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Low Pathogenicity Avian Influenza Viruses Infect Chicken Layers by Different Routes of Inoculation

Mary J. Pantin-Jackwood,[A] Diane M. Smith, Jamie L. Wasilenko, and Erica Spackman

Exotic and Emerging Avian Viral Diseases Research Unit, Southeast Poultry Research Laboratory, Agricultural Research Service, United States Department of Agriculture, 934 College Station Road, Athens, GA 30605

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SUMMARY. In order to develop better control measures against avian influenza, it is necessary to understand how the virus transmits in poultry. In a previous study in which the infectivity and transmissibility of the pandemic H1N1 influenza virus was examined in different poultry species, we found that no or minimal infection occurred in chicken and turkeys intranasally (IN) inoculated with the virus. However, we demonstrated that the virus can infect laying turkey hens by the intracloacal (IC) and intraoviduct (IO) routes, possibly explaining the drops in egg production observed in turkey breeder farms affected by the virus. Such novel routes of exposure have not been previously examined in chickens and could also explain outbreaks of low pathogenicity avian influenza (LPAI) that cause a decrease in egg production in chicken layers and breeders. In the present study, 46-wk-old specific-pathogen-free chicken layers were infected by the IN, IC, or IO routes with one of two LPAI viruses: a poultry origin virus, A/chicken/CA/1255/02 (H6N2), and a live bird market isolate, A/chicken/NJ/12220/97 (H9N2). Only hens IN inoculated with the H6N2 virus presented mild clinical signs consisting of depression and anorexia. However, a decrease in number of eggs laid was observed in all virus-inoculated groups when compared to control hens. Evidence of infection was found in all chickens inoculated with the H6N2 virus by any of the three routes and the virus transmitted to contact hens. On the other hand, only one or two hens from each of the groups inoculated with the H9N2 virus shed detectable levels of virus, or seroconverted and did not transmit the virus to contacts, regardless of the route of inoculation. In conclusion, LPAI viruses can also infect chickens through other routes besides the IN route, which is considered the natural route of exposure. However, as seen with the H9N2 virus, the infectivity of the virus did not increase when given by these alternate routes.

RESUMEN. Los virus de la influenza aviar de baja patogenicidad infectan a gallinas de postura por diferentes vías de inoculación.

Con el fin de desarrollar mejores medidas de control contra la influenza aviar, es necesario entender cómo se transmite el virus dentro de la avicultura comercial. En un estudio previo en el que se examinó la infectividad y la transmisibilidad del virus de la influenza pandémico H1N1 entre diferentes especies de aves, se encontró que la infección es nula o mínima en pollos y pavos inoculados con este virus por vía intranasal. Sin embargo, se ha demostrado que el virus puede infectar pavas reproductoras en producción por las rutas intracloacal e intraoviductal, lo que puede explicar las bajas en la producción de huevos observadas en las granjas de pavos reproductoras afectadas por el virus. Estas nuevas rutas de exposición no habían sido previamente examinadas en los pollos y también podrían explicar los brotes de influenza aviar de baja patogenicidad, que causan una disminución en la producción de huevos en las gallinas de postura y en las aves reproductoras. En el presente estudio, se infectaron aves de postura de 46 semanas de edad libres de patógenos específicos por las rutas intranasal, intracloacal, o intraoviductal con uno de dos virus de la influenza aviar de baja patogenicidad: un virus de origen aviar, el aislamiento denominado A/pollo/CA/1255/02 (H6N2), y un aislamiento obtenido de un mercado de aves vivas, A/pollo/NJ/12220/97 (H9N2). Solamente las gallinas inoculadas por vía intranasal con el virus H6N2 presentaron leves signos clínicos consistentes en depresión y anorexia. Sin embargo, se observó disminución en el número de huevos producidos en todos los grupos inoculados con los virus cuando se compararon con las gallinas del grupo control. Se encontró evidencia de infección en todos los pollos inoculados con el virus H6N2 por cualquiera de las tres rutas y el virus se transmitió a gallinas expuestas por contacto. Por otro lado, sólo una ó dos gallinas procedentes de cada uno de los grupos inoculados con el virus H9N2 eliminaron virus con niveles detectables o seroconvirtieron y no transmitieron el virus a las aves expuestas por contacto, independientemente de la vía de inoculación. En conclusión, los virus de influenza aviar baja patogenicidad pueden infectar a los pollos a través de otras vías, además de la vía intranasal, que se considera la vía natural de exposición. Sin embargo, como se observó con el virus H9N2, la infectividad del virus no aumentó cuando se administró por estas rutas alternas.

