

Detection of influenza viral gene in European starlings and experimental infection

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Background European starlings (*Sturnus vulgaris*) are common, widely distributed birds in North America that frequently come into contact with agricultural operations. However, starlings have been one of the neglected land-based wild bird species for influenza surveillance.

Objectives To study the potential role of starlings in the ecology and epidemiology of influenza virus.

Methods We collected 328 digestive and 156 tracheal samples from starlings in Ohio in years 2007 (July) to 2008 (August) and screened for the presence of influenza virus by real-time RT-PCR, standard RT-PCR and virus isolation using embryonated chicken eggs. In addition, we conducted an experimental infection study to evaluate the replication and induction of antibody response by two low pathogenic avian influenza (AI) viruses in starlings.

Results Although virus isolation was negative, we confirmed 21 influenza positive digestive and tracheal samples by real-time and standard RT-PCR tests. Phylogenetic analysis revealed that five NS genes recovered from Starlings belonged to NS subtype A and were most similar to the NS genes from a wild aquatic bird origin isolate from Ohio. Experimental infection studies using two low pathogenic AI strains showed that starlings could be infected, shed virus, and seroconvert.

Conclusions This study shows that starlings can carry influenza virus that is genetically similar to wild aquatic bird origin strains and may serve as a carrier of influenza virus to domestic animals.

Keywords European starling, infection, influenza, surveillance.

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Introduction

Wild waterfowl, shorebirds, and gulls are regarded as the natural hosts and primary reservoirs of avian influenza (AI) virus. In these hosts, low pathogenic forms of the virus typically cause no apparent disease.¹ However, large quantities of virus can be shed in feces contaminating the environment and increasing the likelihood of subsequent exposure of other avian and non-avian species to AI virus.

European starlings (*Sturnus vulgaris*) were introduced to the United States (US) from Europe, and have since become highly adapted and widely distributed in North America. They are one of the frequently observed bird species reported on livestock operations in the US and typically congregate in large numbers on farms.² Ohio has one of the highest breeding density of European starlings in the

US.³ During the winter in Ohio, it is common to observe flocks of 500 to over 2000 birds, with some large winter roosts containing 400 000–600 000 birds.⁴ Starlings are very versatile and adaptive to multiple habitats that include agricultural operations, wetlands, and human residences. They are known to fly between 24–48 km to feed⁵ and may increase their flying distance from roosting sites to feeding areas farther away if a desirable source of food is plentiful in a more distant location.

Although extensive influenza surveillance studies have been conducted on wild waterfowl and terrestrial poultry, land-based wild birds have been one of the neglected species for influenza surveillance and little is known about the role of those birds including starlings in the ecology and epidemiology of AI virus. In previous studies, very limited surveillance, demonstrated that most of the starlings were

influenza virus or antibody negative.^{6,7} Experimentally, highly pathogenic H7N7 subtype viruses caused 100% mortality in starlings⁸ and, in contrast, Asian-lineage H5N1 isolates caused high levels of virus shedding in oropharyngeal swabs without killing birds,⁹ which suggest that starlings could act as an intermediate host and as a reservoir for highly pathogenic H5N1 influenza viruses that are currently endemic in many countries.

The preference of influenza viruses for different cellular receptors, and the distribution of those receptors in the host, are one of the essential factors involved in determining host range and tissue tropism.¹⁰ For example, AI virus replicates poorly in humans due to restrictions in receptor specificity.¹¹ In contrast, pigs carry both avian and human type of receptors in tracheal epithelium and are postulated to act as intermediate hosts,¹² in which avian and human viruses can re-assort and consequently generate viruses with the ability to overcome the host barrier. Recent evidence indicates that some terrestrial poultry may also provide an environment similar to the one in pigs by displaying both SA α 2, 3-gal and SA α 2, 6-gal receptors. Gambaryan *et al.*¹³ have demonstrated the presence of both SA α 2,3-gal and SA α 2,6-gal receptors in chickens, adding to the notion that chickens could act as a potential intermediate host for the transmission of influenza viruses from aquatic birds to humans. Quail (*Coturnix coturnix*) also express both type of receptors and therefore may also be involved in the interspecies transmission of influenza viruses.^{14,15}

