

# Replication Capacity of Avian Influenza A(H9N2) Virus in Pet Birds and Mammals, Bangladesh

**Brian J. Lenny, Karthik Shanmuganatham, Stephanie Sonnberg, Mohammed M. Feeroz, S.M. Rabiul Alam, M. Kamrul Hasan, Lisa Jones-Engel, Pamela McKenzie, Scott Krauss, Robert G. Webster, Jeremy C. Jones**

Avian influenza A(H9N2) is an agricultural and public health threat. We characterized an H9N2 virus from a pet market in Bangladesh and demonstrated replication in samples from pet birds, swine tissues, human airway and ocular cells, and ferrets. Results implicated pet birds in the potential dissemination and zoonotic transmission of this virus.

Avian influenza A(H9N2) virus is endemic among poultry throughout Eurasia (1–3). In Bangladesh, subtype H9N2 viruses are unique reassortants, containing genes from highly pathogenic avian influenza A(H7N3) viruses. The H9N2 virus poses a substantial infection risk to poultry (2) and has infected pigs and humans (4,5). Its evolution is continually monitored by the World Health Organization ([http://www.who.int/influenza/vaccines/virus/201502\\_zoonotic\\_vaccinevirusupdate.pdf?ua=1](http://www.who.int/influenza/vaccines/virus/201502_zoonotic_vaccinevirusupdate.pdf?ua=1)).

Ongoing influenza surveillance in Bangladesh found H9N2 virus primarily in poultry (5,6); we also surveyed a pet market that sold avian pets (parrots, finches, pigeons) and poultry (quail, turkey, chickens) and obtained isolates from nonpoultry terrestrial birds (6). This mixture of birds and mammals, some for which little associated influenza pathogenesis data exists, provided a unique opportunity to study the ecology, host range, and transmission potential of H9N2 virus.

## The Study

We obtained H9N2 virus isolate A/environment/Bangladesh/9306/2010 (Env/9306) from a fecal sample collected

from a parrot cage. Phylogenetic data are available for other H9N2 viruses isolated in Bangladesh (5), but little phenotypic data exists for this lineage, which represents most H9N2 strains isolated in Bangladesh during 2010–2012. This strain clusters with isolates from Pakistan and India and has mammalian adaptations (2,5). We examined the pathogenicity of Env/9306 in birds commonly found at pet markets and assessed its capacity to replicate in and transmit among mammals by using *ex vivo* and *in vivo* models.

To examine H9N2 replication in bird species, we inoculated 5 finches, 5 parakeets, and 6 chickens oculonasally with  $10^5 \log_{10}$  50% egg infectious doses ( $\log_{10} \text{EID}_{50}$ ) of Env/9306 (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/12/15-1152-Techapp1.pdf>). Oropharyngeal and cloacal swab samples were collected every 2 days postinoculation (dpi) and titrated in eggs. Measurement of donor and contact animal virus shedding is based on the inoculation date of donors; donor and contact animals were kept in the same cage. Inoculated pet birds shed virus oropharyngeally (Figure 1) for 6 days, but not cloacally (data not shown). Chickens, a control H9N2 virus host, shed 2–3 logs more than did pet birds, and for a significantly longer time by area under the curve analysis (up to 10 dpi;  $p \leq 0.001$ ). Finches remained asymptomatic; parakeets and chickens showed sporadic clinical signs (lethargy, hunched posture, labored breathing) at 5–10 dpi. No birds died.

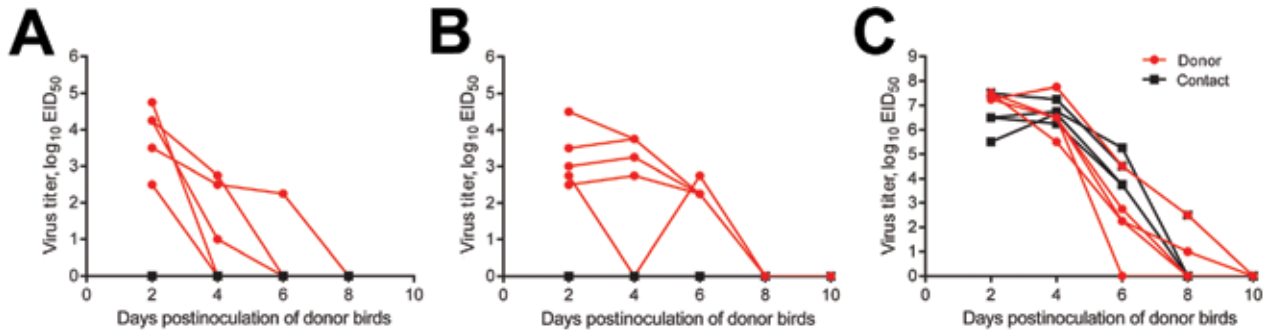
Tissue samples were collected at 3 dpi (Table 1). Virus was isolated from the respiratory tract of 1 parakeet, 2 finches, and all 3 chickens, 2 of which had virus in the gastrointestinal tract. Virus was also isolated from the brain (2 finches, 1 chicken) and eye (1 finch, 1 chicken) (Table 1).

