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Survivability of Eurasian H5N1 Highly Pathogenic Avian Influenza Viruses in Water Varies Between Strains

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SUMMARY.

Aquatic habitats play a critical role in the transmission and maintenance of low-pathogenic avian influenza (LPAI) viruses in wild waterfowl; however, the importance of these environments in the ecology of H5N1 highly pathogenic avian influenza (HPAI) viruses is unknown. In laboratory-based studies, LPAI viruses can remain infective for extended durations (months) in water, but the persistence is strongly dependent on water conditions (temperature, salinity, pH) and virus strain. Little is known about the stability of H5N1 HPAI viruses in water. With the use of an established laboratory model system, the persistence of 11 strains of H5N1 HPAI virus was measured in buffered distilled water (pH 7.2) at two temperatures (17 and 28 C) and three salinities (0, 15,000, and 30,000 ppm). There was extensive variation between the 11 H5N1 HPAI virus strains in the overall stability in water, with a range similar to that which has been reported for wild-bird–origin LPAI viruses. The H5N1 HPAI virus strains responded similarly to different water temperatures and salinities, with all viruses being most stable at colder temperatures and fresh to brackish salinities. These results indicate that the overall stability and response of H5N1 HPAI viruses in water is similar to LPAI viruses, and suggest there has been no increase or loss of environmental survivability in H5N1 HPAI viruses.

RESUMEN.

La supervivencia en el agua de virus euroasiáticos de la influenza aviar altamente patógenos H5N1 varía entre las cepas.

Los hábitats acuáticos juegan un papel fundamental en la transmisión y en el mantenimiento de la influenza aviar de baja patogenicidad (con las siglas en inglés LPAI) en aves acuáticas silvestres, sin embargo, la importancia de estos ambientes en la ecología del virus H5N1 de la influenza aviar altamente patógena (con las siglas en inglés HPAI) es desconocida. En estudios de laboratorio, los virus de baja patogenicidad pueden permanecer infectantes durante períodos prolongations (meses) en el agua, pero la persistencia depende fuertemente de las condiciones del

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agua (temperatura, salinidad, pH) y la cepa del virus. Poco se conoce acerca de la estabilidad de los virus de influenza aviar altamente patógenos H5N1 en el agua. Con el uso de un sistema modelo de laboratorio establecido, se midió la persistencia de 11 cepas de virus de influenza altamente patógenos H5N1 en agua destilada amortiguada (pH 7.2) con dos temperaturas (17 y 28 C) y con tres niveles de salinidad (0, 15,000 y 30,000 ppm). Hubo una amplia variación entre las 11 cepas de virus H5N1 de la influenza aviar altamente patógena con respecto a la estabilidad general en el agua, con un rango similar a lo que se ha reportado con los virus de baja patogenicidad de aves silvestres. Las cepas del virus H5N1 de la influenza aviar altamente patógenos respondieron de manera similar a las diferentes temperaturas del agua y salinidad, y se observó que todos los virus eran más estables a temperaturas más frías y en agua dulce en comparación con salinidades salobres. Estos resultados indican que la estabilidad global y la respuesta de los virus H5N1 de la influenza aviar de alta patogenicidad en el agua es similar a los virus de baja patogenicidad y sugieren que no ha habido un aumento o pérdida de la capacidad de supervivencia en el medio ambiente con los virus altamente patógenos H5N1.

Keywords

avian influenza; H5N1; highly pathogenic; persistence; survivability; water

Avian influenza (AI) viruses are maintained in wild waterfowl populations through an indirect fecal–oral route involving contaminated water on shared aquatic habitats (10). Virus-contaminated waterfowl habitats also are a source for virus spillover into aberrant hosts, such as domestic poultry (9). Several laboratory-based studies have evaluated the stability of wild-bird–origin low pathogenic (LP) AI viruses in water (2,4,13,14,15,19), and collectively, these studies have shown there is a wide range of environmental fitness phenotypes among LPAI viruses maintained in wild birds, including strains of the same hemagglutinin (HA) subtype (2,13). Overall, wild-bird–origin LPAI viruses are relatively stable in water, which presumably facilitates environmental transmission; however, the persistence of these viruses is highly dependent on basic water conditions, including pH, temperature, salinity, and microbial flora (2,15,18).