Key words: low pathogenic avian influenza, H6N2 type, H9N2 type, routes of inoculation, chickens

Abbreviations: AI = avian influenza; BHI = brain heart infusion; C = cloacal; dpi = days postinoculation; EID_{50} = 50% egg infectious dose; HI = hemagglutination inhibition; HPAI = highly pathogenic avian influenza; IC = intracloacal; IHC = immunohistochemistry; IN = intranasal; IO = intraoviduct; LPAI = low pathogenicity avian influenza; OP = oropharyngeal; pH1N1 = pandemic H1N1; qRRT-PCR = quantitative real-time reverse transcriptase-polymerase chain reaction; SEPRL = Southeast Poultry Research Laboratory; SPF = specific-pathogen-free

Avian influenza (AI) continues to be a threat to commercial poultry. AI viruses occasionally transmit from their natural reservoirs, wild aquatic birds, to domestic birds producing subclinical infections and sometimes respiratory disease and drops in egg production. These viruses are typically termed low pathogenicity AI (LPAI) viruses and can be a combination of most 16 hemagglutinin and nine neuraminidase subtypes. Some H5 and H7 LPAI viruses, after circulating in domestic poultry, mutate into highly pathogenic AI (HPAI) viruses and cause high mortality (34). Most LPAI viruses produce subclinical infections in experimental

[A]Corresponding author. E-mail: Mary.Pantin-Jackwood@ARS.USDA.gov

studies but, under commercial rearing conditions and probably complicated by secondary pathogens, environmental stress, and immunosuppression, these viruses can also produce mild to moderate disease. Secondary bacterial, fungal, or viral infections are usually necessary to produce sufficiently severe respiratory damage to result in illness or death; however, some LPAI virus strains, such as certain Asian H9N2 lineages adapted to efficient replication in poultry, can cause more-prominent signs and also significant mortality (1,15,24).

Typically, LPAI viruses have limited local replication in the respiratory and alimentary tracts (33). Rarely, LPAI viruses have spread outside the respiratory or alimentary tract causing infection and damage in epithelial-containing tissues of visceral organs such as kidney, pancreas, and oviduct. Clinical signs will differ with the virus strain, host species, and age of the host and pathophysiologic changes can be found in the respiratory, digestive, urinary, and reproductive systems (18,33).

The intranasal (IN) route of exposure is routinely used to emulate the natural route of infection for AI viruses in experimental settings (31). However, in studies done to evaluate the pathogenicity of the 2009 pandemic H1N1 (pH1N1) virus in breeder turkeys, infection was not produced easily by IN inoculation. Therefore, intracloacal (IC) and intraoviduct (IO) inoculation were tested in an attempt to explain infection of breeder turkeys with the virus and were successful in infecting the turkeys (19,35). In another study, more than 50% of 8-to-10-wk-old turkeys inoculated by the cloacal (C) route with the pH1N1 virus became infected and virus replication was demonstrated in the cecal tonsil and bursa (2). Cloacal exposure has been shown to be important to the transmission of other pathogens in poultry. For example, cloacal contact with feces that have been contaminated with *Histomonas meleagridis* is thought to be one way in which blackhead is transmitted from bird to bird (14), and IC inoculation or vaccination has been used experimentally with Newcastle disease virus and infectious laryngotracheitis virus (4,5,26). Infection following alimentary tract exposure has also been demonstrated in chickens and ducks infected with an H5N1 HPAI virus (11). The initiation of H5N1 HPAI virus infection in birds is still favored by a respiratory route of exposure, but alimentary initiated infection is possible if birds consume high doses of virus, such as by cannibalizing infected carcasses (11). Little is known about the ability of LPAI viruses to infect chickens by alternate routes of exposure.

