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Limited transmission of emergent H7N9 influenza A virus in a simulated live animal market: Do chickens pose the principal transmission threat?

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ABSTRACT

Emergent H7N9 influenza A virus has caused multiple public health and financial hardships. While some epidemiological studies have recognized infected chickens as an important bridge for human infections, the generality of this observation, the minimum infectious dose, and the shedding potential of chickens have received conflicting results. We experimentally tested the ability of domestic chickens (*Gallus gallus domesticus*) to transmit H7N9 to co-housed chickens and to several other animal species in an experimental live animal market. Results indicated that an infected chicken failed to initiate viral shedding of H7N9 to naïve co-housed chickens. The infected chicken did, however, successfully transmit the virus to quail (*Coturnix* sp.) located directly below the infected chicken cage. Oral shedding by indirectly infected quail was, on average, greater than ten-fold that of directly inoculated chickens. Best management practices in live animal market systems should consider the position of quail in stacked-cage settings.

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1. Introduction

Human infections with a newly recognized influenza A virus (H7N9) were first reported from Shanghai and Anhui Province during the late winter/early spring of 2013 (Gao et al., 2013). As of May 2015, over 650 cases of human infections of this virus have been reported in China (ProMED-mail, 2015). For comparison, during the time period of 2003 to July 2015, 844 cases of Asian strain H5N1 highly pathogenic (HP) influenza A virus (IAV) were reported in humans globally, with a case fatality rate of over 50% (WHO, 2015a). Although soon after its emergence the case-fatality rate was considerably lower for H7N9 when compared to H5N1 (Poovorawan et al., 2013), the number of human cases attributed to H7N9 IAV appear to be on pace to surpass those of H5N1 in the near future. Thus, humans appear to be more readily infected with H7N9 and/or are more frequently exposed to environments that are conducive to zoonotic transmission of this virus.

Several of the early human cases of H7N9 are thought to have been associated with poultry contact and/or visiting or working

within live-animal markets (Bao et al., 2013). Thus, some key epidemiological characteristics of human infections have been suggested as histories of exposure to animals and type of exposure to animals, with chickens and visits to live poultry markets yielding some of the highest percentages for case-patients during early periods following the emergence of this virus, respectively (Li et al., 2014). The closure of live poultry markets has been shown to significantly reduce the number of human infections in select Chinese cities soon after the emergence of H7N9 (Yu et al., 2014). Unlike certain highly pathogenic (HP) IAVs (Webster, 2004), the lack of clinical signs of disease and death in H7N9 infected avian species limits potential early warning systems for human cases that are often common for HP IAVs that have zoonotic potential (WHO, 2015b).

Live animal markets have a long history of association with influenza viruses (Webster, 2004). The prevalence of IAVs in live animal markets can be high, as rates of > 25% for some IAVs have been reported for both chickens and ducks in a select region of China (Ni et al., 2015). The carryover of some animals for days to weeks along with the periodic introduction of naïve animals into live animal market settings can provide advantageous conditions for the survival, proliferation, and alteration of IAVs, as well as conditions that are well-suited for zoonotic transmission of these

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viruses (Webster, 2004).

Chickens have been suggested to be an important reservoir for H7N9 viruses in live bird markets, as they are readily infected, shed the virus, and the viruses appear to cause little or no negative effects to the birds (Zhang et al., 2013). Consequently, multiple studies have suggested epidemiological relationships of human infections with the H7N9 virus being associated with chickens in live-bird markets (Bao et al., 2013; Chen et al., 2013). However, a recent study indicated that the 50% infectious dose of H7N9 in chickens was higher than expected, thereby suggesting that this IAV (A/Anhui/1/2013) is not well-adapted to this poultry species and adaptation may be uneven among different isolates (Spackman et al., 2015). This indicates that additional avian species may play an equal or greater role in the transmission and movement of this virus and similar viruses in live animal market settings.