Since its emergence on a domestic goose farm in the Guangdong province of China in 1996 (17), H5N1 highly pathogenic (HP) AI viruses have spread throughout Asia and into Africa and Europe, resulting in over 380 human fatalities and unprecedented losses to the poultry industry in the region (5,8,24). Over the last 17 yr, H5N1 HPAI virus has evolved into a wide diversity of strains that are genetically and biologically distinct. Genetically, the viruses are categorized based on the HA gene into different clades and subclades (23). Biologically, different strains of H5N1 HPAI virus vary in host range and virulence for different avian and/or mammalian hosts (1).

H5N1 HPAI viruses are established in several regions of Asia and Africa. A key factor thought to contribute to the endemicity in parts of Southeast Asia is the large domestic duck farming industry and, specifically, the practice of free-grazing ducks (7). Defining the range of environmental fitness among H5N1 HPAI viruses relative to wild-bird–origin LPAI viruses is a critical step for understanding the risks for H5N1 HPAI viral transmission

within or between domestic and wild waterfowl populations. Multiple laboratory-based trials have examined the persistence of H5N1 HPAI viruses in water (4,6,11); however, the vast majority of these studies have included a single strain or very limited numbers of strains, and little comparative data exist on environmental survivability among H5N1 HPAI viruses. To provide basic insights into this topic, we utilized a standardized laboratory-based model system to measure the persistence of different H5N1 HPAI virus strains in water under varying temperatures and salinities.

MATERIALS AND METHODS

The following 11 H5N1 HPAI virus strains representing six different clades/subclades were used in the experimental persistence trials: A/environment (goose pen)/Hong Kong/485.3/2000 (Subclade 0), A/chicken/Hong Kong/220/1997 (Subclade 0), A/goose/Vietnam/113/2001 (Subclade 0), A/Vietnam/1203/2004 (Subclade 1), A/egret/Hong Kong/757.2/2002 (Subclade 1), A/duck/bac lieu/NCVD 07-09/2007 (Subclade 1), A/West Java/PWT-WIJ/2006 (Subclade 2.1), A/chicken/Nigeria/-228-10/2006 (Subclade 2.2), A/duck/Vietnam/201/2006 (Subclade 2.3), A/muscovy/Ha Nam/NCVD 07-84/2007 (Subclade 2.3), and A/chicken/Korea/ES/2003 (Subclade 2.5). Working stocks for each strain were propagated in 9-to-11-day-old, specific pathogen-free embryonating chicken eggs (21), and resulting viral-infected amnioallantoic fluid (AAF) was titrated in Madin Darby canine kidney (MDCK) cells (2). Stock titers were calculated with the use of the methods described by Reed and Muench (16). All trials were performed with isolates after the second or third passage in eggs. All laboratory work and persistence trials were conducted under biosafety level 3 enhanced containment at the Southeast Poultry Research Laboratory, Agricultural Research Service, United States Department of Agriculture.

