The pathogenesis of H3N8 canine influenza virus in chickens, turkeys and ducks

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Accepted 20 July 2010. Published Online 25 August 2010.

Background
Canine influenza virus (CIV) of the H3N8 subtype has emerged in dog populations throughout the United States where it has become endemic in kennels and animal shelters in some regions. It has not previously been determined whether the canine-adapted H3N8 influenza virus can be transmitted to chickens, turkeys or ducks which are economically important animals that are susceptible to type A influenza virus from numerous species.

Methods
Four-week-old specific pathogen–free (SPF) chickens, 3-week-old SPF turkeys and 2-week-old commercial Pekin ducks were inoculated with $10^{5.2}$ 50% tissue culture infectious doses of CIV per bird by the intra-choanal route. The birds were observed daily, and at 2 and 4 days post-inoculation (DPI), two inoculated birds and one sham-inoculated control bird were euthanized and necropsied to evaluate gross lesions and to collect tissues for microscopic examination. Cloacal and oral swabs were collected at 2, 4 and 7 DPI to evaluate virus shed by real-time RT-PCR (rRT-PCR). Two weeks post-infection, sera were collected from all remaining birds for type A influenza antibody detection by hemagglutination inhibition assay.

Results
Clinical signs and gross lesions were not observed in any of the birds of any species nor did any seroconvert. Oral and Cloacal swab material was negative for virus by rRT-PCR.

Conclusions
Chickens, turkeys and Pekin ducks are not susceptible to infection with CIV by simulated respiratory exposure route with the dose administered.

Keywords
Canine influenza, chickens, ducks, pathogenesis, turkeys.

Introduction
Influenza A virus is known to infect a wide variety of avian and mammalian species.1 In recent years, several natural transmission events of influenza to dogs have been reported, including H3N8 in the United States in 2004,2,3 H3N8 in the UK in 20024 and an H3N2 in South Korea in 2007.5 Based on genetic composition, the H3N8 viruses likely originated by direct transmission from horses2 and the H3N2 virus may have resulted from direct transmission from birds.5 The H3N8 in the United States appears to have become adapted to dogs and has spread among dog populations throughout the country and is seen most often in facilities with dense dog populations such as animal shelters, and kennels. Morbidity is high, and mortality that tends to be low is frequently caused by secondary bacterial pneumonia. Clinical signs such as nasal discharge, fever, lethargy, cough and some fatality because of hemorrhagic lesions in the lungs, particularly in greyhounds, have been reported.2,3,6

It has not previously been determined whether H3N8 canine influenza virus (CIV) can be transmitted to chickens, turkeys or ducks. Although experimental work in species specificity is far from complete, turkeys seem to be susceptible to numerous influenza strains from both avian and mammalian species, chickens and ducks seem to be less susceptible to strains from mammals.7 Influenza infections in poultry are important because they impact animal health and agricultural trade, and control costs are very high. In this study, we experimentally inoculated three common poultry species, chickens, turkeys and Pekin ducks, with H3N8 canine influenza virus to evaluate their susceptibility and the pathogenesis of the virus in these avian species.
Materials and methods

Virus

MDCK-propagated A/Canine/PA/94930,1/06 (H3N8) CIV was provided by Dr. Ed Dubovi at Cornell University and was propagated in MDCK cells one additional time (3 passages total) to prepare the inocula. The virus was titrated in MDCK cells using standard methods.8

Pathogenesis studies in chickens and turkeys

A standard procedure for influenza pathogenesis in poultry was used. Twenty-five 4-week-old specific pathogen-free (SPF) White Leghorn chickens and 25 3-week-old SPF Small White Beltsville turkeys were obtained from in-house flocks. The birds were divided into treatment groups of sham inoculates (n = 5), direct inoculates (n = 10) and contact exposure birds (n = 10). The birds were housed in modified Horsfal isolators with ad libitum access to feed and water. All birds were individually tagged for identification, and serum was collected from 3 to 5 random birds to confirm that the birds had no H3 influenza antibodies by hemagglutination inhibition assay as described in the following sections.

