Survival of the Avian Influenza Virus (H6N2) After Land Disposal

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An integral component in preventing an avian influenza pandemic is containment and disposal of infected bird (poultry) carcasses. Disposal of carcasses in Subtitle D municipal solid waste (MSW) landfills may be an advantageous option due to their large capacities and facility distribution in the U.S. In this study, the survival of H6N2 avian influenza virus (AIV) was measured in a methanogenic landfill leachate and water as a function of temperature, conductivity, and pH. Elevated temperature and nonneutral pH resulted in the quickest inactivation times for AIV in both media, whereas conductivity did not have a significant influence on AIV survival. Media effects were significant and AIV inactivation in leachate was consistently the same or faster than AIV inactivation in water. Based on an initial titer of 10^5 $TCID_{50}$/mL, calculated inactivation times ranged from 30 days to greater than 600 days, indicating that AIV will remain infectious during and after waste disposal. Disposal of infected carcasses in a MSW landfill may be an appropriate option as inactivation times are within the design life of required barrier systems at Subtitle D landfills.

Introduction

Avian influenza virus (AIV), specifically the H5N1 serotype, is a growing public health concern. In 2008, reports of human AIV infection were reported in four countries (*1*). At present, AIV only effectively transfers from poultry to poultry; poultry to human transmission is ineffective (*2*) and human to human transmission is rare (*3*). In the future, it is possible that the species barrier may be overcome by the use of an intermediate host (*2*) and continued mutations could lead to effective human to human transmission of the virus (*3*).

An important component in the mitigation of an infectious animal disease outbreak is containment and disposal of infected carcasses. Disposal options for infected and potentially infected carcasses include burial, incineration, composting, rendering, lactic acid fermentation, alkaline hydrolysis, and anaerobic digestion (*4*). Of the viable disposal options, land disposal by on-site burial, composting, and off-site landfills are three management options recommended by the U.S. Environmental Protection Agency (*5*). To date, hundreds of millions of chicken and ducks have been killed by AIV or been culled in efforts to control disease outbreaks (*6*). Due to the dominance of municipal solid waste (MSW) landfills in the U.S. solid waste infrastructure, landfill disposal is a likely option for carcass disposal following a catastrophic animal mortality event. During a 2002 outbreak of AIV in Virginia, over 4 million poultry carcasses were disposed of in MSW landfills (*7*). To date, few studies have directly investigated the safety of AIV disposal in MSW landfills. Previously, studies of virus survival in landfills or landfill leachate have focused on enteric viruses (*8*−*12*). Carcass disposal can pose a potential public health risk because AIV can remain infectious in a dead host and viral transmission is possible during and after the disposal event (*13*), therefore, it is important to understand the fate of the virus during and after disposal.

The influenza virus is a negative-sense, enveloped, single-stranded ribonucleic acid (RNA) virus with a segmented genome (*14*), and environmental factors including temperature, pH, and the presence of heavy metals have all been demonstrated to influence AIV survival (*15*). Exposure to high temperatures has been repeatedly shown to inactivate AIV in egg fluids or egg and poultry products (*16*−*18*). The influence of pH on AIV inactivation has also been investigated, and pH-induced inactivation has been observed in both low pH (<6.4) and high pH (10 and 14) environments (*15, 19*−*24*). AIV inactivation has been observed in the presence of metals including copper (*25, 26*) and zinc (*26*).

A small number of studies have investigated AIV inactivation in environmental media including water (*15, 27, 28*), domestic wastewater, and drinking water (*29*). To date, no studies have evaluated AIV inactivation in media representative of a land-disposal scenario, such as landfill leachate.

