

# Virus Shedding and Potential for Interspecies Waterborne Transmission of Highly Pathogenic H5N1 Influenza Virus in Sparrows and Chickens<sup>∇</sup>

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**To elucidate the role of sparrows as intermediate hosts of highly pathogenic avian influenza H5N1 viruses, we assessed shedding and interspecies waterborne transmission of A/duck/Laos/25/06 in sparrows and chickens. Inoculated birds shed virus at high titers from the oropharynx and cloaca, and infection was fatal. Waterborne transmission from inoculated sparrows to contact chickens was absent, while 25% of sparrows were infected via waterborne transmission from chickens. The viral shedding and susceptibility to infection we observed in sparrows, coupled with their presence in poultry houses, could facilitate virus spread among poultry and wild birds in the face of an H5N1 influenza virus outbreak.**

The H5N1 influenza A viruses remain a major global concern because of their rapid evolution, genetic diversity, broad host range, and ongoing circulation in wild and domestic birds. H5N1 influenza viruses have swept through poultry flocks across Asia and have spread westward through Eastern Europe to India and Africa since 2003 (1). Sixty-two countries have reported H5N1 influenza virus in domestic poultry/wild birds during the time period 2003 to 2009 ([http://www.oie.int/eng/info\\_ev/en\\_AI\\_factoids\\_2.htm](http://www.oie.int/eng/info_ev/en_AI_factoids_2.htm)), and to date, more than 400 human infections have been documented in 16 countries, with a mortality rate of ~61% ([http://www.who.int/csr/disease/avian\\_influenza/country/cases\\_table\\_2009\\_05\\_22/en/index.html](http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_05_22/en/index.html)). Most human cases of H5N1 influenza have occurred after contact with infected poultry (13).

Some of the more recent isolates of H5N1 highly pathogenic avian influenza (HPAI) virus do not cause overt disease in certain species of domestic and wild ducks; however, these viruses are 100% lethal to chickens and gallinaceous poultry. Because of ducks' ability to "silently" spread H5N1 HPAI virus and their unresolved role as a reservoir, they are the focus of much research (5, 6, 11). In contrast, the possible role of passerine birds has received little attention, despite their widespread interaction with poultry at many sites worldwide ([http://www.searo.who.int/LinkFiles/Publication\\_PHI-prevention-control-AI.pdf](http://www.searo.who.int/LinkFiles/Publication_PHI-prevention-control-AI.pdf)). The order *Passeriformes* includes more than half of all bird species, including sparrows. Since 2001, several outbreaks of H5N1 influenza virus infection have been reported in passerine birds in eastern Asia, often near infected poultry farms (15). Interestingly, the only confirmed presence of asymptomatic infection with HPAI H5N1 in wild birds was in tree sparrows in Henan Province, China. Both tree and house sparrows (*Passer montanus* and *Passer domesticus*, re-

spectively) are members of the Old World sparrow family *Passeridae*, and in fact, the tree sparrow was not recognized as a species separate from that of the house sparrow until 1713 (<http://www.arkive.org/tree-sparrow/passers-montanus/info.html?displayMode=factsheet>). The four avian influenza virus isolates obtained from these asymptomatic infections were of the A/Goose/Guangdong/1/96 lineage and were highly pathogenic to experimentally infected chickens (4, 8).

Under experimental conditions, passerine species have shown varied susceptibility to HPAI H5N1 viruses. Among sparrows, starlings, and pigeons inoculated with HPAI H5N1 virus isolates, only sparrows experienced lethal infection, and transmission to contact birds was extremely rare (2). Similarly, in sparrows and starlings inoculated with the H5N1 HPAI A/chicken/Hong Kong/220/97 virus, clinical signs were observed only in sparrows, and no deaths occurred (9).

To assess the duration and routes of virus shedding and the waterborne virus transmission of HPAI H5N1 virus between sparrows and chickens, we inoculated groups of birds with A/duck/Laos/25/06, which had caused extremely high morbidity and mortality in domestic ducks (7) and was highly pathogenic to chickens, geese, and quail (J.-K. Kim and R. G. Webster, unpublished data). The virus was obtained from our collaborators in Lao People's Democratic Republic and was grown in the allantoic cavities of 10-day-old embryonated chicken eggs (eggs) for 36 to 48 h at 35°C. The allantoic fluid was harvested, titrated (50% egg infective dose [EID<sub>50</sub>] per milliliter), and stored at -80°C. All experiments were approved by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention and were performed in biosafety level 3+ facilities at St. Jude Children's Research Hospital. Wild house sparrows (*Passer domesticus*) were captured locally (Memphis, TN), and specific-pathogen-free outbred White Leghorn chickens (*Gallus domesticus*) were purchased from Charles River Laboratories (North Franklin, CT). All animal experiments were approved by the St. Jude Animal Care and Use Committee and complied with the pol-