The directly exposed birds were inoculated with 10^5.2 50% tissue culture infectious doses (TCID50) per bird in 100 μl by the intra-choanal route (to simulate respiratory transmission) of A/Canine/PA/94930, 1/06 H3N8 CIV. An equal number of contact exposure birds were placed in the same isolator with the direct inoculates at the time of inoculation. Phosphate-buffered saline was administered to sham inoculates in a volume and route identical to that of the direct inoculates.

The birds were observed daily for clinical signs and mortality. Oropharyngeal and cloacal swabs were collected 2, 4 and 7 days post-inoculation (PI) from all birds to evaluate the titers of infectious virus shed by the oral and cloacal routes by quantitative real-time RT-PCR. On days 2 and 4 PI, two birds from each exposure group were euthanatized and necropsied to evaluate gross lesions and to collect tissues for microscopic evaluation and immunohistochemistry. One sham inoculate was necropsied on day 2 PI. The remaining birds were bled for serum to evaluate antibody response and were euthanized 14 days PI.

Pathogenesis in ducks

Eighteen 2-week-old Pekin ducks were obtained from a commercial hobby bird hatchery and were inoculated and housed in an identical manner to the chickens and turkeys with the exception that there were 6 sham inoculates, 12 direct inoculates and no contact exposure ducks because fewer birds were available. Similar to turkeys and chickens, the ducks were observed daily for clinical signs and mortality. Oropharyngeal and cloacal swabs were collected 2, 4 and 7 days PI from all birds to evaluate the titers of infectious virus shed by the oral and cloacal routes by quantitative real-time RT-PCR. On days 2 and 4 PI, two birds from each exposure group were euthanatized and necropsied to evaluate gross lesions and to collect tissues for microscopic evaluation and immunohistochemistry. One sham inoculate was necropsied on day 2 PI. The remaining birds were bled for serum to evaluate antibody response and were euthanized 14 days PI.

Hemagglutination inhibition assay

Serum collected 14 days PI was tested by hemagglutination inhibition (HI) assay using standard methods with chicken red blood cells.9 Negative control serum was normal chicken serum collected from uninfected SPF chickens, and the positive control serum was hyperimmune chicken serum to A/turkey/England/1969 (H3N2). The same stock of A/Canine/PA/94930,1/06 (H3N8) used as inoculum was used as the antigen source. Titers ≤8 were considered negative.

Real-time RT-PCR

Quantitative real-time RT-PCR was run with oropharyngeal and cloacal swabs from days 2, 4 and 7 PI. RNA was extracted from oropharyngeal and cloacal swabs as previously described,10 briefly RNA was extracted from cloacal and OP swabs by adding 750 μl Trizol LS (Invitrogen, Inc., Carlsbad, CA) to 250 μl swab material. The swab material–Trizol LS mixture was mixed by vortexing and incubated at room temperature for 5 minutes, and then 200 μl of chloroform was added. The material was mixed by vortexing, incubated at room temperature a minimum of 10 minutes and centrifuged for 15 min at 12 000 g. Instead of precipitation with 2-propanol according to the manufacturer’s instructions, the RNA extraction was completed by binding and eluting the RNA from the aqueous phase using the MagMAX 96 Al/ND Viral RNA isolation kit (Ambion, Inc., Austin, TX, USA) in accordance with kit instructions using the KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA, USA). A previously described real-time RT-PCR test targeting the matrix gene was used.11 The forward primer and probe matched the sequences of all available North American canine H3N8 influenza isolates 100%; there was one nucleotide mismatch at the 5' end of the reverse primer. The limit of detection per reaction was determined to be approximately 10^5 EID50 per reaction. Reactions were run on the AB 7500 FAST system with the Ambion Ag Path ID one-step RT-PCR kit as per the manufacturer’s instruction with 10 pmol of each primer and 3 pmol of probe. Cycling conditions were 45°C for 10 minutes, 95°C for 10 minutes and 45 cycles of 94°C for 1 second and 60°C for 30 seconds. Reactions were considered positive if there was a cycle...
threshold below 38.0; specimens with cycle thresholds between 38.0 and 45.0 are considered “suspect” and are processed for virus isolation and conventional RT-PCR for confirmation. A 10-fold dilution series of RNA from the stock of A/Canine/PA/94930, 1/06 was run in triplicate to establish a standard curve for virus quantitation.