A better understanding of the transmission of H7N9 IAV in live-animals markets is needed to help inform public health and minimize infections in humans (Bao et al., 2013). Although some early work has suggested an epidemiological link between human cases and chickens (Chen et al., 2013), additional species could play a more significant role in the epidemiology of this virus, as some recent work has suggested that the virus may replicate inefficiently in chickens and this species may be a poor vector of transmission to other species, including humans (Ku et al., 2014).

For these reasons, the objective of this study was to assess the capacity and efficiency of transmission of H7N9 in an artificial multi-species live-animal market. The animals tested included five avian species (three gallinaceous and two peridomestic) and one mammalian species (peridomestic and also sold live in markets), all of which can be found as captive animals sold in or peridomestic species living around live animal markets (Amonsin et al., 2008; Busquets et al., 2010; Cardona et al., 2009; Guan et al., 2013; Webster, 2004).

2. Materials and methods

2.1. Virus

The virus stock used in this experiment was a 2013 human 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 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).

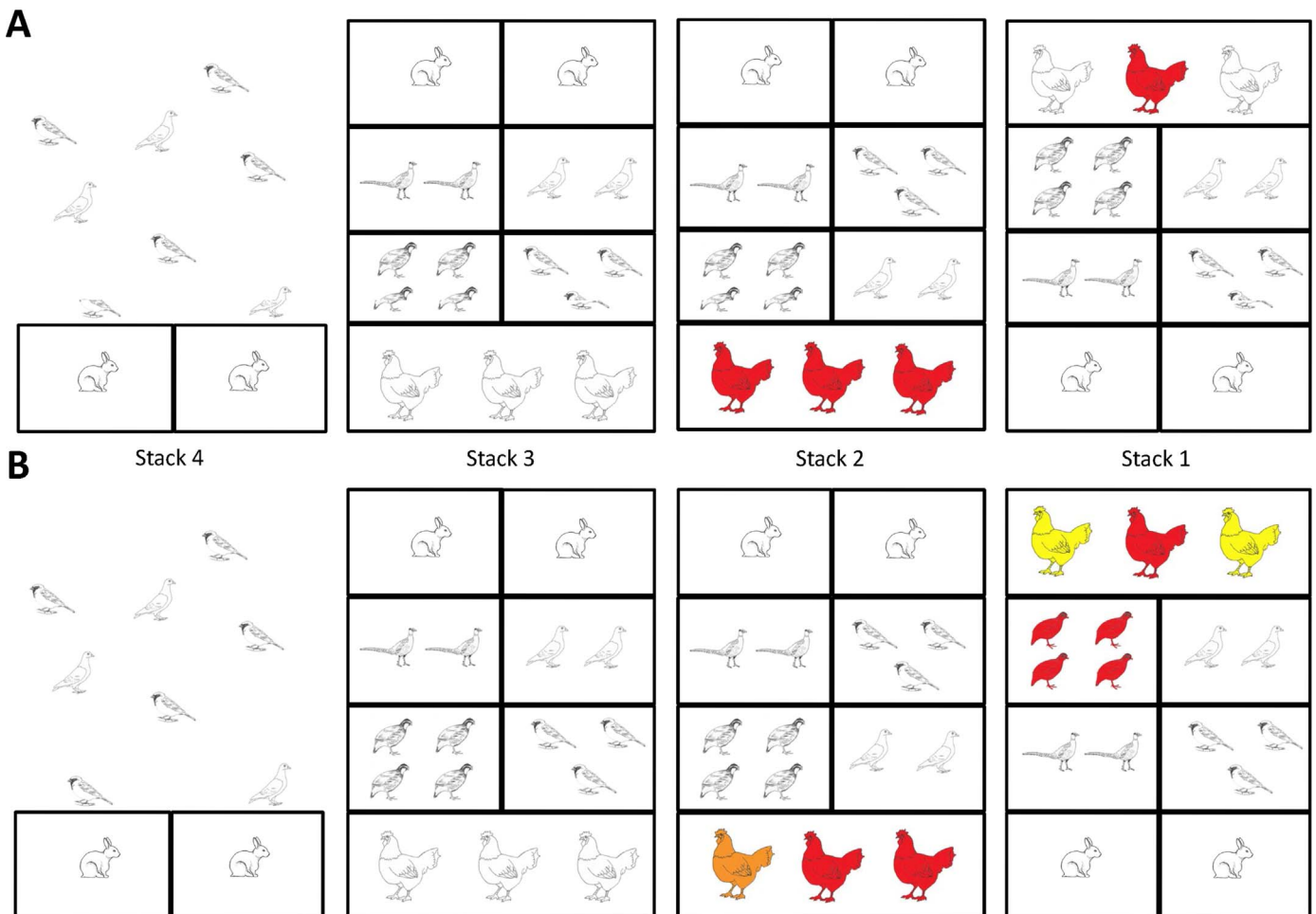


Fig. 1. (A) Experimental setup of four separate stacks of cages for a simulated H7N9 live animal market experiment. Shapes with red fill indicate animals that were experimentally infected at the beginning of this experiment. Shapes that are not associated with a stack were loose-housed in the animal room to assess the potential of these animals to transmit the virus to different stacks. (B) Observed transmission within four separate stacks of cages in a simulated H7N9 live animal market experiment. Shapes with red fill indicate animals that shed virus and seroconverted. Shapes with yellow fill represent animals that seroconverted but did not shed virus. Shapes with orange fill indicate animals that shed small amounts of virus (on a single DPI) but did not seroconvert. Shapes with white fill represent animals that neither shed virus nor seroconverted.