In the present study, we collected 328 digestive and 156 tracheal samples from starlings in Ohio in years 2007–2008 and screened for the presence of influenza virus by real-time RT-PCR, standard RT-PCR and virus isolation using embryonated chicken eggs (ECEs). In addition, we conducted an experimental infection study to evaluate the replication and induction of antibody response by two low pathogenic AI viruses in starlings. Furthermore, we determined the distribution among avian type versus human type receptors in different tissues of starlings.

Materials and methods

Sample collection

Samples were collected from July of 2007 through August of 2008 from captured starlings by trained personnel, under approval from both the Ohio State University Institutional Animal Care and Use Committee and a scientific collection permit issued by the Ohio Department of Natural Resources. Mist nets and decoy traps were employed for live bird capture at different farm sites in nine Ohio counties: Wayne, Holmes, Ashland, Stark, Medina, Knox, Tuscarawas, Huron, and Carroll. All the birds appeared clinically healthy upon capture and were euthanized immediately. Bird carcasses were transported chilled to the

Table 1. Starling samples collected between July 2007 and August 2008

Year	Month	No. of samples		Positive sample*
		Intestine	Trachea	
2007	July	63		#62
	August	39		
	September	48		#110 & #113
	October	60	32	#176 & #212
	November	38	44	
2008	February	30	30	
	March	36	36	
	June	8	8	
	July	6	6	
	Total	328	156	

*Positive samples that NS gene sequences were determined are shown.

laboratory for processing. Entire digestive tract from individual bird were removed and put into 9 ml of buffered peptone water (BPW). The tissues in BPW were placed in stomacher for 2 minutes and 1 ml of aliquot was used for influenza virus detection. Individual tracheal tissues were collected in 1 ml of phosphate buffered saline (PBS) containing gentamicin. Collected tracheal samples were freeze and thaw three times.

A total of 328 digestive and 156 tracheal samples were obtained for this study (Table 1).

Virus isolation

Virus isolation procedure was conducted using ECEs obtained from specific pathogen free (SPF) flock maintained at Food Animal Health Research Program (Wooster, Ohio) as previously described.¹⁶ Briefly, 200 μ l of tissue samples prepared as described above were inoculated into two 9–11 day-old ECEs. At 3 days post-inoculation (DPI), allantoic fluid was collected and the hemagglutination (HA) test was performed to determine the presence of the virus. If the HA test was negative, the pooled allantoic fluid was passaged one additional time in ECE and tested for the presence of virus as described.¹⁶

Real-time and standard RT-PCR

Viral RNA was extracted from 100 μ l of tissue samples described above using the QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA, USA), following manufacturer's instructions. Real-time RT-PCR (RRT-PCR) was performed as previously described using the Qiagen one-step RT-PCR kit (Qiagen) in a 25- μ l reaction mixture containing probe and primers specific for the matrix gene.¹⁷ RRT-PCR was performed with smart cycler (Cepheid, Sunnyvale, CA,

USA). Samples that showed cycle threshold (CT) value of less than 40 were considered potential positives and subjected to standard RT-PCR for further confirmation of presence of influenza viral RNA. Standard RT-PCR was carried out with a one-step RT-PCR kit (Qiagen) with NS gene specific primer set: NS+1 5'-TATTCGTCTCAGGGAG CAAAAGCAGGGTG-3' and NS-890 5'-ATATCGTCTCGT ATTAGTAGAAACAAGGGTGT-3'. The RT-PCR conditions were 50°C for 30 minutes, then 95°C for 15 minutes, followed by 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 1.5 min, and a final 10 minutes incubation at 72°C.