**Results**

No clinical signs were observed in any species throughout the course of the experiment. Necropsies were performed 2 and 4 days PI, and no gross lesions were observed in any species at any time. No antibody to A/Canine/PA/94930, 1/06 (H3N8) was detected in sera from chickens, turkeys or ducks 14 days PI (Table 1). Oropharyngeal and cloacal swabs collected at 2 days PI were negative for virus by rRT-PCR (Table 1). Because there was no detectable antibody and the swabs from days 2, 4 and 7 PI were negative for virus by rRT-PCR, it was determined that examination of tissues for microscopic lesions was unnecessary. It was also concluded that since the birds did not seroconvert, that the virus would not have replicated to levels that could be detected in tissues.

**Discussion**

Type A influenza virus has a broad host range; however, individual isolates and lineages will often be adapted to a few specific host species, where transmission to a new host is relatively rare and sustained transmission in the new host is even less common. Influenza in poultry is highly controlled as infection of commercial stock can lead to severe economic impact, even with viruses that are not highly pathogenic; therefore, it is critical to understand which hosts may serve as potential reservoirs of the virus and what influenza virus lineages will transmit easily to poultry.

H3N8 canine influenza virus, like most avian influenza viruses, has been shown to preferentially bind the α2,3 sialic acid receptor, thus exhibiting a critical characteristic to be able to infect poultry. However, it is not entirely unexpected that H3N8 CIV did not replicate in poultry as infection of poultry with mammalian-adapted viruses often requires a high dose. However, there are exceptions such as some lineages of swine influenza that transmit easily to turkeys, therefore, one cannot predict how transmissible influenza lineages are among different species. Typically pathogenesis studies for influenza in poultry utilize a dose of $10^6$ TCID$_{50}$ or $10^6$ 50% egg infectious doses per bird; however, the CIV isolates used in this study were intentionally low passage in MDCK cells to minimize any adaptation to cell culture and did not grow to sufficient titers to achieve that dose. Importantly, infected dogs generally shed relatively low titers of the H3N8 influenza virus, which supports the improbability of this strain of CIV directly transmitting from dogs to poultry. However, we only evaluated a single isolate so it is possible that other isolates or lineages of H3N8 CIV could more readily transmit to poultry species. Additionally, it is possible that chicken embryo-passaged H3N8 CIV would more readily transmit to poultry; however, our aim was to simulate a natural transmission scenario; therefore, egg-passaged virus may have been artificially well adapted versus virus propagated in a canine cell line.

Although it is not possible to completely reproduce or even predict the conditions for dog-to-poultry transmission, the inability to infect chickens, turkeys or ducks with $10^5$ TCID$_{50}$ administered by a simulated respiratory route indicates that CIV is not easily transmitted to poultry species without direct exposure of birds to high titers of virus. Therefore, dogs should probably not be considered a critical source of influenza virus for poultry.

### Table 1. Detection of antibody to H3 influenza by hemagglutination inhibition assay 14 days post-inoculation and detection of virus by real-time RT-PCR in oral and cloacal swabs collected 2 days post-inoculation

| Species | HI assay | rRT-PCR Oropharyngeal swabs | Cloacal swabs |
|---------|----------|----------------------------|---------------|
|         | Direct inoculates | Contact exposure | Sham inoculates | Direct inoculates | Contact exposure | Sham inoculates | Direct inoculates |
| Chickens | 0/6* | 0/6 | 0/4 | 0/10 | 0/10 | 0/5 | 0/10 |
| Turkeys | 0/6 | 0/6 | 0/4 | 0/10 | 0/10 | 0/5 | 0/10 |
| Ducks | 0/8 | Not done | 0/5 | 0/12 | Not done | 0/6 | 0/12 |

*Number positive/total tested.
Acknowledgements
The authors are grateful to Dr. Ed Dubovi at Cornell University for providing the canine influenza virus. We also thank Mr. Scott Lee for technical assistance with this work.

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