Experimental persistence trials were performed following a previously published study design and protocol (2). Briefly, the persistence of each virus strain was determined in distilled water that was buffered with 10 mM HEPES and adjusted to a pH of 7.2 with 1 N HCl and one of three salinity levels (0, 15,000, and 30,000 parts per million [ppm]) with sea salt. For each virus strain, infective AAF was diluted 1:100 in each of the modified water samples, vortexed, and separated into 2.0-ml aliquots in 5.0-ml polystyrene tubes. The virus-infected water samples for each of the three salinity treatments were divided and placed in incubators set at 17 and 28 C. The viral-infected water samples held at 28 C were sampled 0, 7, 14, and 21 days postinoculation (DPI). The samples held at 17 C were sampled at 0 DPI and at five additional time points, approximately 1–2 wk apart, until 63 DPI. For sampling, duplicate 0.5-ml samples of viral-infected water were diluted 1:1 by addition of 0.5 ml of 2X, serum-free, Eagle's minimum essential medium, and titrated on MDCK cells with standard techniques (2). Results from duplicate titrations were averaged and $\log_{10}$ transformed prior to analyses. Linear regressions were used to calculate the log reduction times (Rt) for each virus strain and water-treatment trial, which is the average number of days required for a 90% reduction in infectivity (i.e., time required for a decrease of the viral titer by 1 $\log_{10}$ median tissue culture infectious dose [$TCID_{50}$]/ml) (18). The calculated Rt values for all strains of H5N1 HPAI virus were compared to wild-bird–origin LPAI viruses that were previously tested under similar water conditions with the use of the same methodology (2,4).

RESULTS AND DISCUSSION

Consistent with previous studies (2,4,18), the loss of infectivity of H5N1 HPAI viruses in water over time was dependent on conditions. Both temperature and salinity strongly influenced viral persistence, with all strains being most stable at the colder water temperature and at freshwater or brackish salinities (Table 1). The rate at which H5N1 HPAI viruses lost infectivity varied between strains, as did the individual viral responses to temperature and salinity (Fig. 1). Although there was not adequate representation of H5N1 HPAI virus strains to evaluate the effect of HA subclade on environmental fitness, our results suggest there is extensive variability within some subclades (i.e., subclade 1 strains A/duck/bac lieu/NCVD 07–09/2007 and A/egret/Hong Kong/757.2/2002 were the least and most persistent strains at 17 C and 0 ppm, respectively). Consequently, the HA subclade may not be a good predictor of environmental phenotype; however, additional studies are needed to evaluate this further.

The range of Rt values among H5N1 HPAI virus strains reported herein and the response trends to water temperature and salinity were consistent with results for wild-bird–origin LPAI viruses tested under similar conditions (Table 2) (2,4,13). A previous study evaluated the persistence of two H5N1 HPAI virus strains (A/duck meat/Anyang/AVL-1/01 and A/whooper swan/Mongolia/244/05) in water under identical conditions with the same laboratory model system (4). Overall, both strains had low Rt values relative to wild-bird–origin H5 LPAI viruses, suggesting these viruses had lost some degree of environmental fitness. However, relative to the 11 H5N1 HPAI virus strains tested in this current study and subsequently evaluated wild-bird–origin LPAI virus strains (2), the two former H5N1 HPAI virus strains (A/duck meat/Anyang/AVL-1/01 and A/whooper swan/Mongolia/244/05) had Rt values that fell within the range of H5N1 HPAI and LPAI viruses, although both were relatively low (Table 2). This would suggest that, in contrast to the conclusions of the earlier study (4), there is no apparent difference in survivability of H5N1 HPAI viruses relative to LPAI viruses and no evidence to indicate H5N1 HPAI viruses have evolved toward a reduced environmental fitness phenotype. Rather, with both HPAI and LPAI viruses, environmental fitness varies between strains and, to date, the viral factors that determine this fitness are not known. As the overall environmental fitness of a strain is not predictable, caution must be exercised when extrapolating results and conclusions generated from a limited number of strains to all H5N1 HPAI viruses.

Although these data suggest strains of H5N1 HPAI and wild-bird-origin LPAI viruses exhibit a similar potential for persistence in water, the involvement of aquatic environments in the transmission and maintenance of H5N1 HPAI viruses in domestic and/or wild aquatic birds is likely dependent on a variety of other factors relating to host, virus, and environment. In particular, existing experimental and field data indicate H5N1 HPAI viruses are preferentially excreted via the respiratory tract of waterfowl (3,12,20) in contrast to LPAI viruses, which are predominately excreted in the feces (22). Such a change in pattern of excretion likely has significant implications for the transmission of H5N1 HPAI virus within and between wild and domestic bird populations, as well as for the role that aquatic environments play in the ecology of this virus.