Naive contacts of inoculated pet birds were not infected, but naive chicken contacts became infected and shed virus as early as 2 dpi (Figure 1). All birds were tested for seroconversion at 16 dpi by hemagglutination inhibition (HI) assay (7; online Technical Appendix). Among finches, 1 of 5 donors and no contacts seroconverted. Among parakeets, 4 of 5 donors and 1 of 5 contacts seroconverted. All chickens seroconverted, and titers exceeded those of pet birds (Table 1).

To determine environmental shedding, we collected swab samples of drinking water, feces, and cages on 1–6 dpi. Virus was detected in water for finches (4 time points) and parakeets (1 time point) but not in fecal or cage swab

Author affiliations: Rhodes College, Memphis, Tennessee, USA (B.J. Lenny); St. Jude Children's Research Hospital, Memphis (B.J. Lenny, K. Shanmuganatham, S. Sonnberg, P. McKenzie, S. Krauss, R.G. Webster, J.C. Jones); Jahangirnagar University, Dhaka, Bangladesh (M.M. Feeroz, S.M.R. Alam, M.K. Hasan); University of Washington, Seattle, Washington, USA (L. Jones-Engel)

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**Figure 1.** Oropharyngeal shedding of influenza A(H9N2) virus isolate A/environment/Bangladesh/9306/2010 (Env/9306) by pet birds and chickens, Bangladesh. Measurement of donor and contact bird virus shedding is based on the inoculation date of donors; donor and contact birds were kept in the same cage or enclosed environment. A) Donor finches ( $n = 5$ ), B) parakeets ( $n = 5$ ), or C) chickens ( $n = 6$ ; red lines) were inoculated with  $10^5 \log_{10}$  50% egg infectious doses ( $EID_{50}$ ) units of Env/9306 and paired with naive birds of the same species ( $n = 4$  or  $5$ ; black lines) in the same cage. Birds were swabbed every 2 dpi and virus titer ( $\log_{10} EID_{50}/mL$ ) was determined in eggs. Individual shedding curves for each animal are provided.

samples, consistent with oropharyngeal shedding patterns (8). All chicken environmental samples contained virus for at least 4 of 6 time points (Table 2).

The H9N2 virus strain Env/9306 contains mammalian-like mutations in genes, including HAQ226L (H3 numbering) (5), which increase H9N2 virus transmissibility to and among mammals (9). We modeled replication in humans (respiratory and ocular routes) by inoculating differentiated normal human bronchial epithelial cells (NHBEs) or primary human ocular cells (cornea and trabecular meshwork) with Env/9306 (multiplicity of infection 0.01) (online Technical Appendix). The Env/9306 strain replicated in NHBEs to  $\geq 7 \log_{10}$  50% tissue culture infectious doses ( $TCID_{50}$ ) per mL and exceeded titers of control human pandemic virus A/California/04/2009 (pH1N1) beyond 48 hours postinoculation ( $p < 0.0001$ ; Figure 2, panel A). Despite the higher titers, Env/9306 did not induce noticeable cytopathology in NHBEs, but pH1N1 did. In corneal and trabecular meshwork cells, Env/9306 replicated to similar titers as did control virus H7N3, a subtype previously shown to replicate in ocular cells (Figure 2, panels B, C) (10).

To assess replication in swine, we inoculated tracheal or lung tissue explants (online Technical Appendix) from 1–2 week old piglets, with  $10^5 EID_{50}/explant$ . Virus replicated  $\geq 6 \log_{10} TCID_{50}/mL$ , comparable to a control swine H2N3 virus (Figure 2, panel D).

