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Experimental Challenge of a Peridomestic Avian Species, European Starlings (*Sturnus vulgaris*), with Novel Influenza A H7N9 Virus from China

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ABSTRACT: In 2013 a novel avian influenza H7N9 virus was isolated from several critically ill patients in China, and infection with this virus has since caused more than 200 human deaths. Live poultry markets are the likely locations of virus exposure to humans. Peridomestic avian species also may play important roles in the transmission and maintenance of H7N9 at live poultry markets. We experimentally challenged wild European Starlings (*Sturnus vulgaris*) with the novel H7N9 virus and measured virus excretion, clinical signs, and infectious dose. We found that European Starlings can be infected with this virus when inoculated with relatively high doses, and we predict that infected birds excrete sufficient amounts of virus to transmit to other birds, including domestic chickens. Infected European Starlings showed no clinical signs or mortality after infection with H7N9. This abundant peridomestic bird may be a source of the novel H7N9 virus in live poultry markets and may have roles in virus transmission to poultry and humans.

Key words: Avian influenza virus, European Starlings, H7N9, live poultry markets, transmission.

In the spring of 2013, a novel avian influenza A virus (A/Anhui/1/2013 [H7N9]), was found in three Chinese patients who ultimately died from the respiratory infection (Gao et al. 2013). Since its discovery, there have been 571 human cases, and 212 people have died from infection of this novel subtype. Human exposure to the A/H7N9 virus has been directly connected to live poultry markets (LPMs) and, to a lesser degree, LPM-associated farms in China, with most cases involving workers and customers at LPMs where poultry and humans come into contact (Lee et al. 2013). Sequence analysis of A/H7N9 and experimental studies in poultry showed that this virus exhibits low pathogenicity in avian species (Pantin-Jackwood et al. 2014). It seems likely that commercial poultry at LPMs serve as virus reservoirs for transmission to other birds and potentially to humans. Peridomestic species of birds, such as House Sparrows (*Passer domesticus*) and Rock Pigeons (*Columba livia*), may also be involved in the transmission of A/H7N9 at LPMs (Jones et al. 2014; Wang et al. 2014). However, many unanswered questions remain regarding the role of peridomestic birds in the maintenance and transmission cycle of A/H7N9 and the risks to poultry and humans at Chinese LPMs.

European Starlings (*Sturnus vulgaris*) are common in the northern hemisphere and can be abundant in peridomestic settings, including agricultural and urban environments. These birds would have high likelihood of contact with influenza-virus-infected poultry and other peridomestic species as well as human populations at LPMs. We challenged European Starlings with three doses of A/H7N9 and determined the amount of virus excreted by infected European Starlings, clinical signs of infection, and serologic responses to infection, and we inferred an approximate infectious dose.

Adult European Starlings (n=22) were locally captured (rural Dane County, Wisconsin, USA; November 2014), placed in HEPA-filtered plastic isolator cages within a BSL-3 Enhanced biocontainment facility (2–3 birds/isolator), and allowed to acclimate for 3–6 wk prior to initiation of the study. The influenza virus isolate, A/Anhui/1/2013 (H7N9), was obtained from the Centers for Disease Control and Prevention (Atlanta, Georgia, USA). Starlings were divided into three treatment

groups and one negative control group. Each group was inoculated intrachoannally (90 μL) and intranasally (10 μL) with one of three doses of virus, $10^{5.9}$ 50% egg infectious dose (EID_{50})/100 μL (high dose, six birds), $10^{3.9}$ EID_{50}/100 μL (medium dose, seven birds), and $10^{1.9}$ EID_{50}/100 μL (low dose, six birds), diluted in brain/heart infusion broth. Three birds were sham-inoculated with brain/heart infusion broth only and housed separately (negative controls). Inoculum titers were determined in embryonated chicken eggs using the method of Reed and Muench (1938).

All birds were weighed daily and observed twice daily to monitor health status. Oropharyngeal and cloacal swabs were collected daily from day postinoculation (DPI) 0 to DPI 8 and also at DPI 10, 12, and 14. Swabs were placed in viral transport media (Hanks Balanced Salt Solution, 0.05% gelatin, 5% glycerin, 1500U/mL penicillin, 1500 μg/mL streptomycin, 0.1 mg/mL gentamicin, 1 μg/mL fungizone) and stored at –80 C. Blood samples (~100 μL) were collected by right jugular venipuncture on DPI 0 and 14 and centrifuged, and sera were stored at –30 C. At the end of the study (DPI 14) all birds were humanely euthanized.

Viral RNA was extracted and reverse transcription-polymerase chain reactions performed using the published procedures, primers, and probe of Spackman et al. (2002). Virus amounts were quantified by comparing cycle threshold values from swabs with those generated from a standard curve of serial dilutions of the same virus stock used as inoculum and expressed as Log_{10} RNA equivalent/mL (Pantin-Jackwood et al. 2014; Spackman et al. 2015). Hemagglutination inhibition (HI) assays for A/H7N9 antibodies were performed using procedures described by Pedersen (2008), titrated using serial twofold dilutions from 1:10 to 1:1280, and titers were recorded as the reciprocal of the highest dilution demonstrating complete inhibition.