Table 1

Oral shedding and contact transmission of chickens (*Gallus gallus domesticus*) experimentally infected with variants of H7N9 (A/Anhui/1/2013) influenza A virus during multiple experimental studies.

Breed	Age	Route	Dose	Highest titer (DPI)	Contact titer (DPI)	Clinical signs	Reference
White leghorn	Adult	Oculonasal	10 ⁶ TCID ₅₀	10 ^{4.5} (4) ^a	10 ^{a,b}	None	Kalthoff et al. (2014)
White leghorn	59 wk	Intranasal	10 ⁶ EID ₅₀	10 ^{6.4} (4) ^c	n/a	None	Pantin-Jackwood et al. (2014)
Not described	4 wk	Intranasal and Intratracheal	10 ^{9.6} EID ₅₀	10 ^{2.5} (1–2) ^d	None detected	Moderate weight loss ^e	Ku et al. (2014)
White leghorn	4 wk	Intratracheal	10 ⁶ EID ₅₀	10 ^{3.6} (4) ^f	10 ^{3.4g} (2 and 5)	None	Spackman et al. (2015)
White plymouth rocks	4 and 10 wk	Intratracheal	10 ⁶ EID ₅₀	10 ^{5.1} (4) ^f	10 ^{3.4g} (2 and 5)	None	Spackman et al. (2015)
Multiple	Approximately 6–8 wk	Intranasal	10 ⁴ PFU	10 ^{3.0} (2)	None detected	None	This study

^a Titers calculated as TCID₅₀ equivalents based on RT-qPCR assays.

^b Contact titer refers to the maximum titer of a contact chicken (i.e., a naïve chicken placed in direct contact with an experimentally infected chicken).

^c Mean titer for that DPI. Results are reported as RRT-PCR log₁₀ EID₅₀ (presumably equivalents)/mL.

^d Mean titer for 1–2 DPI when chickens were shedding virus. Results are reported as log₁₀ EID₅₀/mL.

^e Moderate pathological lesions were discovered during histopathological examination.

^f Mean titer for reported DPI. Results are reported as RRT-PCR Log₁₀ EID₅₀ equivalents/mL. The strain of H7N9 used in this table was from a previous avian experimental infection (e.g., A/Anhui/1/2013) that had been passaged through a bird.

