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Brief Communication

H7N9 influenza A virus transmission in a multispecies barnyard model

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ABSTRACT

Influenza A viruses are a diverse group of pathogens that have been responsible for millions of human and avian deaths throughout history. Here, we illustrate the transmission potential of H7N9 influenza A virus between Coturnix quail (*Coturnix* sp.), domestic ducks (*Anas platyrhynchos domesticus*), chickens (*Gallus gallus domesticus*), and house sparrows (*Passer domesticus*) co-housed in an artificial barnyard setting. In each of four replicates, individuals from a single species were infected with the virus. Quail shed virus orally and were a source of infection for both chickens and ducks. Infected chickens transmitted the virus to quail but not to ducks or house sparrows. Infected ducks transmitted to chickens, resulting in seroconversion without viral shedding. House sparrows did not shed virus sufficiently to transmit to other species. These results demonstrate that onward transmission varies by index species, and that gallinaceous birds are more likely to maintain H7N9 than ducks or passerines.

1. Introduction

Influenza A viruses (IAVs; family *Orthomyxoviridae*), and particularly avian influenza A viruses, are among the most well-known and deadly viruses of humans and animals of the 20th and 21st centuries. In 2013, the novel H7N9 influenza A virus emerged as a product of reassortment from viruses circulating in wild birds with spillover into humans and domestic birds, particularly chickens (Gao et al., 2013; Lam et al., 2013; Pu et al., 2015; Liu et al., 2014; Qi et al., 2014; Yu et al., 2014; Wu et al., 2013). The virus has continued to undergo changes, and the net effect has been continued exposure, transmission, and death in 30–50% of human cases over the course of five distinct human epidemics (Li and Chen, 2021; Pu et al., 2021). The first four epidemics resulted from human infections with low-pathogenic (LP) H7N9, during which time infection in poultry in the absence of disease was linked to spread to humans (Zhang et al., 2013; Pantin-Jackwood et al., 2014). The fifth wave, however, was caused by a combination of the original LP-H7N9 and the emergence of a highly-pathogenic (HP) strain due to viral evolution and mutations in the hemagglutinin (HA) cleavage site (Shi et al., 2017; Qi et al., 2013; Tang and Wang, 2017). This particular HP-H7N9 virus caused more human infections and prompted concerns regarding pandemic potential of the virus; however, deployment of a highly effective poultry vaccine starting in September 2017 in China has since effectively controlled H7N9 infections in both poultry and, consequently, humans (Li and Chen, 2021).

Other IAVs have since emerged and have caused major outbreaks in poultry, including several highly pathogenic H5Nx viruses, with some spillover into humans (Lee et al., 2017; Cui et al., 2022). While much is known about the reassortment potential for IAVs, it is unclear why certain subtypes come to dominate the landscape while others perish. Vaccine campaigns are highly effective as long as the correct viral strains are targeted, but in many cases, reassortment happens too quickly to launch such a campaign, and to date no universal influenza vaccines exist. Thus, our best tool for protection against novel IAVs is prevention of infection by minimizing exposure and, subsequently, the risk of reassortment. To accomplish this requires understanding the transmission dynamics of influenza A viruses, a task made more complex by the fact that each subtype may behave very differently depending upon the infected host. It's long been known that live bird markets where numerous species are co-housed are mixing pools for generating novel subtypes of IAVs, and these markets are present in numerous locations around the world, including China, the origin of H7N9 (Zhang et al., 2013; Han et al., 2014; Cui et al., 2014; Liang et al., 2016; Guan et al., 2019). Furthermore, poultry farms can also serve as a source of infection

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for IAVs, and the ultimate risk of spillover and further transmission by wildlife species remains an ever-present threat. While H7N9 cases were primarily traced back to live poultry markets, the virus was also detected in wild birds, including a tree sparrow (Zhou et al., 2013). More recent IAV outbreaks in poultry, including those in the U.S., have been associated with exposure in wild birds, but the knowledge gap regarding which species or alternative transmission mechanisms are ultimately responsible in each new outbreak remains (Ip et al., 2014; Caliendo et al., 2022). Thus, understanding how transmission dynamics change in the presence of multiple species is important for both epidemiological traceback and risk mitigation.

In this study, we evaluated intra-and interspecies transmission of H7N9 avian influenza virus in an artificial barnyard setting in order to better understand the transmission dynamics between co-mingled birds.