The objectives of this study were to assess the survival of AIV in landfill leachate and the influence of environmental factors, specifically temperature, pH, and conductivity, on AIV survivability. To achieve this objective, a series of assays were performed to investigate survival of a low-pathogenic H6N2 AIV strain in a methanogenic landfill leachate and reverse osmosis (RO) water. The effect of pH, conductivity, and temperature on AIV survival in both media was evaluated. We hypothesized that AIV would survive in landfill leachate for shorter time periods when compared to RO water controls due to the higher conductivity of the leachate. In addition, we hypothesized that higher temperatures and nonneutral pH would result in decreased survival times in both media.

Materials and Methods

Virus Propagation and Titration. A frozen stock of A/CK/CA/101247/01 (H6N2) AIV was received from the National Veterinary Services Laboratories in Ames, IA. The AIV was stored at −80 °C, and thawed on ice prior to use. The virus was propagated in specific pathogen-free (SPF) embryonated chicken eggs (Charles River Laboratories, Storrs, CT) following protocols in Grimes (*30*). Allantoic fluid was harvested using a sterile pipet and tested for the presence of AIV using a hemagglutination assay (*30*). Allantoic fluid samples positive for infectious AIV were stored at −80 °C for use in survivability assays.

Leachate Characterization. A methanogenic landfill leachate was obtained from an operating landfill in Nebraska. The leachate was filtered through a 0.45-μm filter, autoclaved, and stored in the dark at 4 °C until use. The concentrations of anions (F, Cl, NO_3−N, Br, NO_2−N, SO_4, and orthophosphate), total organic carbon, cations (Ca, Mg, Na, and K), and metals (Zn, Fe, Cu, Mn) in the leachate were determined.

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TABLE 1. Leachate Characterization

parameter	value	units
pH	7.5	
conductivity	7.1	mS/cm
calcium	230.1	mg/L
magnesium	289.3	mg/L
sodium	397.1	mg/L
potassium	135.9	mg/L
zinc	27.0	μg/L
iron	598.0	μg/L
manganese	392.1	μg/L
copper	30.0	μg/L
fluoride	0.33	mg/L
chloride	709.30	mg/L
nitrite-n	<0.10	mg/L
bromide	3.11	mg/L
nitrate-N	<0.10	mg/L
ortophosphate-P	<0.10	mg/L
sulfate	1.80	mg/L
TOC	147	mg C/L

Inorganic anions were determined by ion chromatography using a Dionex ICS-90 system with AS14 ion exchange column. Total organic carbon (TOC) was determined by persulfate oxidation infrared detection using an OI Corp model 1010 carbon analyzer. Cations and metals were determined using a GVI Platform XS inductively coupled plasma-mass spectrometer (ICP-MS). Leachate characterization data are presented in Table 1.

Survivability Assay. Survivability assays were conducted to evaluate the effect of three factors on AIV survival: temperature, pH, and conductivity. For temperature assays, equal parts allantoic fluid containing infectious virus and leachate or RO water were mixed together in a 1.5 mL microcentrifuge tube (total sample volume of 900 μL) and incubated in a water bath at the temperature of interest (4, 21, 37, or 60 °C). The final mixture was at pH 8. In pH assays, mixtures of allantoic fluid and water or leachate were prepared as described above, but the pH was adjusted to 4 or 6 using 6 M HCl. For conductivity assays, the initial conductivity of the allantoic fluid/leachate or allantoic fluid/water samples was 9 mS/cm and was adjusted using either NaCl (final conductivity of 30 mS/cm) or RO water (final conductivity of 4 mS/cm). This conductivity range was selected to replicate the range of conductivity values observed in landfill leachate, which have been reported to range from 2.5 to 35 mS/cm (*31*). All pH and conductivity assays were incubated at 21 °C. In all experiments, samples were sacrificed in triplicate at 0, 0.5, 1, 2, 4, 7, 14, 28, and 56 days for analysis by viral titration in cell culture. Each assay was performed two separate times.

Cell Culture Titration. Madin−Darby canine kidney (MDCK) cells were purchased from American type Culture Collection in Manassas, VA. Cell culture was established from frozen stock as monolayers in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2. An initial leachate toxicity assay was performed for MDCK cells prior to viral titration and cytotoxicity was observed only at a leachate: DMEM dilution ratio of 10:1.