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Abbreviations:

AAF	amnioallantoic fluid
AI	avian influenza
DPI	days postinoculation
HA	hemagglutinin
HP	highly pathogenic
LP	low pathogenic
MDCK	Maden Darby canine kidney
ppm	parts per million
Rt	reduction time
$TCID_{50}$	median tissue culture infectious dose

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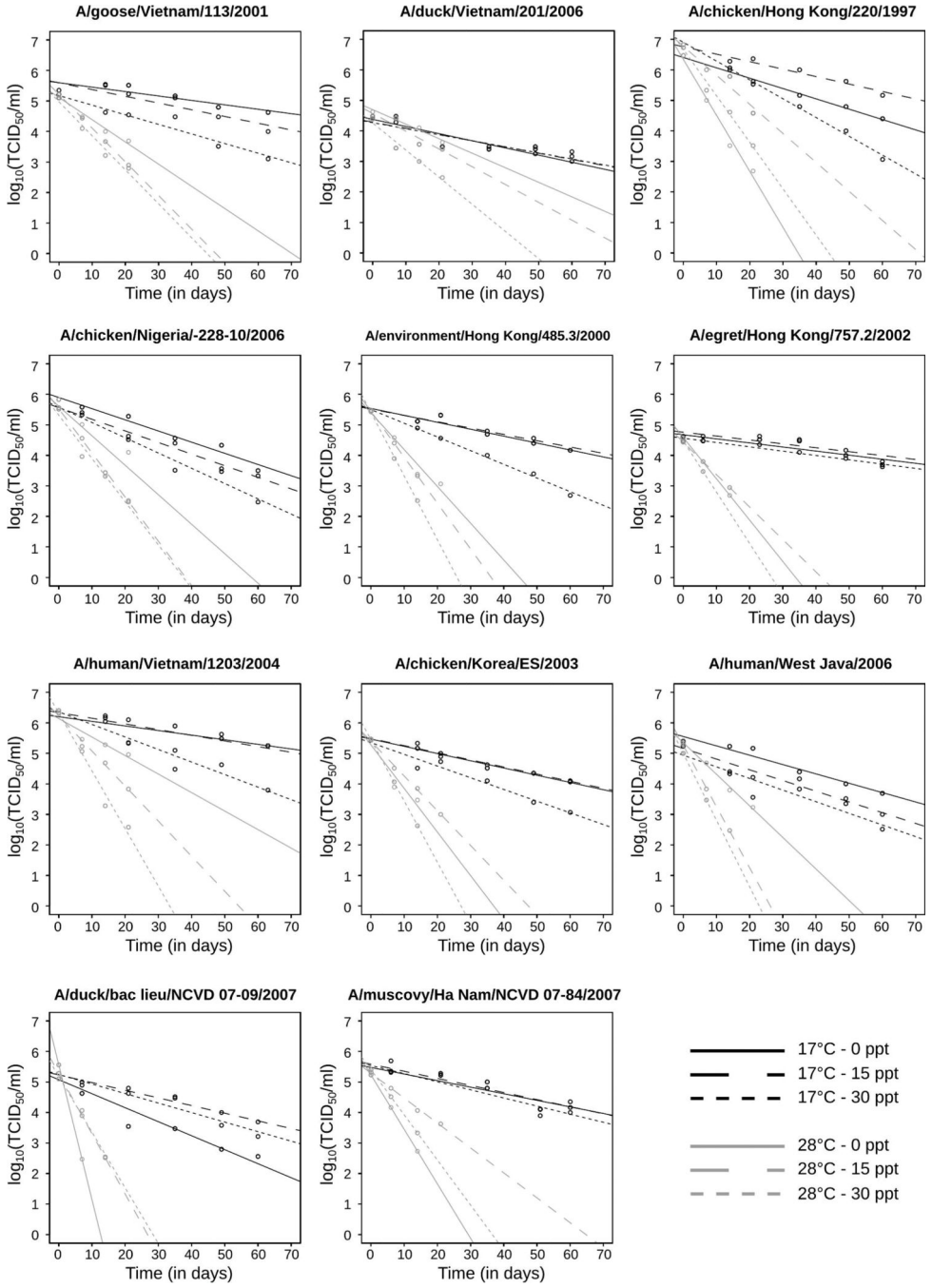