2. Materials and methods

2.1. Virus

The virus stock used in this experiment was a 2013 IAV isolate, A/Anhui/1/2013, obtained from the Centers for Disease Control and Prevention as second egg passage stock. Virus was passaged an additional time in our laboratory by inoculation of day 10 specific pathogen free (SPF) embryonating hen eggs and allantoic fluid was harvested two days later. Stock virus was titrated by plaque assay on MDCK cells as previously described (Achenbach and Bowen, 2011).

2.2. Study animals

All animal work was carried out in an ABSL-3 building. The room dimensions are 12′ X 18′ with 12’ ceilings. Air flow was approximately 15 changes per hour. Animals were loose-housed in a room with shared water and food sources, which allowed animals to freely access the space and directly interact with each other. Four “barnyards” were established using a total of 17 Coturnix quail (*Coturnix* sp.), 17 domestic chickens (*Gallus gallus domesticus*), 18 house sparrows (*Passer domesticus*), and 15 ducks (*Anas platyrhynchos)* (Fig. 1). All gallinaceous birds were less than 1 year old, whereas ducks and house sparrows were of unknown ages, but were presumably adults or subadults. Food and water (poultry feed and seeds) were provided *ad libitum* and were replenished each day in

Fig. 1. Schematic design of each barnyard. Inoculated animals are shown in red in the upper boxes and transmission shown in the lower boxes. In the lower boxes, red indicates animals that shed infectious virus and seroconverted, yellow indicates animals that seroconverted but did not shed infectious virus, and orange indicates animals that shed infectious virus but did not seroconvert by the end of the study. Animals with a strikethrough line indicate those that were removed prior to the end of the study.

each animal room. Animal care and use protocols were approved by the CSU Institutional Animal Care and Use Committee.

2.3. Experimental infection

For each barnyard replicate, the species inoculated with H7N9 was varied so that a different species was introduced as the index species as outlined in Fig. 1. In barnyard 1, two quail were infected via intra-choanal inoculation with 100 μl containing approximately 4 log10 plaque-forming units (PFU) of H7N9 (A/Anhui/1/2013) IAV diluted in PBS and, 4 h post-inoculation, introduced into the room with three naïve quail, five chickens, five ducks, and five house sparrows. In barnyard 2, five house sparrows were inoculated in the same manner and introduced into the room with four quail, four chickens, and four ducks 4 h post-inoculation. In barnyard 3, two chickens were inoculated in the same manner and introduced into the room with four quail, two naïve chickens, four ducks and four house sparrows 4 h post-inoculation. In barnyard 4, two ducks were inoculated in the same manner and introduced into the room with four quail, four chickens, and four house sparrows 4 h post-inoculation.

Oral swabs (and cloacal swabs for ducks) were collected daily for the first five days post-inoculation (DPI) and again on day 7, 9, 12 and 21. All birds were manually restrained for sample collection and swabs were placed into 1 mL of BA-1 viral transport media (Hank's M − 199 salts, 1% bovine serum albumin, sodium bicarbonate [350 mg/L], penicillin [100 units/ml], streptomycin [100 mg/L], amphotericin B [1 mg/L] in 0.05 M Tris, pH 7.6). Blood was collected prior to inoculation and at 21 DPI. In addition, 500 μl water was collected from the shared water source each sampling day (when available) and added to an equal volume of BA-1. Samples were transferred to −80 °C freezers prior to laboratory analyses. Blood samples were centrifuged to separate serum which was stored at −80 °C prior to laboratory analyses.

2.4. Laboratory assays

Pre-exposure and 21 DPI sera samples were analyzed by ELISA with the FlockCheck® Avian Influenza Multi-Screen Antibody Test Kit (IDEXX Laboratories, Inc, Westbrook, ME). All birds were seronegative pre-exposure except one duck in barnyard three. All values considered positive were below a 0.7 sample-to-negative ratio that has been applied to multiple wild avian species (Root et al., 2022; Shriner et al., 2016). While the manufacturer of this assay suggests an S/N ratio of <0.5 as a cutoff value as positive for validated poultry species, an alternative threshold of <0.7 has been proposed for mallards and other wild bird species because it provides a better balance between sensitivity and specificity (Shriner et al., 2016; Brown et al., 2009). In general, all sample-to-negative ratios that did not fall below 0.5 showed large differences between pre- and post-exposure serum samples. Oral swab, cloacal swab, and water samples were tested by plaque assay as previously described (Achenbach and Bowen, 2011). Briefly, confluent monolayers of MDCK cells in 6 well plates were rinsed with PBS and serial dilutions of samples were aliquoted onto cells. Plates were incubated for 1 h at 37 °C prior to the addition of an agarose-minimum essential media (MEM) overlay supplemented with trypsin. Forty-eight hours post-inoculation, plates were fixed by addition of 70% ethanol, agar was removed, and plates stained with crystal violet in methanol. The limit of detection for oral swabs and water samples was 1 log10 PFU/mL (water) or 1 log10 PFU/swab suspended in liquid media.