Ten-fold serially diluted AIV samples were prepared for the viral titration assay. AIV samples were removed from storage at −80 °C, thawed on ice, and DMEM supplemented with 1.5 mg/L TPCK-treated trypsin (Worthington Biochemical Corporation, Lakewood, NJ), 0.2% fungizone, and 0.25% penicillin/streptomycin (Invitrogen, Carlsbad, CA) was added to each tube. A negative control containing only supplemented DMEM was also titrated.

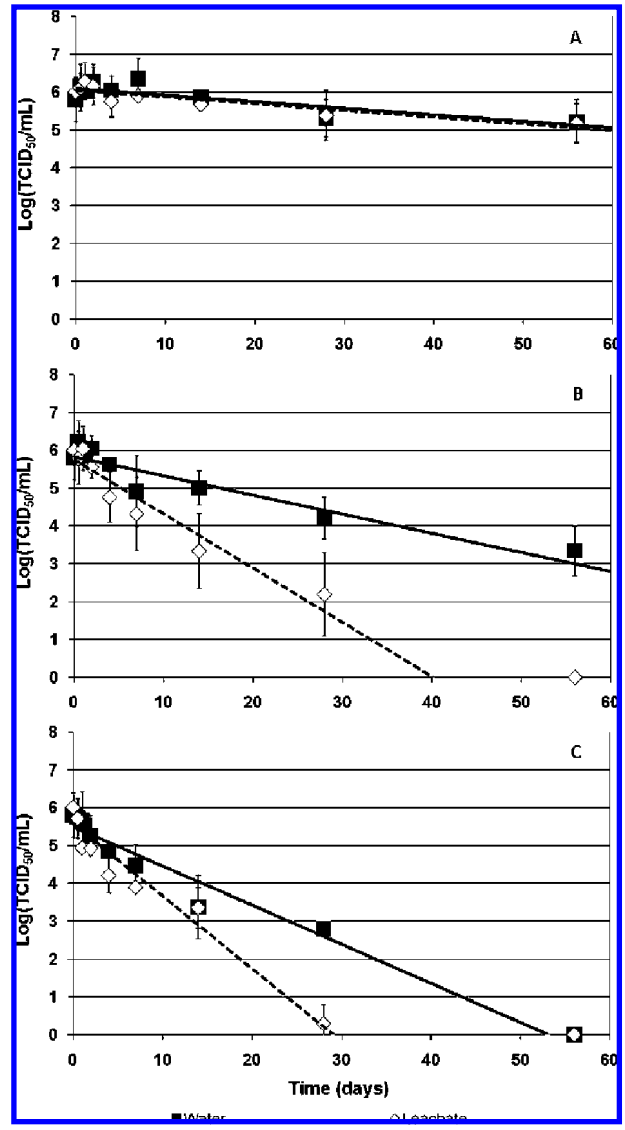

FIGURE 1. AIV survival in water and leachate as a function of temperature at 4 °C (Panel A); 21 °C (Panel B); and 37 °C (Panel C). Error bars represent ± 1 standard deviation.

MDCK cells were added to a 96 well plate and incubated 24 h prior to inoculation in DMEM supplemented with 3% FBS, 0.15% fungizone, and 0.15% gentamicin. The media was removed from the 96-well plates and each well was washed three times with 100 μL of CMF-PBS containing 1.5 mg/L TPCK-treated trypsin (*32*). Virus samples were serially diluted 10-fold, and 50 μL of each dilution was used to infect cells at eight wells per dilution. The plates were incubated for one hour at 37 °C in a humidified incubator with 5% CO_2. After incubation, 100 μL of DMEM supplemented with 0.45% FBS, 1.5 mg/L TPCK-treated trypsin, 0.2% fungizone, and 0.25% penicillin/streptomycin was added to each well. The plates were incubated for 72 h at 37 °C in a humidified incubator with 5% CO_2. Following incubation, the MDCK cells were evaluated for cytopathic effects using an indirect enzyme-linked immunoassay following a modified version of the protocol presented by Brodersen and Kelling (*33*). Virus titer was calculated using the method of Reed and Muench (*34*).