^g Highest mean titer reported among all contact chickens co-housed with focal chickens infected with 10⁶ EID₅₀/mL (Spackman et al., 2015). Additional inoculation doses used in the experiment produced different titers. Contact transmission was only observed in chickens co-housed with animals infected with high doses of 10⁶ (1/3 and 0/3) and 10^{7.6} (3/3 and 1/3) for two strains of the virus that had been passaged through a chicken during previous studies. Table is unclear which breed(s) is being presented.

2.2. Study animals

All animal work was carried out in an ABSL-3 building. The room dimensions were 12' × 18' with 12' ceilings. Air flow was approximately 15 changes per hour. Animals were housed in multiple stacks of animal cages to assess viral transmission from multiple routes (Fig. 1). A total of 6 ring-necked pheasants (*Phasianus colchicus*), 12 quail (*Coturnix* sp.), 9 domestic chickens (*Gallus gallus domesticus*), 9 rock pigeons (*Columba livia*), 14 house sparrows (*Passer domesticus*), and 8 cottontail rabbits (*Sylvilagus* sp.) were used in this experiment. All gallinaceous birds were young-of-the-year, whereas house sparrows, pigeons, and cottontail rabbits were of unknown ages, but were presumably adults. Stacks 1, 2, and 3 all contained 3 chickens, 4 quail, 2 pigeons, 3 house sparrows, 2 pheasants, and 2 cottontail rabbits with animals situated in different locations within the stacks (See Fig. 1 for stack set up). Stack 4 consisted of 2 cottontail rabbits housed separately in a single row that was level with the bottom level of stack 3 (Fig. 1). In addition, 3 rock pigeons and 5 house sparrows were loose in the animal room to potentially vector the virus between infected (stacks 1 and 2) and uninfected stacks of cages (stacks 3 and 4; Fig. 1). Food and water were provided *ad libitum* and were replaced each day when needed in each animal pen as well as in multiple feeding stations within the animal room to accommodate loose-housed birds. Water dishes held between 500–1100 mL, with the largest dishes in the chicken cages. Loose-housed birds had opportunities to move among all stacks of cages and food and water stations for these animals were placed both on the floor of the room and on top of cage stacks; however, these birds did not have access to the same water as the caged animals. Animal care and use protocols were approved by the NWRRC (protocol number 2209) and CSU (protocol number 14-5220A).

2.3. Experimental infection

On day 0 post infection (DPI), 1 chicken in stack 1 (top level) was intranasally inoculated with 100 µl containing approximately 10⁴ PFU of H7N9 (A/Anhui/1/2013) IAV diluted in PBS (Fig. 1). In addition, 3 chickens in stack 2 (bottom level) were infected with the same dose of the same virus (Fig. 1). These were the only animals that were deliberately infected during this study. Consequently, this design allowed the assessment of viral transmission

occurring downward among cage levels (stack 1), upward among cage levels (stack 2), and through animals vectoring the virus between infected and uninfected stacks (e.g., loose animals to stacks 3 and 4; Fig. 1).

Samples were collected during odd days of the experimental infection from stack 1 from 1 to 21 DPI, during even days from stack 2 from 2 to 20 DPI, from stacks 3 and 4 on 7, 14, and 21 DPI, and from loose-housed birds on 21 DPI. All birds were manually restrained for sample collection of oral swabs and cottontail rabbits were anesthetized (intramuscular injection of a 5:1 ratio of ketamine/xylazine at approximately 100 mg/kg and 20 mg/kg, respectively) prior to nasal flush collection using 1 mL of BA-1 diluent. In addition, a water and a fecal swab sample were collected during each day (when available) from cages of processed animals and on 21 DPI (all cages). All swab samples were stored in 1 mL of BA-1 viral transport media and water samples (500 µl) were stored in 500 µl of BA-1. Samples were transferred to –80 °C freezers prior to laboratory analyses. Animals were bled on the following days: 13 and 21 DPI (stack 1), 14 and 21 DPI (stack 2), and 21 DPI (stacks 3, 4, and loose birds). Blood samples were centrifuged to separate serum which was stored at –80 °C prior to laboratory analyses.