Fig. 1.

The infectivity of 11 strains of H5N1 highly pathogenic avian influenza virus in distilled water over time at two temperatures (17 and 28 C), three salinities (0, 15,000, and 30,000 ppm), and a pH of 7.2. Different shaded lines represent the least-squares regression lines for each temperature and salinity treatment.

Table 1.

Average virus $\log_{10}$ reduction times (Rt) for 11 strains of H5N1 highly pathogenic avian influenza virus in distilled water at two temperatures (17 and 28 C), three salinities (0, 15,000, and 30,000 ppm), and a pH of 7.2. The Rt values are the average number of days required for a decrease of viral titer by 1 $\log_{10}$ $TCID_{50}$/ml based on linear models. The hemagglutinin subclade for each virus strain is listed in parentheses behind the virus identification.

	0 ppm		15,000 ppm		30,000 ppm	
Virus	**17 C**	**28 C**	**17 C**	**28 C**	**17 C**	**28 C**
A/chicken/Hong Kong/220/1997 (0)	29	5	41	11	16	7
A/chicken/Korea/ES/2003 (2.5)	42	7	43	9	26	5
A/chicken/Nigeria/-228-10/2006 (2.2)	27	10	26	7	20	7
A/duck/bac lieu/NCVD 07-09/2007 (1)	22	2	40	5	32	5
A/duck/Vietnam/201/2006 (2.3)	43	21	49	17	50	11
A/egret/Hong Kong/757.2/2002 (1)	75	7	78	9	71	6
A/environment/Hong Kong/485.3/2000 (0)	44	8	48	7	22	5
A/goose/Vietnam/113/2001 (0)	69	14	45	9	32	9
A/Vietnam/1203/2004 (1)	66	16	54	9	24	5
A/West Java//PWT-WIJ/2006 (2.1)	33	10	28	5	26	5
A/muscovy/Ha Nam/NCVD 07-84/2007 (2.3)	46	6	43	12	38	7

Table 2.

Average virus $\log_{10}$ reduction times (Rt) for wild-bird-origin low pathogenic avian influenza (LPAI) viruses (roman type) and H5N1 highly pathogenic avian influenza (HPAI) viruses (italic type) in distilled water at two temperatures (17 and 28 C), three salinities (0, 15,000, and 30,000 ppm), and a pH of 7.2. The Rt values are the average number of days required for a decrease of viral titer by 1 $\log_{10}$ $TCID_{50}$/ml based on linear models. Viruses are listed from top to bottom in relative decreasing persistence in water. The Rt values for all LPAI viruses and two H5N1 HPAI virus strains (A/duck meat/Anyang/AVL-1/01 and A/whooper swan/Mongolia/244/05) were obtained from published studies conducted with the use of the same laboratory model system (references are provided below the table).