3. Results

Clinical signs of disease associated with influenza A virus infection were not observed in any of the animals during the course of the study, although we did remove four chickens and one quail in total due to unrelated illness (suspect heart failure based on gross necropsy and breed predilections of Leghorn chickens and poor body condition of one quail). Onward transmission and seroconversion varied depending upon index species as summarized in Fig. 1. In barnyard 1, where two quail were inoculated, all remaining quail, all surviving chickens (2 were removed prior to the end of the study for unrelated health reasons), and 2/5 ducks shed infectious virus and seroconverted. Timing of shedding indicates several waves of infection, with directly inoculated quail shedding peak titers between 1 and 3 DPI, the remaining quail reaching peak shedding on days 5–7, and peak shedding for the chickens and ducks on day 9 post-exposure; one individual chicken was still shedding virus on day 12 (Fig. 2). Additionally, the remaining three ducks seroconverted in the absence of detectable viral shedding. In barnyard 2, house sparrows were the index species, and while all five of the directly inoculated birds shed infectious virus, there was no onward transmission in this room and none of the contact animals seroconverted. In barnyard 3, where two chickens were inoculated, the singular remaining chicken and 2/3 quail became infected and shed infectious virus, while all quail, chickens and ducks seroconverted. As with barnyard 1, the directly inoculated animals exhibited peak shedding 1–3 DPI while contact animals shed the most virus between days 7–9 (Fig. 2). Finally, in barnyard 4, where two ducks were inoculated, none of the animals shed infectious virus, including the inoculated ducks, although both ducks and 2 of 4 chickens seroconverted. Notably, we observed direct physical contact between ducks, chickens and quail in every iteration of the barnyard, and while sparrows were not seen directly interacting with other animals, they were observed perching on the shared food and water sources in very close proximity to other birds.

Generally, quail and chickens tended to shed infectious virus for the longest period of time (3–5 days) and quail shed more virus (mean peak titer 4.6 log10 pfu/swab) compared to chickens (mean peak titer 4.2 log10 pfu/swab); however, the difference was not significant when compared using an unpaired two-tailed *t*-test ($p = 0.22$) (Fig. 2). Ducks and house sparrows shed for no longer than 2 days at titers 3 log10 pfu/swab or lower (Fig. 2). The level and duration of shedding had a direct impact on the number of animals infected downstream, with the infected quail serving as the most proficient source of infection to other animals compared to other species. In several cases, animals seroconverted in the absence of shedding infectious virus; likely, this is attributable to infection with viral shedding below the limit of detection of our assay.

Infectious virus was recovered from the water for 5 days following inoculation of quail in barnyard 1 and for one day following inoculation of chickens in barnyard 3. No infectious virus was detected in water from barnyards 2 or 4, which used house sparrows and ducks as index animals, respectively.

4. Discussion

Certain influenza A viruses are the cause of significant infections in poultry world-wide and have led to disease in humans and other animals in multiple instances. Interspecies transmission is one of the most likely mechanisms by which IAVs undergo reassortment, which can lead to rapid emergence of novel influenza strains with the capacity to cause severe disease in animals and humans (Lam et al., 2013; Pu et al., 2021; Guan et al., 2019; He et al., 2022). The near constant threat of emerging and re-emerging IAVs demands that research into surveillance, prevention, and mitigation efforts be ongoing. H7N9 influenza virus emerged in 2013 and has been responsible for five epidemics in humans with a 30–50% case fatality rate (1,8,9). In 2017, a recombination event led to the emergence of HP-H7N9, which sparked fear about the pandemic potential of the virus (Qi et al., 2013). Fortunately, vaccination of poultry has proven successful in controlling the spread of this particular strain (Li and Chen, 2021). However, the emergence of HP H5N1 in Europe and North America in 2021 has led to a massive outbreak in poultry, with spread of this virus largely linked to wild birds (Caliendo et al., 2022). Consequently, the need to understand transmission dynamics of avian influenza viruses remains of paramount importance if we are to ultimately mitigate infections.