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TABLE 1. Transmission rates, mortality rates, and mean peak titers of A/duck/Laos/25/06 (H5N1) virus in inoculated and contact birds

Group	Type of bird (no.)	Infection route	Transmission rate (%)	Mortality rate (%)	Mean peak virus titer (log <sub>10</sub> EID <sub>50</sub> /ml) <sup>a</sup>	
					Oropharyngeal	Cloacal
1	Chickens (5)	Inoculation	100	100	6.45	5.95
	Sparrows (8)	Contact <sup>b</sup>	25	25	3.88	4.25
2	Sparrows (8)	Inoculation	100	100	4.56	4.03
	Chickens (5)	Contact <sup>c</sup>	0	0	NA	NA

<sup>a</sup> Swab samples were taken daily after virus inoculation and after introduction of infective water to contacts. NA, not applicable.

<sup>b</sup> Contact sparrows were given 1 liter of water containing 1 ml resuspended fecal material (10<sup>6.5</sup> EID<sub>50</sub>/ml) obtained from infected chickens on day 2 p.i.

<sup>c</sup> Contact chickens were given 3 liters of a 1:3 dilution of water from the trough used by inoculated sparrows.

icies of the National Institutes of Health and the Animal Welfare Act.

Before inoculation, oropharyngeal and cloacal swabs were collected from sparrows, and baseline blood samples were collected from chickens to exclude preexisting H5N1 influenza virus infection. Eight sparrows were inoculated intranasally with 10<sup>6</sup> EID<sub>50</sub> of virus in a volume of 100 μl, and five chickens were inoculated with 10<sup>2</sup> EID<sub>50</sub> of virus in a volume of 1 ml (0.5 ml intranasally, 0.5 ml intratracheally, and 1 drop per eye). All birds in each experimental group were housed in a single cage. Inoculated sparrows were provided with 1 liter of water in a shallow stainless steel pan at the bottom of the cage, and chickens were given 3 liters of water in a trough inside the cage. Twenty-four hours after inoculation, 1 liter of water was removed from the inoculated chickens' cage and placed undiluted in a cage housing 8 contact sparrows; similarly, 1 liter of water was taken from the inoculated sparrows' cage, mixed with 2 liters of fresh water, and placed in a cage housing 5 contact chickens. Clinical disease signs, including depression, huddling at the cage bottom, and ruffled feathers, were monitored through daily observation, and oropharyngeal and cloacal swabs obtained from all birds were collected daily for 14 days. Swab samples were titrated in eggs and expressed as log<sub>10</sub> EID<sub>50</sub>/ml (10). The lower limit of detection was 0.75 log<sub>10</sub> EID<sub>50</sub>/ml.

Blood samples were taken from all surviving contact birds on day 14 of the study. Sera were treated with a receptor-destroying enzyme (Denka Seiken, Campbell, CA), as instructed by the manufacturer, and heat inactivated at 56°C for 30 min. Hemagglutination inhibition (HI; using 0.5% packed chicken red blood cells) titers were determined as the reciprocal of the highest serum dilution that inhibited 4 hemagglutinating units

of virus. HI titers of ≥10 were considered suggestive of recent influenza virus infection.

Inoculation with A/duck/Laos/25/06 was lethal to all birds (Table 1). While chickens succumbed to infection within 2 days postinoculation (p.i.), the mean time until death for sparrows was 4.1 days; mortality occurred rapidly (overnight) without prior observation of clinical signs. Expected clinical signs, should they have occurred, included moderate to severe depression, huddling at the cage bottom, and ruffled feathers (9). All inoculated birds shed virus from the oropharynx and, to a lesser extent, from the cloaca (Fig. 1A and B). The mean virus titers of inoculated chickens and sparrows were comparable on day 1 p.i.; however, on day 2 p.i., the mean oropharyngeal and cloacal viral titers of chickens were approximately 2 and 2.5 times greater, respectively, than those of sparrows (Fig. 1A and B). The virus titer in water used by inoculated sparrows was 10<sup>0.75</sup> EID<sub>50</sub>/ml at 1 day p.i. and peaked at 10<sup>1.75</sup> EID<sub>50</sub>/ml on days 2 and 4 p.i. (Fig. 1C). No virus was detected in water from the inoculated chickens' cage.

Virus was not isolated from the swab samples obtained from contact chickens, suggesting the absence of waterborne virus transmission from sparrows (Table 1). Further, HI testing of the contact chickens detected no virus-specific antibodies (data not shown). Because virus was not detected in the water from the inoculated chickens' cage, we generated a contaminated water source for the contact sparrows by creating a suspension of fecal material in phosphate-buffered saline (PBS; 10<sup>6.5</sup> EID<sub>50</sub>/ml), using swabs obtained from all five infected chickens at 2 days p.i.; we added 1 ml of this mixture to 1 liter of fresh water for a final concentration of 10<sup>3.5</sup> EID<sub>50</sub>/ml. Waterborne virus was transmitted to 2 of 8 contact sparrows, whose deaths occurred at 5 days and 10 days postcontact, respectively.