2.4. Laboratory assays

Pre-exposure serum samples were analyzed by ELISA with the FlockCheck[®] Avian Influenza MultiS-Screen Antibody Test Kit (IDEXX Laboratories, Inc, Westbrook, ME) or by standard hemagglutination inhibition (HI) tests. The ELISA was used to pre-screen quail and pheasants. All sample-to-negative ratio values were above a 0.5 threshold that has been suggested for some species by the manufacturer and were also greater than an alternative threshold aimed at balancing sensitivity and specificity (Shriner et al., 2016). Chickens, pigeons, sparrows, and rabbits were pre-screened using HI as previously described (Pedersen, 2014). All samples were negative at the lowest sera dilution (< 1:8). Oral swab, fecal swab, and water samples were tested by plaque assay as previously described (Achenbach and Bowen, 2011). The limit of detection for oral swabs, fecal swabs, and water samples was 10^{1.0} PFU/mL. Post-experimental serology was conducted with standard HI assays using the inoculation virus on sera collected during 13 DPI (stack 1), 14 DPI (stack 2), and 21 DPI (all

animals). Serum dilutions of $\geq 1:8$ were considered positive for assessment of serologic responses for all animals tested (Table 1).

3. Results

The viral shedding of experimentally inoculated chickens in this study was minor. The sole chicken experimentally infected in stack 1 yielded moderate viral shedding only on 1 and 3 DPI with titers of $10^{2.1}$ and $10^{2.3}$ PFU/mL on those DPI, respectively (Table 2). The chickens in stack 2, in which all three birds were experimentally inoculated, also yielded only minor viral shedding. Shedding was observed during 2 DPI ($n=1$) and 4 DPI ($n=3$), with titers ranging from a maximum of $10^{3.0}$ on 2 DPI to $10^{2.5}$ on 4 DPI (Table 2).

Evidence of viral transmission was limited to stack 1 in this study. Although the 2 naïve chickens co-housed with an inoculated chicken yielded evidence of serologic activity on at least one occasion (Chicken two 13 DPI=1:256; Chicken three 13 and 21 DPI=1:256), neither of these birds yielded evidence of detectable viral shedding. This suggests that these animals were exposed to sufficient levels of virus to elicit an immune response, but insufficient virus to produce a productive infection associated with detectable levels of virus in oral swab samples.

Of interest, all quail housed directly below the chickens located in stack 1 shed virus and seroconverted (Table 2), while none of the naïve chickens co-housed with the inoculated chicken shed virus in this stack. Quail shed virus as early as 3 days post contact (DPC) and as late as 11 DPC, with all birds yielding evidence of shedding on 7 DPC (Table 2). The highest titer of virus detected from an oral swab of a quail was $10^{3.7}$ PFU/mL. No other animals located in this stack ($n=9$) yielded evidence of shedding or seroconversions (Table 2). Notably, pigeons were housed in similar location as were quail within the stack (directly below the chickens; Fig. 1) but failed to show evidence of infection.

Although 120 fecal samples and 101 water samples were collected during multiple time points of the experiment, none were positive for live virus by plaque assay. This lack of environmental contamination likely influenced and ultimately limited

transmission within and between the cage stacks. However, the potential role of environmental contamination from other species is unknown at this time.

4. Discussion

Chickens have been suggested to be important host species for H7N9 viruses in live bird markets (Zhang et al., 2013). However, some recent studies indicate that contact transmission among co-housed chickens is dependent on the inoculation dose of the infected chicken(s), with higher doses supporting more transmission (Spackman et al., 2015). Nonetheless, failures of contact transmission have been reported for naïve chickens co-housed with chickens infected with $10^{9.6}$ EID₅₀ (Ku et al., 2014), thereby suggesting that chickens infected with high doses, even at levels that are unlikely in natural settings, may not be the most significant species of epidemiological interest in live bird markets. However, the generality of this statement may be influenced by age and reproductive stressors of individual birds, as a higher infection rate was noted for older chickens (i.e., 59 week-old) when compared to younger birds (4 and 10 week-old) (Pantin-Jackwood et al., 2014; Spackman et al., 2015). Overall, the low levels of viral shedding of H7N9 by chickens we observed is consistent with some previously reported work (Ku et al., 2014), but inconsistent with the higher levels of shedding that have been reported by others (Pantin-Jackwood et al., 2014). It should be noted that A/Anhui/1/2013 is a human isolate, and while some human origin H7N9 viruses have human-associated mutations such as Q217L in the hemagglutinin and PB2 E627K, several of these strains have been tested in chickens and replicate well, suggesting that these mutations do not halt replication in poultry (Spackman et al., 2015).