Sequencing and phylogenetic analysis of NS genes

The RT-PCR product was separated on an agarose gel by electrophoresis, and amplicons of the appropriate size were subsequently excised from the gel and extracted with a Gel Extraction Kit (Qiagen). Depending on the concentration of the sample, gel purified products were either sequenced directly or after cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA, USA). BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST>) were conducted on each sequence to identify related reference NS gene. Sequence comparisons to selected NS genes were conducted by using the Megalign program using the Clustal V alignment algorithm (DNASTAR, Madison, WI, USA), and phylogenetic relationships were estimated through a bootstrap trial of 1000. The nucleotide sequences of NS genes from starling samples have been deposited in the GenBank database (accession numbers HM212769–HM212771).

Experimental infection study in starlings

Based on NS gene sequence data which showed close relationship between sequences from starling and other birds in Ohio, two AI viruses, A/chicken/OH/494832/2007 (H2N3) and A/Northern Shoveler/OH/28926-3/2007 (H4N2), isolated from back yard poultry and wild aquatic birds, respectively, in Ohio were used in an experimental infection study. Eighty-one starlings were captured as described above in March of 2009 and confirmed to be seronegative for influenza virus by agar gel immunoprecipitation and hemagglutination inhibition (HI) tests against 13 HA subtypes (H1–H13).^{16,18}

Birds were held for a week of quarantine period, wherein they were observed daily and judged to be in good health on the basis of food intake, behavior, and absence of clinical signs. The birds were divided into three groups and housed in three different BSL-2 biocontainment rooms. Two groups of 12 birds each were inoculated intranasally with 10⁶ 50% egg infectious dose (EID₅₀) of H2N3 and H4N2 viruses, respectively. One additional group of eight birds were inoculated with PBS and served as negative control. Tracheal and cloacal swab samples were collected in

2 ml of PBS containing gentamicin on 2, 4, and 7 days post-infection (DPI) from all birds. RNA was extracted from 100 µl of swab samples as described above and quantitative RRT-PCR was performed as described previously.¹⁹ Virus isolation was conducted as described above.

Serum samples were collected at 14 DPI and HI antibody titers were determined according to standard methods using 1% turkey erythrocytes and four HA units of homologous virus.¹⁶

Lectin immunostaining for receptor detection in tissues

Tissue sections (trachea, lung, small and large intestine, muscle and kidney) from 10 starlings were collected, fixed in 10% neutral buffered formalin, and embedded in paraffin. Tissues were cut into 3–5 µm thick sections, mounted on 3-aminopropyltriethoxy-silane-coated slides, deparaffinized in xylene, and rehydrated in alcohol, then followed by incubating with 3% H₂O₂ in methanol for 30 minutes and washing with water and PBS each for 1 minutes. Subsequently, the sections were blocked with 1% BSA in PBS or PBS with Tween 20 (PBST) for 40 minutes. For detection of receptors, the sections were incubated with digoxigenin (DIG)-labeled *Maackia amurensis* agglutinin (specific for SA α 2,3-gal) or *Sambucus nigra* agglutinin (specific for SA α 2,6-gal) (DIG Glycan Differentiation kit; Roche Applied Science, Mannheim, Germany) overnight at 4°C. After washing with TBS, the sections were incubated with peroxidase-conjugated anti-DIG Fab fragments (Roche Applied Science) containing 1% BSA for 90 minutes. After washing with PBS, the sections were developed in a solution of diaminobenzidine for colorization for 1–2 minutes, counterstained with Meyer's hematoxylin for 5 minutes, washing with running tap water over 30 minutes, mounted and observed with a light microscope.

Statistical analyses

To determine the statistical significance of viral shedding titer differences between tracheal and cloacal swab samples and also between groups of birds infected with H2N3 or H4N2 viruses, two-sample *t*-test was performed. Results were considered to be statistically significant if the comparison gave a *P*-values of <0.05.