We modeled replication and transmission in vivo by inoculating 3 donor ferrets with strain Env/9306 ( $10^6 EID_{50}$  units); each was co-housed with a separate naive, direct contact. Donors shed  $4 \log_{10} TCID_{50}/mL$  in nasal washes for 6 dpi; 2 of 3 donors displayed lethargy, swollen sinuses, sneezing, or a combination of these during this period. No virus was shed by naïve direct contacts. One donor ferret displayed lethargy (4–8 dpi) and 1 sneezing (10–12 dpi) (Figure 2, panel F, data not shown). To examine whether the lack of transmission correlated with virus tropism, ferret tracheal and lung explants (online Technical Appendix) were inoculated with  $10^5 EID_{50}/explant$  of Env/9306 or pH1N1. Env/9306 replicated in ferret tracheal explants to titers  $> 5 \log_{10} EID_{50}/mL$  (72 hours postinoculation), statistically lower than the rate for pH1N1 (Figure 2, panel E). No replication of either virus was observed in lung explants.

## Conclusions

We demonstrated replication of a nonpoultry avian influenza A(H9N2) virus in finches and parakeets with limited environmental shedding (water), but no transmission to cage mates. Shedding routes were more limited, virus titers lower, and clinical signs less frequent in pet birds than in chickens. Nevertheless, the potential for pet birds to act as vectors of the virus should not be underestimated. We recently showed that novel influenza A(H7N9) virus

**Table 1.** Replication of avian influenza A(H9N2) virus in organs and seroconversion of inoculated birds, Bangladesh\*

Bird	Organ titer†						HI titer‡	
	Brain	Eye	Trachea	Lung	Small intestine	Large intestine	Donor	Contact
Finch	2.9 (2/3)	3.5 (1/3)	3 (2/3)	3.5 (1/3)	–	–	4.3 (1/5)	– (0/5)
Parakeet	ND	ND	3.5 (1/3)	–	–	–	6.1 (4/5)	5.3 (1/5)
Chicken	5.5 (1/1)	4.25 (1/1)	4.5 (3/3)	5.1 (3/3)	3 (2/3)	4.5 (1/3)	10.8 (6/6)	10.9 (4/4)

\*HI, hemagglutination inhibition; –, below the limit of detection ( $< 0.75$  50% egg infectious doses [ $EID_{50}$ ]/mL or serum dilution  $< 1:20$ ); ND, not determined.

†Tissues were harvested 3 days postinoculation and titrated in embryonated chicken eggs, and were reported as  $\log_{10} EID_{50}/mL$ . Data are the means of positive samples ( $> 0.75 EID_{50}/mL$ ) (no. birds shedding/total birds sampled at given time point).

‡Mean reciprocal values ( $\log_2/50\mu L$ ) of the highest titer that inhibited 4 hemagglutinating units of homologous virus (no. seropositive animals/total no. sampled).

**Table 2.** Detection of avian influenza A(H9N2) virus in swab samples from environment of inoculated birds, Bangladesh\*

Bird	Sample	Swab titer†					
		1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi
Finch	Water	3.25	2.5	2.5	1	–	–
	Cage	–	–	–	–	–	–
	Feces	–	–	–	–	–	–
Parakeet	Water	–	–	–	–	1	–
	Cage	–	–	–	–	–	–
	Feces	–	–	–	–	–	–
Chicken	Water	3.25	2.25	4.5	3.5	5.5	–
	Cage	3.5	4.5	4.5	4.5	3.25	2.5
	Feces	3	<	3.25	3.75	2.5	–

\*dpi, days postinoculation; –, below the limit of detection (<0.75 50% egg infectious doses [EID<sub>50</sub>]/mL).

