days. Persistence trials for A/duck/Memphis/546/74 (21) involved virus propagated in the intestines of experimentally infected ducks rather than infective AAF. In addition, fecal material present in the water may have created conditions unfavorable to persistence of this virus.

Differences in results from those reported for A/duck/Memphis/546/74 (21) may also relate to inherent variation among AIV strains. This possibility is supported by observations that AIVs differ significantly in their ability to persist in water at both 17 C and 28 C.

Results are compatible with observations of prolonged infectivity of the H5N2 viruses responsible for the 1983–84 AIV epornitic in Pennsylvania. Environmental sampling of premises from which infected poultry had been depopulated confirmed AIV persistence to 105 days in a liquid manure pit during winter (2).

Prolonged infectivity in water at temperatures reflecting field conditions provides support for several proposed mechanisms for maintenance of virus within waterfowl populations and their habitats. Results for A/green-winged teal/LA/169GW/88 (H10N7) at 4 C suggest that these viruses can overwinter in frozen or cold lake water in northern waterfowl habitats. Virus, therefore, may be available locally to ducks returning to breeding areas during spring.

Results also support a maintenance cycle involving transmission within duck populations on the wintering grounds. Although susceptibility of flocks and prevalence of AIV are low during the overwintering period following migration (6), high population density among wintering ducks may provide sufficient numbers

of susceptible individuals to maintain virus. This is supported by isolation of AIV during winter (11,19) and by isolations of AIV from adult birds (1,9). The availability and subsequent transmission of AIV to susceptible birds during the overwintering period would be increased by infected birds shedding large quantities of infectious virus (21), shedding virus for extended periods of up to 30 days (6), and persistence of virus in the environment for over 200 days.

Decreased persistence at higher water temperatures, which was observed in the 28 C treatment in the present study, may also indirectly enhance survival of viruses during winter. High temperatures may limit transmission among resident species on the wintering ground during summer. This would increase the proportion of susceptible birds in the population when conditions for transmission become favorable during winter.

Persistence in the environment during the overwintering period alsd provides a means for transmission of AIV among unassociated avian species and populations using distinct but proximal habitats. Recent research on AIV in Charadriiformes (10) suggests that these viruses may be maintained in separate cycles in different avian orders.

The possibility of long-term transmission between unassociated populations has direct application to prevention of disease in commercial poultry. Results from the present study and other reports (5,8,16,18) confirm that domestic poultry should not be allowed to share a common water source with free-living ducks. The extent of AIV persistence in water under natural conditions, however, is not known.

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