Results

Detection of influenza virus in tracheal and intestinal samples

Eighteen digestive tract (out of 328) and three tracheal (out of 156) samples tested positive by RRT-PCR screening and confirmation by standard RT-PCR. The CT values obtained from RRT-PCR ranged between 29.75 and 38.65. More than 80% of the positive samples (15 out of 18)

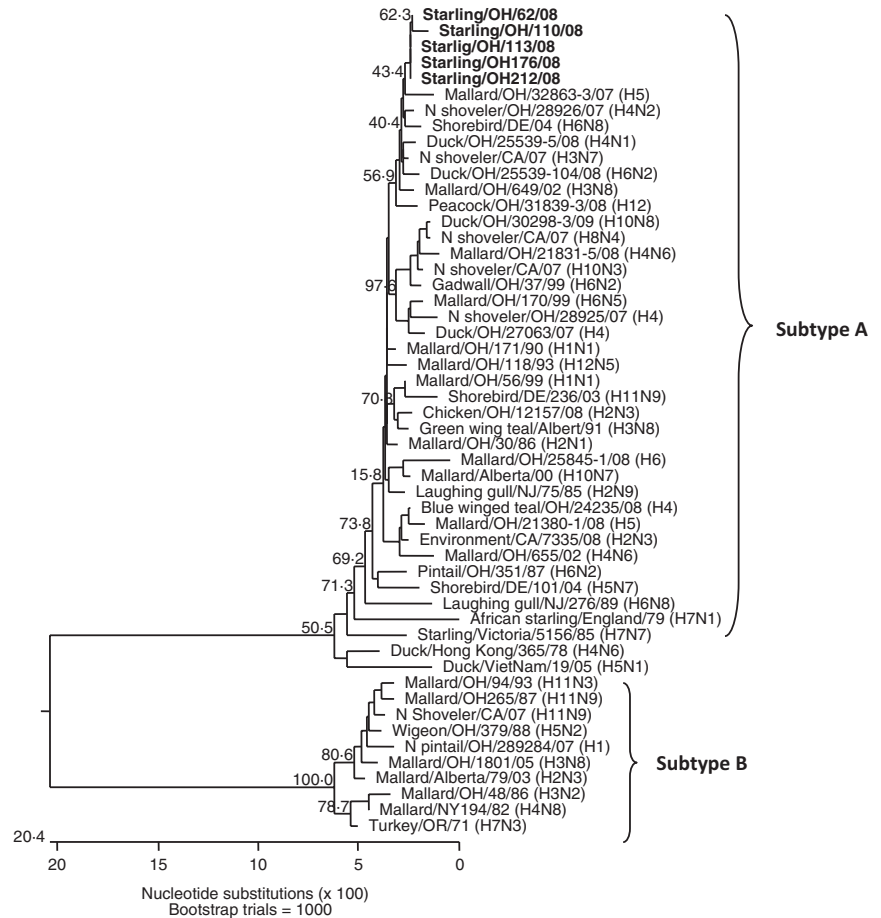


Figure 1. Phylogenetic tree based on nucleotide sequences of the NS gene from starling samples and other reference strains obtained from the GenBank Database. Bootstrap values on major nodes are shown. Standard two-letter abbreviations are used for states in the United States.

showed CT values higher than 36.00 which approximately correlate to EID_{50} titer of 10 or less. Despite the two blind passages of samples in SPF ECEs, virus isolation was unsuccessful.

NS gene sequence analysis

We were able to determine the sequences of NS genes (852 bp from position 21 to 872) recovered from five starling samples. All samples, in which we were able to determine the NS gene sequences, were collected from farms within same county in Ohio. Sequence analysis revealed that three NS genes were identical (113, 176, 212) and shared more than 99% similarity with two other NS genes (62 and 110). Starling origin NS genes were most similar (at least 97% similarity) to NS genes from wild aquatic bird origin influenza viruses isolated from Ohio [mallard/OH/32863-3/07 (H5), duck/OH/25539-5/08 (H4N1) and Northern shoveler/OH/28926/08 (H4N2)]. Phylogenetically all starling NS genes belong to NS subgroup A and cluster with wild bird origin NS genes from different states in the US (Figure 1). NS genes from two previously identified starling isolates, African Starling/England/79

(H7N1) and Starling/Victoria/5156/85 (H7N7), were distantly located from American wild bird NS gene cluster.