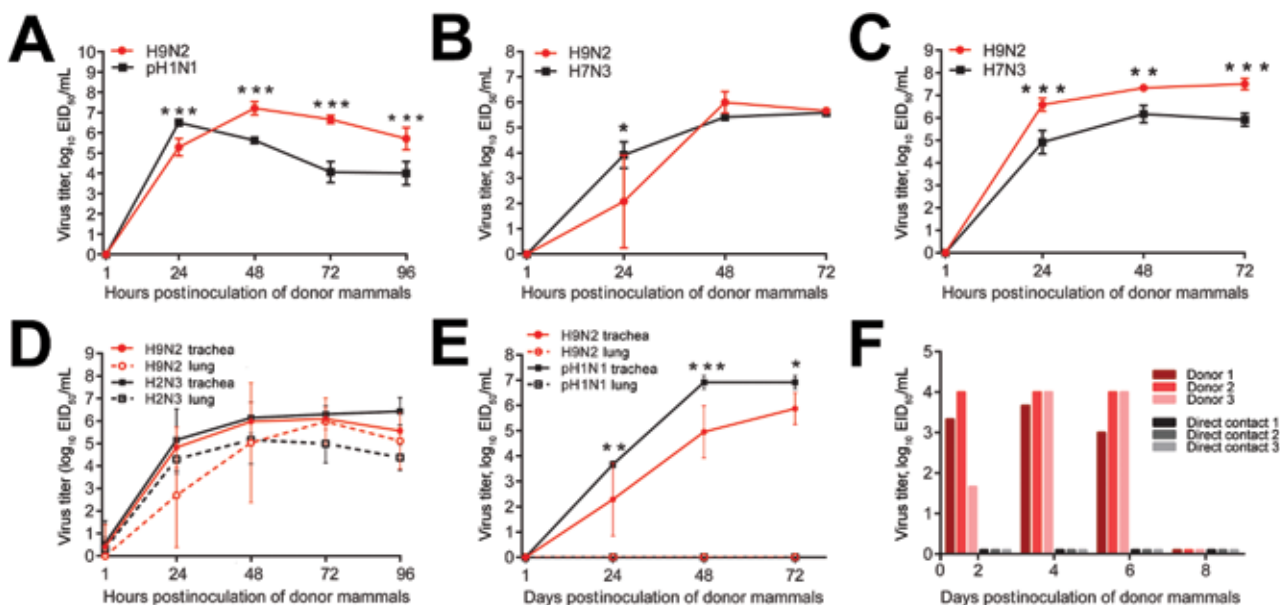
†Samples were titrated in embryonated chicken eggs and reported as log<sub>10</sub> EID<sub>50</sub>/mL.

transmits between passerines, which include finches, and poultry by water despite a lack of intraspecies transmission (8); H9N2 virus has also been isolated from wild, finch-like birds in China (11).

Interspecies transmission of the Env/9306 strain remains a risk to mammals because of adaptation mutations (5,9) and is supported in this study by replication in ferrets and in human and swine tissues. Physical contact between pet birds and their owners, as well as shedding of virus into the environment (water), could be transmission sources.

Live bird markets are crucial to zoonotic spread of avian influenza viruses (AIVs) (12). However, our data suggest transmission potential in pet markets and vendor sites other than poultry markets; these sites may house birds infected with AIVs and should be included in future

surveillance. Our results may also inform surveillance sample collection. Oropharyngeal samples were collected from pet birds; collecting environmental swabs alone may yield lower isolation rates or fail to detect this virus. H9N2 virus replication in pet birds also has implications for viral spread. Poultry are a major source of dissemination, but our data show domesticated or pet birds can harbor H9N2. Pet trading can extend across international borders and greatly expand the range of AIVs, as when H9N2 virus was repeatedly imported into Japan in infected parakeets (13). Finally, the unique influenza varieties among pet birds may provide more opportunities for H9N2 virus to gain novel genetic elements; this subtype has had remarkable levels of reassortment activity with influenza A(H7N9) and highly pathogenic avian influenza A(H5N1) viruses (14,15).



**Figure 2.** Pathogenesis of influenza A(H9N2) virus isolate A/environment/Bangladesh/9306/2010 (Env/9306) in ex vivo and in vivo mammalian models, Bangladesh. Replication kinetics of Env/9306 or a virus control are shown in A) primary normal human bronchial epithelial cells, B) primary human corneal epithelial cells, C) primary human trabecular meshwork cells, D) swine respiratory tissue explants, and E) ferret respiratory tissue explants. Error bars indicate mean  $\pm$  SD of the combined results of 2 individual experiments of  $n = 3$  inserts, wells, or tissue explants per virus group. Env/9306 replication is indicated in red, and control virus replication in black. F) Replication of Env/9306 in ferrets ( $n = 3$ ; red bars) and transmission to naive, direct contact ferrets ( $n = 3$ ; black bars) housed in the same cage. Statistical significance of replication between virus groups at a given time point was determined by performing a 2-way analysis of variance. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.0001$ . EID<sub>50</sub>, 50% egg infectious doses.