LPAI viruses are known to affect lay of eggs and replicate in the reproductive tract of turkeys and, as with turkeys infected with the pH1N1 virus, drops in egg production are commonly reported with LPAI virus outbreaks in turkey breeders (8,13,15,22,29,32,37). Decreases in egg production have also been observed in chicken breeders and layers infected with LPAI viruses (3,12,15,24,40). In 2000, an outbreak of H6N2 LPAI occurred in California and 12 separate incidences were reported primarily involving layer-type birds, but the virus was also isolated from backyard chickens and a primary broiler breeder (7,39). Drops in egg production and increased mortality were among the clinical signs reported in the layer flocks. Yolk peritonitis was a feature also described (39).

The IO route of exposure provided an explanation of how turkey breeders became infected with the pH1N1 virus through the practice of artificial insemination and highlighted the importance of exploring alternate routes of infection for AI viruses in poultry (19). In 1970, Samadieh and Bankowski showed that egg production was severely affected in laying turkey hens inseminated with semen contaminated with a turkey influenza virus (25), but no other studies have been published exploring the transmission of AI virus by semen. The presence of virus antigen has been found in testicles of birds infected with HPAI viruses (6; Pantin-Jackwood, pers. obs.) but has not been reported for LPAI viruses.

To evaluate whether LPAI viruses could infect chickens through the cloacal route and reproductive tract, we inoculated chicken layers with two different LPAI viruses given by three different routes, IN, IC, and IO, and examined them for the presence of clinical signs, gross and microscopic lesions, virus in tissues, virus shedding, seroconversion, and transmission to contacts.

MATERIALS AND METHODS

Viruses. Two LPAI viruses from the Southeast Poultry Research Laboratory (SEPRL) repository were used in this study: A/chicken/CA/1255/02 (H6N2) and A/chicken/NJ/12220/97 (H9N2). The H6N2 virus was isolated from 118-week-old layers (39) and the H9N2 virus was a chicken isolate from a live bird market. The H6N2 virus was chosen because of its history of producing drops in egg production in layer flocks and its known ability to infect chickens experimentally (10,39). The H9N2 isolate had not been previously examined in chickens and was chosen to see if the route of inoculation would have an effect on infectivity in hens. The viruses were propagated in 9-day-old specific-pathogen-free (SPF) embryonating chicken eggs and titrated as described (27,38). Allantoic fluid was harvested from the eggs 48 hr postinoculation and diluted in brain heart infusion (BHI) medium (BD Bioscience, Sparks, MD) in order to obtain 10^6 50% egg infectious dose (EID_{50}) per 0.1 ml/bird. A sham inoculum was made using allantoic fluid from noninfected eggs diluted 1:300 in BHI. All experiments were performed in biosecurity level-2 enhanced facilities at SEPRL.

Experimental design. Seventy 46-wk-old, SPF white leghorn chickens (*Gallus domesticus*) in lay, from SEPRL's in-house flock, were used in this study. The hens were housed in self-contained isolation units that were ventilated under negative pressure with inlet and exhaust HEPA-filtered air and maintained under continuous lighting. Feed and water were provided with *ad libitum* access. Hens were cared for in accordance to SEPRL's Institutional Animal Care and Use Committee-approved animal use protocol. Hens were acclimated to the isolators for 3 days before the beginning of the study to ensure that they were all laying eggs.

Hens were divided into 7 groups of 10 birds each. Hens from groups 1–3 were inoculated with 0.1 ml of inoculum containing 10^6 EID_{50} of the H6N2 virus IN via the choanal slit ($n = 10$), IC by applying the inoculum to the cloacal lips ($n = 10$), and IO by depositing the virus inside the vagina ($n = 10$). Hens from groups 4–6 were similarly inoculated: IN ($n = 10$), IC ($n = 10$), and IO ($n = 10$) with 0.1 ml of inoculum containing 10^6 EID_{50} of the H9N2 virus. Hens from group 7 were IN inoculated with sham inoculum ($n = 10$; Table 1).