At the two lower inoculum doses ($10^{3.9}$ and $10^{1.9}$ EID_{50}/100 μL) as well as the negative controls, no birds became infected based on lack of excretion of detectable amounts of viral RNA and no seroconversion to A/H7N9 (data not shown). At the highest dose ($10^{5.9}$ EID_{50}/100 μL), two birds (nos. 4 and 6) oropharyngeally excreted moderate amounts of virus by DPI 1 or 2 (Table 1). The duration of excretion lasted for 7 and 6 d, respectively, and both birds excreted up to 10^5 to 10^6 EID_{50} RNA equivalent/mL. One high dose bird (no. 2) excreted 10^3 EID_{50} RNA equivalent/mL on DPI 3; however, no viral RNA was detected in any other sample, no virus was isolated in embryonated eggs from this sample, and this bird did not develop detectable antibodies to A/H7N9. Therefore, we cannot confidently state that this bird became infected with A/H7N9. No viral RNA was detected in cloacal swabs taken from any bird at any time point, including from the birds orally excreting virus (data not shown). None of the challenged or control birds exhibited any weight loss, behavioral issues, or any other overt clinical sign of infection.

Another high dose bird (no. 3) began to orally excrete detectable viral RNA on DPI 5 and continued to shed virus through DPI 8 (Table 1). This shedding began 3 d later than its cage-mate no. 4 that excreted up to10^6 EID_{50} RNA equivalent/mL for 7 d began excreting virus. Thus it may be that bird no. 3 was not infected from the original inoculation, but became infected by bird-to-bird transmission from its infected cage mate.

Hemagglutination inhibition analysis of preinoculation sera revealed no detectable AIV H7N9 antibodies in any bird, indicating no prior exposure to A/H7N9. Analysis of DPI 14 sera from birds nos. 3, 4, and 6 revealed the presence of hemagglutination-inhibiting antibodies to A/H7N9 (Table 1), albeit at relatively low titers (20–40). No other birds developed any postinoculation HI activity.

While we could not calculate an exact infectious dose, our data (2/6 European Starlings infected by inoculation with $10^{5.9}$ EID_{50} RNA equivalent/mL; 1/6 infected by transmission from infected cage mate excreting ~10^6 EID_{50} RNA equivalent/mL) suggest the European Starling 50% infectious dose is approximately 10^6 EID_{50} RNA equivalent/

TABLE 1. Oropharyngeal excretion of H7N9 avian influenza virus for 10 days postinfection (DPI) and hemagglutination inhibition (HI) titers in European Starlings (*Sturnus vulgaris*) experimentally challenged with a high dose ($10^{5.9}$ EID_{50}/100 μL) of A/Anhui/1/2013 (H7N9). No virus excretion was observed in any bird beyond 10 DPI, and none of the challenged birds exhibited cloacal virus excretion. At medium and low doses, no birds became infected (excreted virus or seroconverted). Excreted viral RNA quantities expressed as $\log_{10}$ 50% egg infectious dose RNA equivalent/100 μL. HI detection threshold was 1:10 serum dilution. No Ct indicates no influenza virus RNA detectable by PCR.

Bird ID	DPI 0	DPI 1	DPI 2	DPI 3	DPI 4	DPI 5	DPI 6	DPI 7	DPI 8	DPI 10	HI titer (DPI 14)
1	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	<1:10
2	No Ct	No Ct	No Ct	1.25×10^3	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	<1:10
3	No Ct	No Ct	No Ct	No Ct	No Ct	9.28×10^2	5.99×10^3	9.20×10^4	1.43×10^5	No Ct	1:20
4	No Ct	No Ct	5.73×10^4	5.48×10^5	1.48×10^6	6.49×10^5	7.16×10^5	3.09×10^5	8.51×10^4	No Ct	1:40
5	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	<1:10
6	No Ct	3.97×10^5	4.37×10^5	6.53×10^5	1.13×10^5	9.87×10^3	2.48×10^3	No Ct	No Ct	No Ct	1:20

mL, which is similar to the results found in A/H7N9 experimentally infected chickens (*Gallus gallus domesticus*; Spackman et al. 2015).

Infected European Starlings exhibited no clinical signs of disease even though they orally excreted sufficient amounts of virus ($\sim10^6$ EID_{50} RNA equivalent/mL) to transmit to other European Starlings and potentially to other species including domestic poultry (Pantin-Jackwood et al. 2014; Spackman et al 2015). Thus A/H7N9 infection in European Starlings has characteristics of low pathogenic avian influenza virus infection; however, we found no evidence of cloacal excretion typically seen with low pathogenic avian influenza virus infection (Webster et al. 1992). Other possible routes of transmission could include contaminated water or food supplies, aerosol droplet, or direct physical contact such as mutual preening (Delogu et al. 2010).

In summary, European Starlings can be infected by relatively high doses of A/H7N9 with no overt adverse health effects. Infected birds can excrete virus for up to 7 d in quantities sufficient to infect other European Starlings, poultry, and potentially humans. The role of this, and other peridomestic species at LPMs, and in the transmission of A/H7N9 and other AIV is largely unknown, and continued research is warranted.

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