Experimental infection of starlings with H2N3 (chicken strain) and H4N2 (shoveler strain) AI viruses

Two AI viruses isolated from Ohio were used to test the susceptibility of the starlings. No birds showed either clinical signs or mortality during 14 day observation period after infection. Influenza virus was detected from tracheal and cloacal swabs from most of the infected starlings although the average titers from different time points of sample collections were less than $10^{2.76}$ EID_{50} per 0.2 ml (Table 2). H4N2 infected birds shed higher average titer of virus compared to H2N3 infected birds from swab samples collected at three different time points and the difference at 7 DPI samples was statistically significant. In both infection groups, we observed higher viral shedding titer from cloacal swab samples compared to tracheal swab samples and the difference was statistically significant at 2 and 4 DPI. The shedding of infectious virus was further confirmed by virus isolation in eggs. Approximately 40% and 60% of the

Table 2. Replication of low pathogenicity avian influenza viruses in European starlings

Virus	Log EID ₅₀ /0.2 ml + SD (no. positive/no. tested)***				Log ₂ HI titer + SD [†]
	Swabs	2 DPI	4 DPI	7 DPI	
H2N3	Trachea	1.26 + 0.08 (3/12)*	1.06 + 0.44 (4/12)	0.42 + 0.33 (8/12)**	4.92 + 1.16
	Cloaca	2.22 + 0.48 (7/12)*	1.66 + 0.44 (3/12)	0.87 + 0.46 (5/12)**	
H4N2	Trachea	1.32 + 0.99 (5/12)*	1.20 + 0.77 (9/12)*	1.25 + 0.33 (9/12)**	5.42 + 0.90
	Cloaca	2.76 + 0.94 (10/12)*	2.37 + 0.99 (3/12)*	1.72 + 0.42 (5/12)**	

HI, hemagglutination inhibition; DPI, days post infection.

* $P < 0.05$ between viral titers of tracheal and cloacal samples obtained at same DPI.

** $P < 0.05$ between viral titers of H2N3 and H4N2 infected groups.

***Virus titer is expressed as log₁₀ mean 50% egg infectious dose per 0.2 ml + standard deviation.

[†]The HI titer is expressed as the log₂ reciprocal of the endpoint in a twofold dilution of sera.

RRT-PCR positive tracheal and cloacal swabs from H2N3 and H4N2 infected birds, respectively, were found to be virus isolation positive (data not shown).

Serum samples were collected at 14 DPI and HI test showed that all birds were seroconverted. The average HI antibody titer was 4.92 and 5.42 log₂ for H2N3 and H4N2 infected groups, respectively, which were not statistically different.

Receptor distribution in starlings

The receptor distribution in different tissues was determined as the average percentage of positive staining observed by visual examination of three different fields of the tissue observed under 200× magnification of light microscope (Figure 2). On the tracheal epithelium, positive staining (>70%) for both α2,3SA-gal (avian-type) and α2,6SA-gal (human type) receptors were observed. On the contrary, in bronchi, the majority of epithelial cells (70%) were positive for avian type receptors, but negative for human type receptors. Among the different parts of intestines examined, the small intestinal sections showed sporadic and negligible staining for α2,3SA-gal and α2,6SA-gal receptors whereas sections of large intestine consistently showed >40% positive staining for α2,3SA-gal receptors and no or negligible staining for α2,6SA-gal receptors. The tubular cells of the kidney strongly expressed both avian and human type receptors (40–50%).