		17 C, pH 7.2			28 C, pH 7.2		
Virus	**H5N1 HPAI subclade**	**0 ppm**	**15,000 ppm**	**30,000 ppm**	**0 ppm**	**15,000 ppm**	**30,000 ppm**
A/laughing gull/DE/AI00-2455/00 (H7N3)[B]	NA[A]	111	24	18	11	4	4
A/N.Shoveler/NC/1523546/05 (H7N6)[C]	NA	79	43	27	5	7	3
A/egret/Hong Kong/757.2/02 (H5N1)	*1*	*75*	*78*	*70*	*7*	*9*	*6*
A/mallard/MN/182742/98 (H5N2)[B]	NA	71	21	11	20	10	5
A/goose/Vietnam/113/01 (H5N1)	*0*	*69*	*45*	*32*	*14*	*9*	*9*
A/ruddy turnstone/NJ/828219/01 (H5N7)[B]	NA	38	63	14	9	8	4
A/Vietnam/1203/04 (H5N1)	*1*	*62*	*51*	*25*	*17*	*9*	*5*
A/mallard/MN/355790/00 (H5N3)[B]	NA	53	56	19	14	9	4
A/blue-winged teal/TX/421717/01 (H2N4)[C]	NA	37	53	26	8	7	4
A/green-winged teal/LA/213GW/87 (H1N1)[C]	NA	52	37	12	6	4	3
A/environment/Hong Kong/485.3/00 (H5N1)	*0*	*44*	*48*	*22*	*8*	*7*	*5*
A/ruddy turnstone/NJ/828227/01 (H5N8)[B]	NA	48	21	10	6	7	7
A/muscovy/Ha Nam/NCVD 07-84/07 (H5N1)	*2.3*	*47*	*45*	*37*	*6*	*12*	*6*
A/chicken/Hong Kong/220/97 (H5N1)	*0*	*29*	*46*	*17*	*5*	*11*	*7*
A/duck/Vietnam/201/06 (H5N1)	*2.3*	*43*	*45*	*40*	*21*	*16*	*10*
A/chicken/Korea/ES/03 (H5N1)	*2.5*	*42*	*43*	*26*	*7*	*9*	*5*
A/mallard/MN/199057/99 (H4N6)[C]	NA	25	41	27	10	5	5
A/duck/bac lieu/NCVD 07-09/07 (H5N1)	*1*	*22*	*40*	*32*	*2*	*5*	*6*
A/mallard/MN/182761/98 (H7N3)[B]	NA	36	22	15	12	5	5
A/mallard/MN/346250/00 (H5N2)[C]	NA	21	35	11	3	4	2

Virus	H5N1 HPAI subclade	17 C, pH 7.2			28 C, pH 7.2		
		0 ppm	15,000 ppm	30,000 ppm	0 ppm	15,000 ppm	30,000 ppm
A/ruddy turnstone/NJ/1016409/03 (H9N2)[C]	NA	34	22	34	10	5	4
A/West Java/PWT-WIJ/06 (H5N1)	*2.1*	*33*	*28*	*26*	*10*	*5*	*5*
A/northern pintail/TX/421716/01 (H8N4)[C]	NA	32	32	10	10	3	2
A/mallard/MN/199036/99 (H3N2)[C]	NA	32	32	12	6	5	3
A/ruddy turnstone/DE/650635/02 (H7N3)[B]	NA	32	23	29	4	4	4
A/duck meat/Anyang/AVL-1/01 (H5N1)[B]	*1*	*16*	*30*	*19*	*5*	*5*	*3*
A/blue-winged teal/TX/578597/02 (H7N4)[B]	NA	29	22	18	10	3	5
A/chicken/Nigeria/-228-10/06 (H5N1)	*2.2*	*27*	*26*	*22*	*10*	*7*	*7*
A/whooper swan/Mongolia/244/05 (H5N1)[B]	*2.2*	*26*	*14*	*14*	*4*	*5*	*3*
A/mallard/MN/355788/00 (H12N5)[C]	NA	21	19	14	7	4	2
A/red knot/DE/AI00-1329/00 (H10N7)[C]	NA	16	21	16	8	2	3
A/ring-billed gull/421733/01 (H6N4)[C]	NA	12	12	7	3	4	4
A/dunlin/DE/AI00-1459/00 (H11N6)[C]	NA	9	9	9	4	4	1

[A] Subclade designation not applicable for LPAI viruses.

[B] Reference 4.

[C] Reference 2.