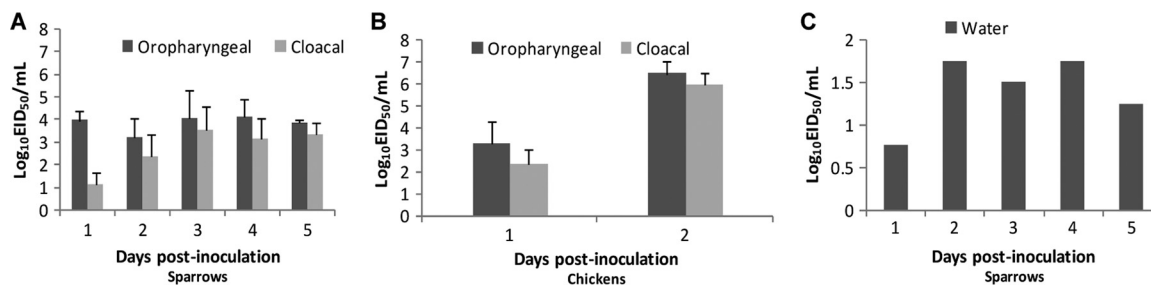


FIG. 1. Mean oropharyngeal and cloacal virus titers in sparrows (A) and chickens (B) inoculated with a lethal dose of A/duck/Laos/25/06 (H5N1) virus. (C) Virus titers in the drinking water of inoculated sparrows. Sparrows were inoculated with 10<sup>6</sup> EID<sub>50</sub>/ml of virus, and chickens were inoculated with 10<sup>2</sup> EID<sub>50</sub>/ml of virus. The lower limit of detection was 0.75 log<sub>10</sub> EID<sub>50</sub>/ml.

Our results showed that sparrows were susceptible to the A/duck/Laos/25/06 (H5N1) virus at a wide range of doses, as demonstrated by the 100% mortality of both inoculated sparrows ( $10^6$  EID<sub>50</sub> of virus intranasally) and infected contact sparrows (water contained  $10^{3.5}$  EID<sub>50</sub>/ml of virus). The 100% lethality of the virus to sparrows supports the report of Boon et al. (2) stating that more recent (2005–2006) H5N1 isolates appear to be more pathogenic to passerine birds than earlier isolates, such as A/chicken/Hong Kong/220/97 (H5N1).

While the duration and route of virus shedding clearly varied between infected sparrows and chickens, results also suggested that transmission rates may be different between the two species, as transmission occurred only from chickens to sparrows via artificially contaminated water (and not vice versa). Virus transmission from sparrows to chickens may require direct contact and/or aerosol transmission rather than ingestion of waterborne virus, seeing as water titers were as high as  $10^{1.25}$  EID<sub>50</sub>/ml (on days 1 and 3 postcontact) after dilution with fresh water, and this dose was 100% lethal to experimentally infected ducks (7). Additionally, in our experiment, A/duck/Laos/25/06 was rapidly lethal to naturally infected chickens at a dose of  $10^2$  EID<sub>50</sub>/ml. Alternatively, transmission from infected sparrows to chickens may require a higher virus titer in the water. Future studies are indicated to determine the concentration of contaminated sparrow water necessary to infect chickens with A/duck/Laos/25/06 and to determine transmissibility of HPAI H5N1 virus from infected chickens to contact sparrows via naturally contaminated water.

The undetectable level of virus in the water trough of inoculated chickens, all of which shed high levels of virus from the oropharynx and cloaca, may reflect rapid disease progression that caused the chickens to stop drinking water by day 1 p.i. and succumb to infection on day 2 p.i. These results may indicate that sparrows are unlikely to be infected under normal circumstances during an H5N1 virus outbreak. Our findings could also be attributed to the extremely high lethality of A/duck/Laos/25/06 to chickens and the reduced period of time for shedding, compared to those of other recent HPAI H5N1 virus isolates where mortality occurred as late as day 5 p.i. in experimentally infected chickens (12, 14). In contrast, the sparrows shed virus for several days, and their drinking water was rapidly contaminated with virus. The long-term shedding we observed in sparrows was also seen by Brown et al. in house sparrows infected with A/whooper swan/Mongolia/244/05 (H5N1) HPAI virus (3). These findings, in view of the widespread intermingling of land-based wild birds with wild and domestic waterfowl and poultry (2, 3), suggest that passerine birds can facilitate the spread of H5N1 virus.