The current study suggests that quail may pose more of a transmission threat and subsequent public health threats as compared to chickens. Although both quail and pigeons were in cages located directly under the chickens in stack 1 (which housed one shedding chicken), only the quail shed virus and produced serologic responses. This suggests that quail are highly susceptible to this virus, and likely require a small infectious dose for productive infections, most likely through the oral route. The premise that quail require a low infectious dose is supported by a previous experimental infection study in which high infection rates in group-housed quail were achieved by 6 DPI with a low inoculum dose of 10^2 EID₅₀ (Pantin-Jackwood et al., 2014). Similarly to the chickens used in the current study, the quail were also young animals at the initiation of the experiment. Thus, if there is indeed an effect of age and reproductive status on infection and infectious dose in chickens, the observation does not appear to be ubiquitous to all gallinaceous birds, as young but reproductively active quail (e.g., multiple quail produced eggs during the experiment) were susceptible to infection from presumably low doses of inoculum during this experiment. An additional finding of this study is that quail significantly extended the period during which infectious virus was excreted in the experimental wet market setting. For example, chickens were only noted to shed virus up to 4 DPI (3 DPI for stack 1 and 4 DPI for stack 2). A single quail yielded evidence of viral shedding on 3 DPI and was shedding at higher levels as compared to the sole chicken shedding on that day, thereby suggesting that this quail (Quail 32; Table 2) may have been responsible for infections of the additional quail ($n=3$) with which it was co-housed. This is supported by serological data from this study, as the two quail that did not initiate viral shedding until 7 DPC had a negative or lower titer antibody response during 13 DPC, as compared to the two quail that initiated shedding earlier in this stack (Table 2). Following the infection of a single chicken in stack 1, quail extended the time period that virus was shed in the

Table 2

Viral shedding and antibody titers of birds that seroconverted or shed virus on at least one occasion during a simulated live animal market experimental transmission study. No other animals yielded evidence of viral shedding/serologic activity during the duration of the study.

Animals		Serology			Virus isolation	
ID and location		Days post infection (DPI)			Shedding characteristics	
Bird ID	Stack ^b	13 DPI ^c	14 DPI	21 DPI	Highest titer ^d (DPI)	Shedding duration DPI
Chicken 1 ^a	1	< 8 ^e	n/a	512	2.3 (3)	1–3
Chicken 2	1	256	n/a	< 8	None	n/a
Chicken 3	1	256	n/a	256	None	n/a
Quail 28	1	64	n/a	512	3.9 (9)	5–9
Quail 29	1	32	n/a	128	3.3 (7)	7–9
Quail 32	1	64	n/a	128	3.4 (3 and 5)	3–7
Quail 35	1	< 8	n/a	512	3.7 (7)	7–11
Chicken 4 ^a	2	n/a	< 8	< 8	1.9 (4)	4 only
Chicken 5 ^a	2	n/a	256	128	3.0 (2)	2–4
Chicken 6 ^a	2	n/a	< 8	128	2.5 (4)	4 only

^a Animals that were intentionally infected at the initiation of the study.

^b Stack refers to the stack of cages an individual animal was associated with (see Section 2 and Fig. 1).

^c DPI=days post infection of chickens. Quail were not directly infected.

^d Titer refers to log₁₀ PFU/mL of oral swab samples. The limit of detection for oral swabs was $10^{1.0}$ PFU/mL.