Statistical analyses were completed with Graphpad Prism software (Graphpad Software, Inc., La Jolla, CA 92037). Slopes of the inactivation models were determined using linear regression and the slopes were compared with a two tailed *t* test with an α value of 0.05.

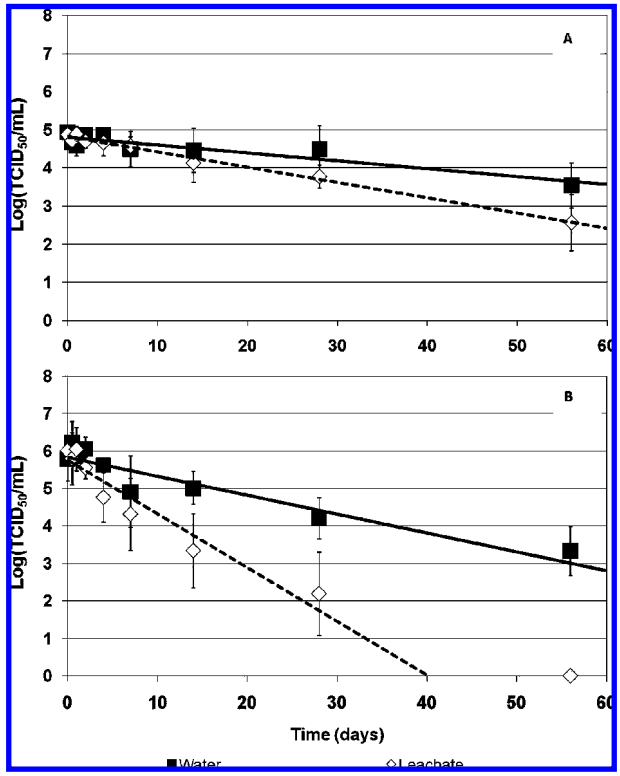

FIGURE 2. AIV survival in water and leachate as a function of pH at pH 6 (Panel A) and pH 8 (Panel B). pH 4 data not shown. Error bars represent ± 1 standard deviation.

Results

Assay results of AIV survival in water and leachate as a function of temperature are presented in Figure 1. No quantifiable virus was detected in any sample from the 60 °C assays (data not shown). Temperature proved to have a significant ($p < 0.01$) effect on AIV inactivation, with higher temperature resulting in an increased inactivation rate for both media.

At 4 °C, there was no significant difference in AIV inactivation rates between water and leachate (Figure 1, Panel A). At 21 and 37 °C, AIV inactivation rates in leachate were significantly faster than inactivation rates in water ($p < 0.01$).

Results for survivability assays conducted at variable pH are shown in Figure 2. At pH 4, no infectious virus was recovered at any time point, and sampling was discontinued after 14 days (data not shown). Significantly higher inactivation rates were observed in both media at pH 8 (Figure 2, panel B) compared to data obtained at pH 6 (Figure 2, panel A) at a p value of <0.01. For both pH levels, AIV inactivation rates were significantly greater in leachate than in water ($p < 0.01$).

The results of conductivity assays are shown in Figure 3. In water at 4 mS/cm (Figure 3, Panel A), AIV inactivation was not significantly different from water at 9 mS/cm (Figure 3, Panel B) or 30 mS/cm (Figure 3, Panel C). In leachate, conductivity did have a significant effect on AIV inactivation rates. AIV inactivation rates at a conductivity of 4 mS/cm were significantly lower than 9 mS/cm ($p < 0.01$) but not significantly different than 30 mS/cm. AIV inactivation in leachate at a conductivity level of 9 mS/cm was significantly higher than the calculated inactivation rate at 30 mS/cm ($p < 0.01$). Between the two media, the only significant difference in inactivation rates were found in water and leachate at 9 mS/cm ($p < 0.01$).