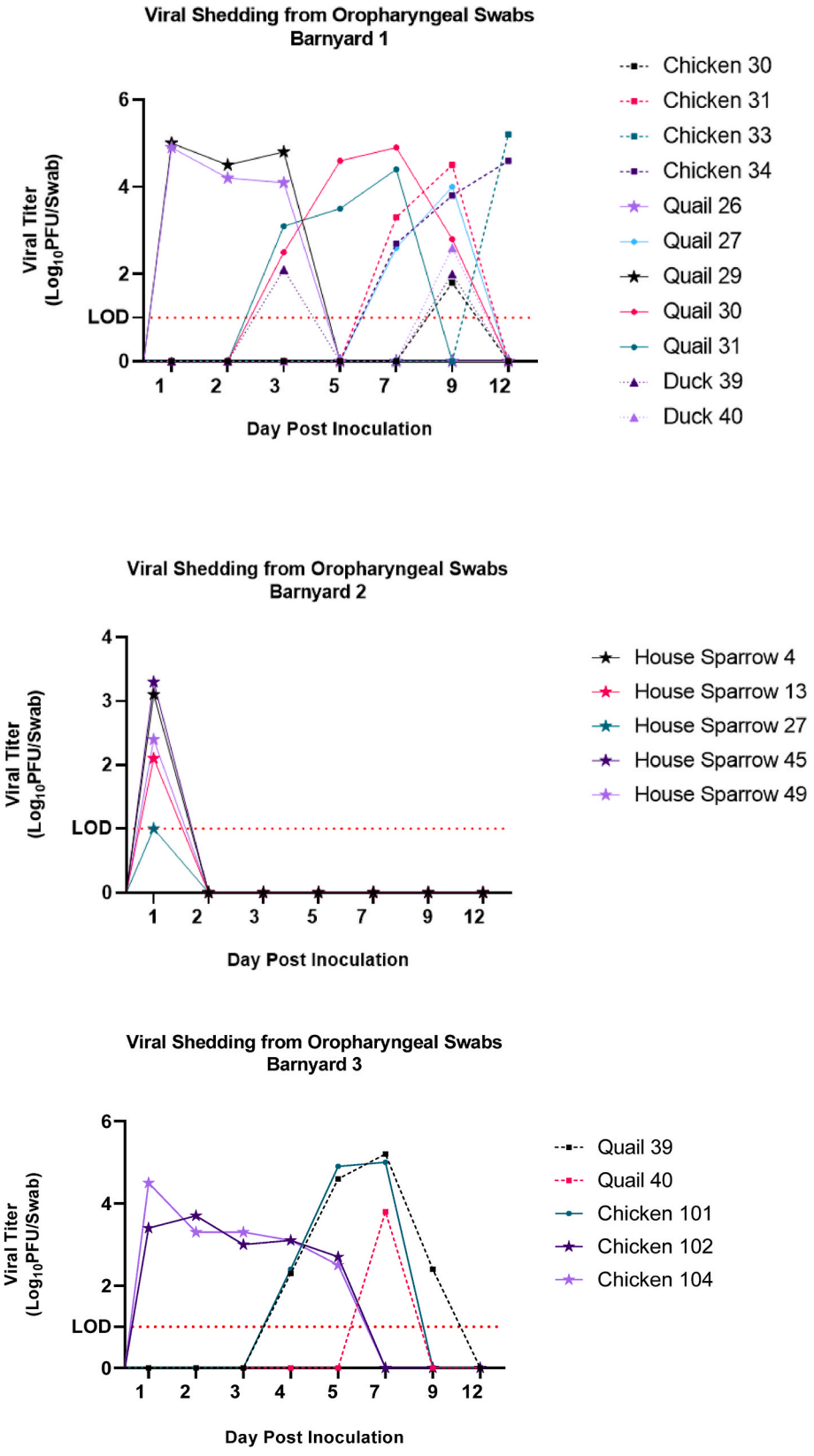

Fig. 2. Oropharyngeal shedding of individual animals in each barnyard. Viral titers in log10 PFU/swab of all individuals that shed infectious virus. LOD = Limit of detection of the assay (1.0 log10 PFU/swab). Index animals are indicated by stars. Barnyard 4 was not included in this figure as no viral shedding was detected in any animals.