Hens were observed daily for clinical signs of disease and laid eggs were counted. Eggs were collected at 4 days postinoculation (dpi) for virus detection. Oropharyngeal (OP) and C swabs were collected at 2, 4, 7, and 14 dpi from all birds to determine viral shedding by quantitative real-time RT-PCR (qRRT-PCR). Swab samples were suspended in 2 ml sterile BHI broth containing an antibiotic-antimycotic mixture and frozen at −70 C until processing. Two hens from each group were euthanatized at 3 dpi with 0.2 ml sodium pentobarbital (5 g/ml) per bird, gross lesions recorded, and the following tissues: lung, spleen, heart, kidney, and oviduct, were collected separately in BHI for virus isolation and stored frozen at −70 C. Trachea, lungs, bursa, kidneys, adrenal gland, thymus, brain, liver, heart, ventriculus, pancreas, intestine, spleen, ovary, oviduct, beak, and thigh tissue were also collected at necropsy. Tissues were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. Immunohistochemistry (IHC) was used to stain duplicate sections to determine influenza viral antigen distribution in individual tissues as described previously (17). Two new hens were added to each group at 3 dpi, serving as contacts to study virus transmission. At 14 dpi, all the hens were bled, euthanatized, and necropsied to examine the oviduct.

Table 1. Number of eggs laid by hens inoculated through different routes with 10^6 EID_{50}/0.1 ml of H6N2 or H9N2 viruses or by sham-inoculated controls.

Groups[A]	No. eggs laid 1 to 7 dpi	No. eggs laid 8 to 14 dpi	Total no. of eggs	No. of hens with active oviducts at 14 dpi
1. H6N2 IN	21	6	27	3/10
2. H6N2 IC	26	15	41	4/10
3. H6N2 IO	19	10	29	4/10
4. H9N2 IN	19	11	30	6/10
5. H9N2 IC	21	15	36	6/10
6. H9N2 IO	25	10	35	7/10
7. Controls	33	23	56	8/10

[A]IN = intranasal; IC = intracloacal, IO = intraoviduct.

Serology. Hemagglutination inhibition (HI) assays were performed with serum collected from chickens 14 days after virus challenge to quantify the antibody response. The assay antigens were prepared by inactivating the allantoic fluid from virus-infected chicken eggs with 0.1% beta-propriolactone and adjusting the pH to 7.0 with sodium-bicarbonate. The HI assays were performed in accordance with standard procedures (20). Titers were calculated as the reciprocal of the last HI-positive serum dilution, and samples with HI titers of 4 (2^2) or below were considered negative. A one-way ANOVA with a Tukey's post-test was used to analyze HI titers, after confirming normality, using Prism v.5.01 software (GraphPad Software Inc., La Jolla, CA) and values expressed as the mean ± SD ($P < 0.05$).

Virus detection. RNA was extracted from OP and C swabs using a previously described combination of Trizol LS reagent (Invitrogen Inc., Carlsbad, CA) and the MagMax AI/ND RNA isolation kit (Ambion, Inc., Austin, TX) (9). A qRRT-PCR targeting the influenza M gene (30) was performed using the SmartCycler® 2 (Cepheid Inc., Sunnyvale, CA) and the OneStep RT-PCR kit (Qiagen®, Valencia, CA). A standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of the viruses in duplicate and results reported as EID_{50}/ml equivalents (21). The calculated qRRT-PCR detection limit was 10^2 EID_{50}/ml per reaction.

Tissues (lung, spleen, heart, kidney, and sections of the oviduct) were normalized by weight and homogenized in BHI medium, and titers of infectious virus were subsequently determined in embryonated chicken eggs as previously described (36). Similarly, albumin collected from eggs laid on 4 dpi was also examined for the presence of virus. The minimal detectable titer was $10^{0.9}$ EID_{50}/ml from swabs and $10^{1.97}$ EID_{50}/g from tissues.

RESULTS

Clinical signs and gross lesions. Only hens IN-inoculated with the H6N2 virus presented clinical signs consisting of mild depression and a decrease in feed consumption which lasted until 7 dpi. The hens from all groups were laying at the beginning of the experiment and eggs were collected from all groups until the end of the study. However, a decrease in the number of eggs laid was observed in most groups during the second week of the study (Table 1). A decrease in total eggs laid was observed in all virus-inoculated groups when compared to sham-inoculated controls. Hens inoculated with the H6N2 virus produced, in total, 27%–52% fewer eggs and hens inoculated with the H9N2 virus produced 36%–46% less eggs. Hens inoculated with the H6N2 virus by the IN and IO route had the largest decrease in eggs laid. When oviducts were examined at the end of the experiment, 6–7 out of 10 hens per group inoculated with the H6N2 virus had stopped laying eggs *vs.* 3–4 hens per group from the H9N2-inoculated hens and two from the sham-inoculated hens.