Calculated inactivation rates and R^2 values for linear inactivation model fits are found in Table 2. Calculated R^2 values for linear inactivation ranged from 0.30 to 0.92, which compare favorably with previously reported values of 0.15 (*15*) and 0.12 to 0.94 (*28*) for linear models of AIV survivability.

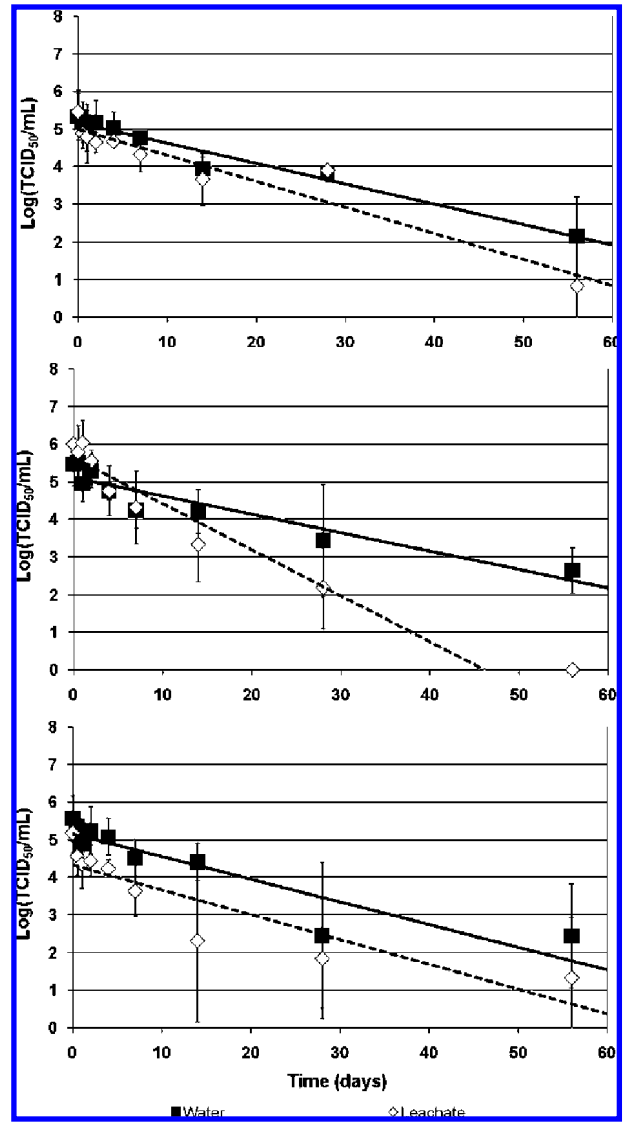

FIGURE 3. AIV survival in water and leachate as a function of conductivity at 4 mS/cm (Panel A); 9 mS/cm (Panel B); and 30 mS/cm (Panel C). Error bars represent ± 1 standard deviation.

Discussion

The results from this study represent the first published data investigating AIV survival in a land disposal scenario and confirm previously reported results that AIV is heat sensitive (*15*−*18, 27, 28, 35*). In this study, the influence of temperature on virus inactivation in both leachate and water was found to be statistically significant with higher temperatures resulting in faster inactivation rates. At 4 °C, the inactivation rate determined from data collected in this study (0.0175 d^{-1}) is higher than the previously reported value of 0.004 d^{-1} for AIV inactivation in water at 4 °C (*15*). At 21 °C, the AIV inactivation rates in water and leachate from this study are within the range of inactivation rates previously reported for water at 17 °C, which vary from 0.009 to 0.410 d^{-1} (*15, 28*). Similarly, inactivation data obtained in this study at 37 °C are comparable to results previously reported for AIV inactivation in water at 28 °C