Previously, we demonstrated that transmission of H7N9 influenza virus in an artificial wet market setting is dependent upon placement of species in relation to one another and is more likely a result of direct contact with contaminated spilled food, water, and/or feces than aerosol transmission (Bosco-Lauth et al., 2016). While chickens have largely been implicated as the major source of exposure of humans to H7N9, quail tend to have a lower infectious dose and a longer duration of shedding than chickens (Pantin-Jackwood et al., 2014; Bosco-Lauth et al., 2016). Here, we expand upon the mixed species model and demonstrate that transmission is dependent upon the infection kinetics of the index species and that gallinaceous birds (chickens and quail) are more likely than ducks or wild passerines to become infected, shed virus, and transmit H7N9 to other animals. Interestingly, direct inoculation of ducks did not result in infectious viral shedding by any contact bird, whereas contact with infected quail and/or shedding into the environment did allow for transmission and shedding in ducks. This suggests that continued exposure to high-titer H7N9 virus is more likely to propagate an infection in this species than a single exposure. The significance of this finding is that wild birds, which are more likely to transiently be in contact with domestic birds, may not always be the most likely source of exposure, at least for certain influenza A viruses such as the H7N9 strain used for the artificial barnyard studies described herein. In this study, house sparrows were directly exposed but only shed infectious virus for 1 day at low levels, which was not sufficient to infect any of the remaining animals, despite shared environments. Conversely, infected quail readily transmitted to other quail, chickens, and ducks, resulting in both viral shedding and seroconversion. Infected chickens did infect contact quail, chickens, and ducks, but to a lesser degree than quail as some individuals seroconverted in the absence of viral shedding. Importantly, exposure of quail to H7N9 led to continuous shedding of both quail and other birds in the barnyard for a minimum of 12 days. This is consistent with previous studies showing efficient transmission from quail to other birds in an artificial wet market setting (Bosco-Lauth et al., 2016). This long-term shedding, onward transmission and virus isolation from the water was limited to the study timeframe and the lack of additional naïve hosts; however, had naïve birds been introduced to the barnyard at any time during those 12 days, they would have been exposed and, likely, infected. This illustrates how difficult it is to control influenza transmission once a population of susceptible hosts has been exposed and infected, and also shows that persistent contamination of an environment is facilitated by the presence of multiple species and shared resources.

There are several limitations to this study, namely the lack of diversity of the wild bird population (represented only by house sparrows in this study), lack of introduction of additional naïve hosts to further demonstrate transmission, and use of a virus that was isolated from a human, which could behave differently than an avian isolate. The age of the birds may have also contributed to variability in shedding, as older birds tend to be less susceptible and the ducks and sparrows in this study were of unknown age. Notably, a recent study reported that relatively large numbers (N = 10, N = 20, and/or N = 30) of IAV infected European starlings (*Sturnus vulgaris*) collectively had the ability to transmit a LP IAV to bobwhite quail (*Colinus virginianaus*; (Root et al., 2022)). We used a LP IAV strain to demonstrate viral kinetics in susceptible hosts; a more recent HP strain could have looked very different in this model. However, we assert that generally, influenza A virus transmission occurs readily between susceptible birds and that certain avian species may be more likely to propagate a long-term infection among multiple species than others. Practically speaking, this confirms what we already know about the risk of intermingling animals and emergence of zoonotic pathogens: interspecies transmission is common and can have devastating consequences (Webster, 2004; Bao et al., 2013). Yet, it is still difficult to implement management practices to limit exposures, particularly when wildlife are involved. Intensive poultry farms have some of the best biosafety practices in the agricultural world, and yet are still at risk of influenza A virus exposure. Backyard flocks are common globally and present the ultimate challenge in disease mitigation. However, some practical knowledge can be applied, such as keeping food and water sources covered/unavailable to wild birds, physical separation of species and their food/water sources, and instituting a quarantine period for any new birds. Broadly, these findings indicate that transmission dynamics are dependent upon shedding kinetics of the index hosts, and that certain species are more prone to spreading influenza viruses than others. While every influenza virus strain behaves differently, it is increasingly clear that limiting the comingling of species may aid in minimizing onward transmission and the opportunity for reassortment.

5. Conclusions

Interspecies transmission of H7N9 avian influenza virus readily occurs when birds are co-mingled in an artificial barnyard setting, particularly when the index species are quail or chickens.

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Ethics statement

The animal study protocol was approved by the Institutional Animal Care and Use Committee for Colorado State University, protocol #963.

Data availability statement

All research data is either presented in this manuscript or can be made available upon request.

CRediT authorship contribution statement

Angela Bosco-Lauth: Conceptualization, Methodology, Formal analysis, Investigation, Resources, writing—original draft preparation, writing—, Supervision, Funding acquisition. **Anna Rodriguez:** Conceptualization, Methodology, Formal analysis, Investigation, writing—. **Rachel M. Maison:** Formal analysis, writing—. **Stephanie M. Porter:** Investigation, writing—original draft preparation, writing—, All authors have read and agreed to the published version of the manuscript. **J. Jeffrey Root:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, writing—original draft preparation, writing—, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

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