Gross and microscopic lesions and viral antigen staining in tissues. Two hens per group were euthanatized at 3 dpi and necropsied. No gross lesions were observed in sham-inoculated control hens. As for the virus-inoculated hens, the only changes observed consisted of congestion of the oviduct. All hens examined had active oviducts. Tissues collected from the hens were also examined for microscopic lesions and viral antigen staining. No, or minimal, lesions were observed in most hens with the exception of hens IN-inoculated with the H6N2 virus in which the trachea presented mild to moderate degenerative changes of the epithelium and the lungs had mild congestion, mild interstitial inflammation with mixed mononuclear cells, and mild catarrhal bronchitis. Mild to moderate hyperplasia of the intestinal epithelium and mild proliferation of gut-associated lymphoid tissues was also present in many hens. Remaining organs, including the oviduct, lacked significant histopathologic lesions. No viral antigen staining, detected by IHC, was present in tissues of any of the hens examined.

Virus detection and serology. No virus was detected in swabs or tissues from the sham-inoculated control birds. The virus shedding and seroconversion data for the virus-inoculated groups is presented in Table 2. All hens became infected with the H6N2 virus when exposed by any of the three routes; IN, IC, or IO. This was demonstrated by seroconversion, with hens from all three groups showing antibodies to the virus at 14 days after inoculation. No statistical difference in HI titers was found between the groups (data not shown). However, differences in numbers of birds shedding virus were observed between hens inoculated with the H6N2 virus by the three different routes. Six to 8 out of 8–10 hens inoculated shed virus through the oropharyngeal route at 2, 4, and 7 dpi, but only 3 of 8 hens shed virus through the cloaca and only at 4 dpi. In hens IC-inoculated, most viral shedding occurred at 4 dpi, and by

Table 2. Virus detection in OP and C swabs and seroconversion in hens inoculated through different routes with 10^6 EID_{50}/0.1 ml of H6N2 or H9N2 viruses.

	Virus shedding at 2 dpi; no. of positive hens/total hens (log_{10} titer)[B]		Virus shedding at 4 dpi; no. of positive hens/total hens (log_{10} titer)		Virus shedding at 7 dpi; no. of positive hens/total hens (log_{10} titer)		Seroconversion at 14 dpi
Groups[A]	OP	C	OP	C	OP	C	HI positive hens/ total hens (titer[C])
H6N2 IN	8/10 (4.8±.5[D])	0/10	8/8 (5.3 ± 0.5)	3/8 (3.0 ± 0.5)	6/8 (1.2 ± 0.3)	0/8	8/8 (6.6 ± 1.9)
H6N2 IC	1/10 (4.2)	3/10 (4.3 ± 0.8)	5/8 (4.3 ± 1.7)	4/8 (4.4 ± 0.1)	1/8 (2.1)	1/8 (1.8)	8/8 (5.5 ± 1.5)
H6N2 IO	1/10 (4.0)	2/10 (4.7 ± 0.4)	4/8 (4.1 ± 1.6)	6/8 (4.3 ± 1.1)	5/8 (2.9 ± 0.6)	4/8 (1.5 ± 0.6)	8/8 (6.8 ± 1.8)
H9N2 IN	–	–	1/8 (3.2)	0/8	0/8	0/8	1/8 (7)
H9N2 IC	–	–	0/8	0/8	0/8	0/8	2/8 (2)
H9N2 IO	–	–	0/8	0/8	0/8	0/8	2/8 (3.0±.7)

[A]IN = intranasal; IC = intracloacal; IO = intraoviduct.
[B]OP = oropharyngeal; C = cloacal.
[C]Mean log_2 HI titer of the sera ± standard deviation.
[D]Mean virus titer ± standard deviation for positive samples as determined by qRRT-PCR and reported as log_{10} EID_{50}/ml.