H9N2 virus will remain a threat in the foreseeable future. Efforts are needed to identify its presence in poultry and nonpoultry avian species. Phenotypic properties of these viruses, including replication *ex vivo* and *in vivo*, are a valuable supplement to existing genotypic data and further inform the risk for spread within avian and human populations.

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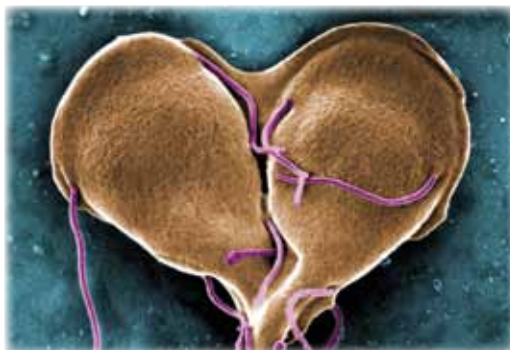
Mr. Lenny is a student and researcher in the St. Jude Children's Hospital–Rhodes College Research Fellowship Program. His research interests include viral pathogenesis and epidemiology of zoonotic diseases.

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Address for correspondence: Jeremy C. Jones, Department of Infectious Diseases, Virology Division, St. Jude Children's Research Hospital, 262 Danny Thomas Pl, MS330, Memphis, TN 38120, USA; email: [Jeremy.jones@stjude.org](mailto:Jeremy.jones@stjude.org)

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## Technical Appendix

All animal experiments were approved by the St. Jude Children's Research Hospital Hospital Animal Care and Use Committee (Memphis, TN, USA) and performed in compliance with the policies of the National Institutes of Health and the Animal Welfare Act.

## Viruses

All viruses were propagated and titrated in 10-day-old, embryonated chicken eggs, with 50% egg infectious doses (EID<sub>50</sub>) determined by the method of Reed and Muench (*Am. J. of Hygiene*, v27, 1934, pp.493–497). A/Environment/Bangladesh/9306/2010 (parrot, H9N2) was subjected to 2 rounds of treatment with Newcastle disease virus (NDV) hyper-immune sera and a final seed stock prepared from a limiting dilution series in eggs. RNA was isolated from the final virus stock preparation and subjected to reverse transcription (RT)PCR (Qiagen, Valencia, CA) by using 2 pairs of NDV gene specific primers as described (*J. of Vir. Meth.* 86(1), 2000, pp.71–83) to confirm the absence of NDV. Several control viruses were used to demonstrate productive replication in NHBE and ferret explants (A/California/04/2009, pandemic H1N1), swine explants (A/swine/Missouri/2124514/2006, H2N3), or ocular cells (A/LaughingGull/DE/42/2006, H7N3).

## Cell Lines

Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) were cultured in modified Eagle's medium (MEM; CellGro, Herndon, VA) supplemented with 5% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT) at 37°C in 5% CO<sub>2</sub>. Virus titers from NHBE, ocular cell, explant, and ferret nasal wash infections were titered in MDCKs in MEM supplemented with 1% bovine serum albumin (BSA) and 1 µg/mL tosyl phenylalanyl

chloromethyl ketone (TPCK) treated trypsin. Titers in MDCKs were expressed as 50% tissue culture infectious doses (TCID<sub>50</sub>) as calculated by the method of Reed and Muench.

Primary normal human bronchial epithelial (NHBE) cells (Lonza, Walkersville, MD) from a 4-year-old healthy male donor were cultured in an air-liquid interface (ALI) system on Transwell inserts (Corning, NY). The apical surfaces were exposed to a humidified 95% air and 5% CO<sub>2</sub> environment for a minimum of 6 weeks. Basal surfaces were cultured in BEBM medium (Lonza) supplemented with SingleQuot growth factors (Lonza). Basal medium was changed every 48 h along with an apical surface wash to remove mucus.

Primary human corneal epithelial and trabecular meshwork cells (Science Cell, Carlsbad, CA) were cultured in manufacturer supplied media supplemented with FBS at 37°C in 5% CO<sub>2</sub>. During virus infections, cells were switched to serum free-medium supplemented with 1% bovine serum albumin and 0.5 µg/mL TPCK treated trypsin.