Discussion

Our surveillance of starlings in Ohio revealed that starlings do carry influenza viral genes. Several studies have shown that starlings may contribute to the epidemiology and spread of bacterial pathogens. In one study, *Campylobacter jejuni* strains isolated from starlings were indistinguishable from isolates obtained from humans.²⁰ In another study,

indistinguishable *Escherichia coli* O157 subtypes were isolated from two feedlots approximately 100 km apart and wild birds were determined to be potential vehicles shared between the two feedlots.²¹ Furthermore, molecular comparisons of *Salmonella* strains isolated from wild birds on commercial poultry operations demonstrated a shared common strain types between the wild birds and the laying hens.²²

The evidence cited above and scarcity of literature on influenza in starlings has led us to investigate the potential role of European starlings in the dissemination of influenza virus. However, it is unclear whether efficient viral replication occurs in starlings in natural conditions. The low level of viral RNA detected from starlings was approximately equivalent to <10 EID₅₀ of virus and explains in part the failure to isolate the live virus in this study. This also indicates that starlings may not serve as biological amplifier of the influenza viruses at least for those strains they encountered. Previous experimental studies showed that starlings shed large amounts of virus upon challenge with highly pathogenic AI (HPAI) virus and demonstrated clinical signs and even high mortality.^{8,9} However, no HPAI virus has been recovered from starlings in nature and due to extreme difference in pathobiology between HPAI and low pathogenicity AI (LPAI) viruses, experimental HPAI data in starlings cannot be used to predict the potential role of starlings in the epidemiology of AI viruses which are predominantly LPAI viruses. Our study involved a one-time serologic analysis of 81 starlings captured for an *in vivo* experiment and extensive serologic surveillance is necessary to complement the virologic surveillance in order to evaluate the potential role of starlings as biological amplifier of influenza virus.

Based on NS gene sequence analysis which showed close relationship between starling NS genes and recent wild waterfowl-origin NS genes from Ohio, we speculate that