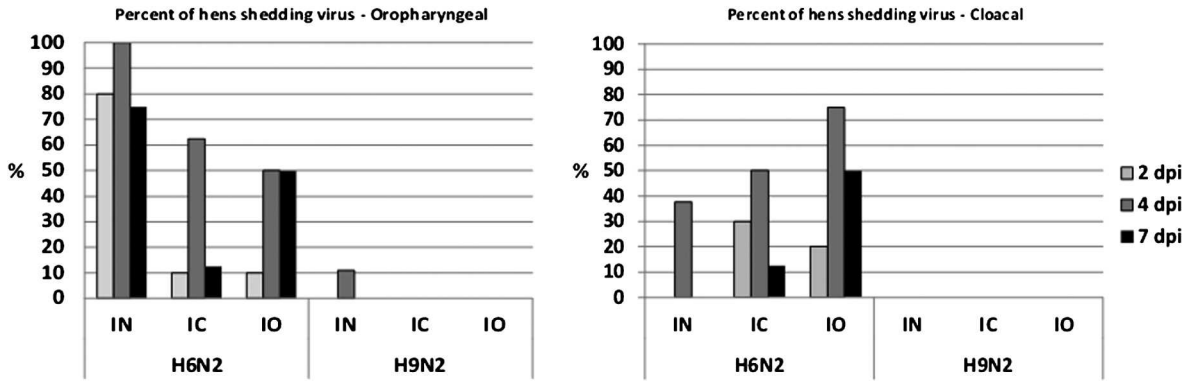

Fig. 1. Percentage of hens shedding virus at 2, 4, and 7 days after being inoculated through different routes with H6N2 or H9N2 viruses.

both the OP and the C route, but at 7 dpi only one hen was shedding virus. In hens IO-inoculated, virus was shed by half or more of the hens at both 4 and 7 dpi and by both routes. The highest virus titers were shed by the OP route from hens IN-inoculated. The percentage of hens shedding viruses at each time point can be better appreciated in Fig. 1. More than 75% of the hens IN-inoculated shed virus through the OP route at all time points, with 37% of hens shedding virus though the cloaca at only 4 dpi. Only 30% of the hens IC-inoculated shed virus initially though the cloaca, but virus was also detected in the oropharynx at 2, 4, and 7 dpi in 10%, 62.5%, and 12.5% of the hens, respectively. In addition, only 10%–20% of hens IO-inoculated shed virus at 2 dpi but more than 50% shed virus through both routes at 4 and 7 dpi. As for hens inoculated with the H9N2 virus, only 1 or 2 hens from each group shed detectable levels of the virus or seroconverted.

The two contact hens from the H6N2 IN- and IC-inoculated groups were positive for virus shedding at 4 days after being introduced into the isolators and seroconverted (Table 3). Only one of the contacts from the IO-inoculated group got infected. None of the contacts from the H9N2 groups showed evidence of infection.

Virus replication was also examined at 2 dpi in tissues collected from hens inoculated with the H6N2 LPAI virus. Virus was only detected in the lung of the two hens IN-inoculated (titers of 1.97 and 2.7 EID_{50}/g), in one hen IC-inoculated (titer of 1.97 EID_{50}/g), in the oviduct of one hen IN-inoculated (titer of 1.97 EID_{50}/g), and in one hen IO-inoculated (titer of 2.7 EID_{50}/g). No virus was detected in albumin samples from all eggs collected at 4 dpi. Tissues and eggs from hens inoculated with the H9N2 virus were not examined because most did not show evidence of infection by virus shedding and seroconversion.

Table 3. Virus detection in OP and C swabs of contact hens, examined at 4 days after introduction into the different groups, and HI titers measured at 12 days.

Groups[A]	Virus shedding; no. of positive hens/total hens (titer)[B]		Seroconversion; no. of positive hens/total hens (titer)
	OP	C	
H6N2 IN	2/2 (3.7, 3.1[C])	0/2	2/2 (7.0, 7.0[D])
H6N2 IC	2/2 (4.5, 3.2)	0/2	2/2 (6.0, 5.0)
H6N2 IO	1/2 (3.7)	0/2	1/2 (10.0)
H9N2 IN	–	–	0/2
H9N2 IC	–	–	0/2
H9N2 IO	–	–	0/2

[A]IN = intranasal; IC = intracloacal; IO = intraoviduct.
[B]OP = oropharyngeal; C = cloacal.
[C]Mean virus titers as determined by qRRT-PCR and reported as $\log_{10}$ EID_{50}/ml.
[D]Mean $\log_2$ HI titer of the sera ± standard deviation.