^e The limit of detection for the hemagglutination inhibition assay was 1:8.

current study (Table 2). For obvious reasons, the additional quantities of virus shed into this system along with the greater duration of live virus that was detected certainly pose a greater zoonotic and agricultural threat. This is supported by the results of earlier experimental infection studies of H7N9 in multiple avian species in which directly inoculated quail were shown to shed the highest titer of oral swabs (as high as $10^{7.6}$ log₁₀ number of EID₅₀/mL) and to shed as long or longer than any of the other six species tested (up to 11 DPI) (Pantin-Jackwood et al., 2014).

Considering that pigeons were located under the same infected chicken as were quail, the lack of evidence of infection in pigeons suggests that they are far less susceptible to infection with this virus than are quail. This result is supported by previous observations suggesting that pigeons, although susceptible to IAVs, do not typically shed in sufficient quantities to support transmission cycles of the viruses (Abolnik, 2014). In a recent experimental infection study, although some pigeons were susceptible to IAV infection with H7N9, their viral shedding was limited in quantities and of a short duration as compared to chickens and quail (Pantin-Jackwood et al., 2014). Similar observations have been noted for HP IAVS, as the few pigeons that shed following inoculations with HP H5N1 IAV did so at very low titers (Yamamoto et al., 2012).

Prior studies indicate that shared water sources between poultry and passerines is a likely source of transmission (Jones et al., 2014, 2015). While the animals in this experiment did not share water sources between species, the lack of detectable live virus in any of the environmental samples (i.e., water and fecal samples) suggests that water or fecal transmission were not the primary transmission mechanisms in this study. Thus, one possible scenario for transmission between chickens and quail is through poultry feed contaminated with an infectious dose of an oral secretion falling to the cage below containing quail and the subsequent consumption of the feed by quail. No pigeons shed virus nor seroconverted during the current study, even though they were housed directly below the same chicken that infected quail that were housed directly adjacent to the pigeons. This suggests that a higher dose of virus, such as 10^6 EID₅₀ (Pantin-Jackwood et al., 2014), may be needed to infect this species or that this species is more susceptible by limited routes of infection as compared to the presumed environmental contamination we observed in quail.

The results of this study suggest that inter-cage transmission from experimentally infected chickens to naïve chickens is inefficient, at least at the dose that and the isolate we used to inoculate chickens, which is consistent with previous studies suggesting a high inoculation dose is needed to consistently achieve contact transmission among co-housed chickens (Spackman et al., 2015). However, the observed infections in quail warrant further scrutiny, as row-to-row transmission of H7N9 among quail cages would appear plausible based on the preliminary results of this study. Taking into account the results of the current study and a previous experimental transmission study suggesting that quail require a small infectious dose of H7N9 in index animals to reach high infection rates (Pantin-Jackwood et al., 2014), quail may represent a key species in susceptibility to and transmission of this virus in live animal markets when they are present in these settings.

Although none of the cottontail rabbits acquired IAV infections during the current study, their roles in inter-species transmission in live animal market settings should not be discounted. In an earlier study assessing the shedding capacities of peridomestic mammals experimentally infected with the same H7N9 as used in this study, most mammals shed several orders of magnitude greater than any of the infected birds in the current study (Root et al., 2016). As such, select mammalian species could produce a more significant transmission threat for H7N9 in live animal

markets than some commonly associated avian species.

Overall, a key question associated with IAV transmission in live animal market settings is which index species produce the most risk for intra- and interspecific transmission. In addition, key characteristics of various animal species and experimental procedures, such as age, breed, inoculation dose, and viral strain (Pantin-Jackwood et al., 2014; Spackman et al., 2015), as well as other potential contributing factors, such as sex and experimental locations of animals in stacked-cage settings need to be addressed. Once elucidated, this information could help to inform best management practices in these types of market settings and could be valuable knowledge for advances in the complex epidemiology of public health issues associated with emergent H7N9 IAV.

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