## **Explant Preparation**

Piglets (1–2 week old, kindly provided by C. Leffler and E. Parfenova [University of Tennessee, Memphis, TN]) and ferrets (6–8 months old [Triple F Farms, Sayre, PA]) and were euthanized. The trachea and lung lobes were removed intact and washed with PBS containing 10× penicillin-streptomycin-amphotericin B (Sigma, St. Louis, MO). The tracheas were opened by a single lengthwise incision, and explants (5 mm diameter) were prepared with a biopsy punch. One lower lung lobe was inflated and washed through an incision in the branching bronchus with PBS containing 10× antibiotics/antimycotic and then inflated again with 1.6% low-melt agarose (Sigma). After the agarose had solidified, 2×1×1 cm segments were cut from lung and embedded in a 4% agarose plug. Thin slices were cut from the agarose/lung plug and explants (5 mm diameter) were prepared from these slices. Tracheal and lung explants were maintained on transwell inserts, and 1 mL of medium (as described for NHBE culture) was added to the basal chamber. Tissues were incubated at 37°C in 5% CO<sub>2</sub> for at least 18 h before infection.

## **NHBE and Explant Infection**

The apical surfaces of NHBEs or explants were washed with PBS and equilibrated at 37°C for 30 min with infection medium (BEBM supplemented with 0.5% BSA). Cells and tissues were inoculated with 100 µL of virus inoculum at MOI 0.01 (NHBEs) or containing 10<sup>5</sup> EID<sub>50</sub> units (explants) for 1 hr. Apical surfaces were given 2 acid washes (PBS, pH 2.2) and 3 pH neutral PBS washes to remove unbound virus. At each time point, 300 µL of infection was added to the apical surface for 30 min and harvested for titration in MDCKs. NHBE cell data are representative of combined data from 2 independent tests of 3 inserts per virus group. Explant data are representative of 2 independent tests with 3 explants per tissue per virus group.

## **Hemagglutination Inhibition Assay**

The presence of anti-influenza hemagglutination protein antibody titers were determined by preparing serial 2-fold dilutions of receptor destroying enzyme (Denko Seiken, Campbell, CA) animal sera in PBS in 'v-bottom' shaped plates. Virus was standardized to 4 hemagglutinating units by using horse erythrocytes (Rockland, Limerick, PA) and added to the sera dilutions for 30 minutes. Horse erythrocytes were then added and incubated for 60 minutes. HI values were recorded as the reciprocal of the highest dilution of sera that inhibit virus-induced erythrocyte agglutination.

## **Avian Species Infection**

Mixed age and sex zebra finches (n = 5, *Taeniopygia guttata*) and parakeets (n = 5, *Melopsittacus undulates*) (Birds Express, South El Monte, CA), 8–10 week old mixed sex white leghorn chickens (n = 5, *Gallus gallus domesticus*) (McMurray Hatchery, Webster City, IA) were quarantined for at least 1 week before experiments. Swabs from each bird were verified influenza negative by egg isolation. Food and water was provided ad libitum. Birds were inoculated intranasally, intraocularly, and orally with 10<sup>5</sup> log<sub>10</sub> EID<sub>50</sub> units of virus in 100 µL (finches, parakeets) or 500 µL (chickens) of phosphate buffered saline. All bird species were housed with a naïve contact animals (n = 4–5) to assess transmission. Oropharyngeal and cloacal swab samples were collected on days post inoculation (dpi) 2, 4, 6, 8, 10, and 13. Water samples

(500  $\mu$ L), feces and cage swabs were obtained 1–6 dpi from each species' housing. Samples were titrated in eggs.

## **Ferret Infection**

Male ferrets, 6–8 months of age (Triple F Farms), were verified seronegative for influenza A (H1 and H3 subtypes) by HI assay. Three donor ferrets in individual cages were lightly anesthetized by isoflurane and intranasally inoculated with  $10^6$  EID<sub>50</sub> units of virus in a total volume of 500  $\mu$ L. Twenty-four hours later, a naïve cage mate (direct contact) was introduced into the cage of each donor ferret. Every 48 hr, the ferrets' temperatures and weights were recorded before they were sedated with ketamine (25 mg/kg of bodyweight) and induced to sneeze by intranasal instillation of PBS. Virus titer in the nasal washes were titrated in MDCK cells.

## **Statistics**

Mean infectious titers were compared by using the 1-tailed Student t-test in Excel (Microsoft, Redmond, WA, USA) or 2-way ANOVA GraphPad Prism v6 (La Jolla, CA, USA). Area under the curve analysis for cumulative shedding was performed by using GraphPad Prism v6.