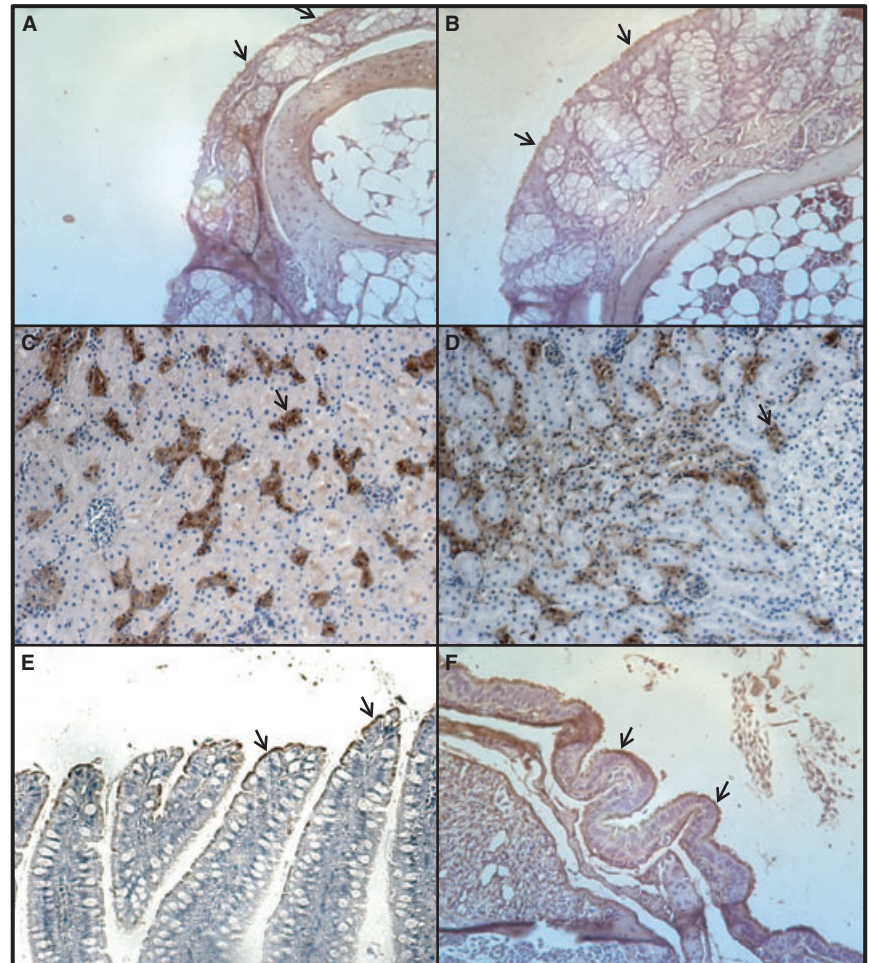


Figure 2. Distribution of α 2,3SA-gal (avian-type) receptors along the tracheal epithelium (A), kidney (C), colon (E), and bronchial epithelium (F) and α 2,6SA-gal (human-type) receptors along the tracheal epithelium (B) and kidney (D), respectively. Plant lectins, *M. amurensis* agglutinin and *S. nigra* agglutinin, were used for the detection of α 2,3SA-gal and α 2,6SA-gal receptors, respectively. Positive stainings are shown as dark brown color and some of them are indicated by arrow.

the starlings may have close contact with those birds. However, due to an extensive reassortment occurring in influenza viruses, it is also possible that only NS gene may have been derived from wild aquatic bird viruses and other genes from a totally different origin.

To address some of the questions raised by surveillance data, experimental infection studies were conducted using two LPAI strains of different origin as challenge viruses: one isolated from wild aquatic birds and the other from poultry. All the infected birds seroconverted and showed relatively high HI antibody titer (Table 2). Although not statistically significant, Shoveler strain (H4N2) infected birds showed slightly higher HI antibody titer than birds challenged with chicken strain (H2N3). In agreement with the serologic results, more viral shedding in the trachea and cloaca was observed from H4N2 infected birds than H2N3 infected birds. In our previous studies, we observed that ducks in general produced lower level of antibody upon LPAI infection compared to turkeys or chickens. In addition, ducks supported low level of virus replication

mainly in the digestive tract upon LPAI virus challenge (unpublished data).^{23,24} Although limited strains both in origin and subtype were used in this study, the level of virus replication and humoral immune response in starlings upon LPAI infection is similar to what we observe in ducks upon LPAI infection compared to those in chickens or turkeys.

Influenza viruses attach to host cells through interactions of the viral hemagglutinin with sialic acid terminated oligosaccharide residues on host cells. Using specific sialic acid determinants generated by sialyltransferases, human and avian viruses were found to preferentially bind to α 2,6SA-gal (human type) and α 2,3SA-gal (avian type) receptors, respectively.^{25,26} The presence of avian and human type receptors on the tracheal epithelium of the starling (Figure 2) indicate that they may be susceptible to infection with both avian and mammalian influenza viruses. Similar receptor distribution was also observed in chicken, turkey, duck, and quail which indicate although diverse influenza viruses may succeed in initial infection of all these species,

other host factors may prevent or restrict the sustained replication of certain viruses in specific hosts. In all these species, avian type receptors were observed in the trachea and bronchial epithelium and their absence in other parts of lung might explain the localization of LPAI viruses in the upper respiratory tract.

In accordance with receptor distribution in turkeys and ducks,²⁷ only avian type receptors were predominant and were mostly restricted to the large intestines. In contrast to chickens and quail, no or sporadic human type receptors, which may be due to non-specific binding, were observed in intestinal tissue sections. In the experimental infection study (Table 2), although birds were infected through the intranasal route significantly greater amounts of virus were recovered from cloacal swabs than tracheal swabs. Both receptor distribution and replication data indicate that the intestine is the major target for influenza virus replication in starlings as in wild aquatic bird species.

In summary, our study demonstrated that starlings can carry influenza virus. We also demonstrated experimentally that they could be infected with LPAI viruses, shed virus, and seroconvert. European starlings form aggregations with other bird species, and become more and more adapted to living in an urban environment and animal agricultural settings. Consequently, starlings that acquired AI virus in a marsh from scavenging a diseased bird or feed contaminated with feces from other wild birds could easily transport and transmit the virus to poultry and swine operations and to residential areas. The risks associated with starlings in influenza virus transmission to other animals and human are unknown, but clearly warrant further investigation.

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