DISCUSSION

As demonstrated in this study, LPAI viruses can also infect chickens through other routes besides the IN route, which has been traditionally considered the natural route of exposure. The H6N2 virus infected all hens regardless of the route of inoculation and, although most hens didn't show signs of infection when given the H9N2 virus, one or two hens got infected when inoculated by any of the three routes. Hens inoculated IN with the H6N2 virus presented mild clinical signs and shed higher titers of the virus through the OP route at 2 and 4 dpi than did hens inoculated by the IC or IO routes. Interestingly, in hens inoculated with the virus by the IC or IO routes, the respiratory tract eventually got infected, reflected by virus shed by the OP route at 4 dpi. Likewise, hens IN-inoculated shed virus by the cloaca at 4 dpi. This most likely indicates infection by re-exposure to the virus by the different routes, probably by virus present in the isolators after being shed by the hens. The virus infected the hens when given through the cloaca or the oviduct; however, it is not clear in what tissues or cells the virus replicated in, as no virus staining was observed in tissues collected at 3 dpi. Nevertheless, the virus did not initially replicate as well when given by the IC or IO route when compared to the IN route, as more hens inoculated by this route were shedding virus at the earlier time point.

The H6N2 virus was originally isolated from 118-wk-old layers presenting with drops in egg production, increased mortality, and decrease of feed consumption (39). In our study, we saw a decrease in egg production in hens inoculated with this virus but only found evidence of virus replication in the oviduct of two of the hens examined at 3 dpi. However, at the end of the study, half of the hens inoculated with the H6N2 virus had stopped laying eggs compared to eight out of 10 of the sham-inoculated controls. Hens inoculated with the H9N2 virus also laid fewer eggs than did the controls, in spite of the lack of evidence of infection in most of the hens inoculated with this virus. A study examining the pathogenesis of an H4N8 LPAI virus in SPF laying hens indicated that this virus had a tissue tropism for the respiratory and urogenital systems, but lesions were only observed in the oviduct of some of the virus-inoculated hens at 5 and 8 dpi and none at 1.5 and 3 dpi (28). Similar to our study, no virus was isolated from the internal contents of eggs; however, virus was recovered from ovarian and oviduct tissues (28). Those authors concluded that AI virus isolated from the kidney,

magnum, and ovary of hens resulted from a localized infection of the respiratory tract and was not the result of systemic influenza infection but was probably the result of viral infection of the air-sac epithelium associated with the serosal surface of the internal organs. Our study indicates a second alternative: the direct or retrograde infection of the intestine and the oviduct by exposure to the virus by the IC or IO routes. Environmental exposure to the virus by the cloacal route is feasible, and IO exposure is a possibility in chicken breeders if the reproductive organs of the males are infected with the virus or via transmission by semen as reported previously for turkeys (25).

When conducting controlled experiments, it is difficult to reproduce the conditions present in the farms and many factors, including the number of birds used and the presence of secondary infectious agents, could explain the differences observed in the presentation of the disease. For instance, in breeder turkeys, LPAI infections commonly result in decreased egg production, and co-infection with other agents can worsen the clinical presentation and, in some cases, induce complete cessation of egg production (8,13,16,22,29,32,37). Environmental factors such as animal density and housing can also contribute to the differences in infection and transmission observed between the field and experimental situations (2). On the other hand, Pillai *et al.* showed that a triple reassortant H3N2 virus associated with drops in egg production in turkey breeders can, by itself, cause drastic reduction or complete cessation of egg production and pathology of the reproductive tract in IN-inoculated 26-wk-old breeder turkeys (22) and that this virus could be detected from internal egg contents following experimental infection (23). In our study, we did not observe virus staining in the oviduct as seen in studies in turkeys (19,22), and virus isolation from the oviduct or egg albumin was low or under the levels of detection. Therefore, it is not clear why the hens infected with the LPAI viruses produced fewer eggs than did the controls. One explanation could be that the mild disease produced by infection caused distress or affected feed and water consumption enough to affect lay. More studies are needed to better understand the effect of LPAI on the reproductive tract of hens.

In conclusion, LPAI viruses can also infect chickens when given by the IC or IO routes, can locally replicate, and can re-infect by the respiratory route, which is considered the natural route of exposure. This has implications in the transmission and pathogenesis of AI viruses in poultry and can inform future management decisions for control of the virus, among them better cleaning and disinfection of the premises and examination of alternate routes of transmission, including through AI virus-infected males.

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