Throughout the United States, sparrows and starlings are commonly found in low-biosecurity poultry housing, where they often eat and drink from the feed and water troughs. We used a shallow stainless steel basin in our sparrow enclosures to simulate these poultry watering troughs, which allow flocks of wild birds, such as sparrows, to bathe, defecate, and drink. Although we did not observe sparrows bathing in the water basin during the study, seed and fecal droppings were present in the water, indicating that the sparrows were either perching on the water basin or standing in the water. In the face of an

H5N1 outbreak, these birds could spread virus within or among poultry facilities and the wild bird population by contaminating food and/or water with feces and/or oropharyngeal secretions. Our findings on the shedding of HPAI H5N1 virus in infected sparrows, when taken together with the ethological knowledge of these birds, suggest that the behavior of infected sparrows may be a critical determinant of their ability to act as an intermediate host for influenza. Understanding the importance of influenza infection in nonwaterfowl and nonpoultry species is therefore an area that necessitates further research.

To our awareness, this is the first experimental study to illustrate interspecies transmission of H5N1 virus between poultry and wild birds. The transmission of waterborne virus to 25% of sparrows provides further evidence that they can serve as intermediate hosts of H5N1 viruses. Although we did not observe waterborne virus transmission from sparrow to chicken, further studies are needed to investigate the transmission of other H5N1 virus strains and to examine the role of direct contact.

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#### REFERENCES

- Alexander, D. J. 2007. Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. *Avian Dis.* 51:161–166.
- Boon, A. C., M. R. Sandbulte, P. Seiler, R. J. Webby, T. Songserm, Y. Guan, and R. G. Webster. 2007. Role of terrestrial wild birds in ecology of influenza A virus (H5N1). *Emerg. Infect. Dis.* 13:1720–1724.
- Brown, J. D., D. E. Stallknecht, R. D. Berghaus, and D. E. Swayne. 2009. Infectious and lethal doses of H5N1 highly pathogenic avian influenza virus for house sparrows (*Passer domesticus*) and rock pigeons (*Columba livia*). *J. Vet. Diagn. Investig.* 21:437–445.
- Feare, C. J. 2007. The role of wild birds in the spread of HPAI H5N1. *Avian Dis.* 51:440–447.
- Kida, H., R. Yanagawa, and Y. Matsuoka. 1980. Duck influenza lacking evidence of disease signs and immune response. *Infect. Immun.* 30:547–553.
- Kim, J. K., N. J. Negovetich, H. L. Forrest, and R. G. Webster. 2009. Ducks: the “Trojan horses” of H5N1 influenza. *Influenza Other Respir. Viruses* 3:121–128.
- Kim, J. K., P. Seiler, H. L. Forrest, A. M. Khalenkov, J. Franks, M. Kumar, W. B. Karesh, M. Gilbert, R. Sodnomdarjaa, B. Doungneun, E. A. Govorkova, and R. G. Webster. 2008. Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. *J. Virol.* 82:11374–11382.
- Kou, Z., F. M. Lei, J. Yu, Z. J. Fan, Z. H. Yin, C. X. Jia, K. J. Xiong, Y. H. Sun, X. W. Zhang, X. M. Wu, X. B. Gao, and T. X. Li. 2005. New genotype of avian influenza H5N1 viruses isolated from tree sparrows in China. *J. Virol.* 79:15460–15466.
- Perkins, L. E., and D. E. Swayne. 2003. Varied pathogenicity of a Hong Kong-origin H5N1 avian influenza virus in four passerine species and budgerigars. *Vet. Pathol.* 40:14–24.
- Reed, L. J., and H. Muench. 1938. A simple method for estimating fifty percent endpoints. *Am. J. Hyg. (London)* 27:493–497.
- Songserm, T., R. Jam-on, N. Sae-Heng, N. Meemak, D. J. Hulse-Post, K. M. Sturm-Ramirez, and R. G. Webster. 2006. Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerg. Infect. Dis.* 12:575–581.
- Tian, G., S. Zhang, Y. Li, Z. Bu, P. Liu, J. Zhou, C. Li, J. Shi, K. Yu, and H. Chen. 2005. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. *Virology* 341:153–162.
- Uyeki, T. M. 2008. Global epidemiology of human infections with highly pathogenic avian influenza A (H5N1) viruses. *Respirology* 13(Suppl. 1):S2–S9.
- Webster, R. G., R. J. Webby, E. Hoffmann, J. Rodenberg, M. Kumar, H. J. Chu, P. Seiler, S. Krauss, and T. Songserm. 2006. The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. *Virology* 351:303–311.
- Webster, R. W., Y. Guan, M. Peiris, and H. Chen. 2006. H5N1 influenza continues to circulate and change. *Microbe* 1:559–565.