TABLE 2. Inactivation Rates and Model Fits for Experimental Survivability Data

medium	environmental variable	parameter level	inactivation rate (d^{-1})	R^2	estimated persistence[a] (d)
water	temperature	4 °C	0.02 ± 0.005	0.30	184−696
		21 °C	0.05 ± 0.007	0.64	78−136
		37 °C	0.10 ± 0.007	0.87	43−56
	pH	4	NA[b]	NA	NA
		6	0.02 ± 0.004	0.50	173−413
		8	0.05 ± 0.007	0.64	78−136
	conductivity	4 mS/cm	0.05 ± 0.006	0.79	76−117
		9 mS/cm	0.05 ± 0.007	0.64	78−148
		30 mS/cm	0.06 ± 0.011	0.56	61−175
leachate	temperature	4 °C	0.02 ± 0.004	0.38	192−526
		21 °C	0.14 ± 0.014	0.75	29−44
		37 °C	0.19 ± 0.011	0.92	23−29
	pH	4	NA	NA	NA
		6	0.04 ± 0.003	0.85	106−151
		8	0.12 ± 0.011	0.75	29−44
	conductivity	4 mS/cm	0.07 ± 0.007	0.78	59−93
		9 mS/cm	0.14 ± 0.014	0.75	29−44
		30 mS/cm	0.07 ± 0.013	0.49	54−129

[a] Calculated for an initial titer of 10^5 $TCID_{50}$/mL. Average inactivation rates ± 1 SD were used to develop persistence estimates. [b] Data not available.

(0.05−0.59 d^{-1}) reported in Stallknecht et al. (*15*) and Brown et al. (*28*). Care should be taken when comparing results from previous studies as differences in serotype and experimental design may account for the observed variation in the inactivation rates (*36*).

AIV inactivation has been previously demonstrated to be pH dependent and the results of this study are in agreement with the results from previous research (*15, 24, 35*). Scholtissek (*35*) showed that below pH 4.6, none of the tested AIV strains remained infectious. In this study, samples obtained from leachate and water assays at pH 4 resulted in nondetectable titers. AIV was determined to be most stable at pH 6 and was less stable at pH 8. It should be noted that pH treatments investigated in this study were not within the range determined to be optimal for AIV inactivation (pH 7.2−7.4).

The influence of conductivity on AIV survival was less clear than the other variables examined. It is possible that the composition of the media is more important in predicting AIV survival than the overall conductivity of the media. For example, previous research has shown that metals can be effective antiviral agents (*25, 26*).

AIV inactivation rates calculated in this study yield theoretical persistence times ranging from approximately 30 to >600 days (based on initial titer of 10^5 tissue culture infection $dose_{50}$ ($TCID_{50}$)). This indicates that AIV could remain infectious both during and after waste placement. Variables evaluated in this study that had the most influence on AIV survival were elevated temperature (37−60 °C) and nonneutral pH. Based on data collected in this study, codisposal of infected carcasses with an acidic or alkaline material might be suggested to alter waste pH. Other recommended practices might include covering the carcasses as quickly as possible and enhancing waste decomposition in the cell containing infected carcasses to increase temperatures within the landfill. In comparing data obtained in this study on AIV inactivation in landfill leachate and water, calculated inactivation rates in leachate were as much as 2 times faster than inactivation rates in water. Thus, extrapolating information on AIV survival in a landfill based on published data in water may result in conservative estimates of AIV survival. However, it should be noted that certain factors that were not evaluated as part of this study, such as the presence of solids or microbial populations in landfill leachate could potentially influence AIV survival in landfills. Data obtained from this study indicate that landfilling is an appropriate method for disposal of carcasses infected with AIV. Inactivation times calculated in this study (<2 yrs) were well within the design lifetimes of composite barrier and gas and leachate collection systems at typical Subtitle D landfills.

Acknowledgments

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