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BRIEF REPORT

Cottontail rabbits shed clade 2.3.4.4 H5 highly pathogenic avian influenza A viruses

J. Jeffrey Root[1] · Angela M. Bosco-Lauth[2] · Nicole L. Marlenee[2] · Richard A. Bowen[2]

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Abstract
During 2014-2015, clade 2.3.4.4 H5Nx highly pathogenic (HP) avian influenza A viruses (IAV) were first detected in North America and subsequently caused one of the largest agricultural emergencies in U.S. history. Recent evidence has suggested that cottontail rabbits can shed multiple IAV subtypes. We experimentally infected cottontail rabbits with three HP H5Nx IAVs. All rabbits tested shed virus on at least one day by at least one route. Cottontail rabbits appear to be an exception to the limited capacity for replication that has been previously reported for certain other mammalian species inoculated with clade 2.3.4.4 HP H5Nx avian influenza A viruses.

During late 2014, highly pathogenic (HP) H5N8 avian influenza A virus (IAV) and reassortant H5N2 viruses were identified in Washington, USA from wild and/or captive birds fed wild waterfowl [6]. The H5N2 virus spread rapidly in the U.S. during the winter and spring of 2015, reaching Iowa by mid-April [17]. The effects of these viruses on the U.S. poultry industry were tremendous, and while multiple states were affected, in Minnesota and Iowa alone, approximately 40 million birds either died from HP IAV infections or were culled [17]. In Iowa, the economic impact of this outbreak to the state has been estimated to be greater than 1 billion U.S. dollars [3].

The potential roles of peridomestic wildlife in the epidemiology of avian IAVs have received increasing attention over recent years [5, 9, 16]. The 2015 HP avian IAV outbreaks in the U.S., as well as other emergent, high consequence strains (both low pathogenic and HP that have high zoonotic or agricultural health implications) elsewhere (e.g., Asian H7N9 and H5N1), have motivated researchers to pursue several studies associated with questions involving the replication competence of some of these synanthropic animals [2, 4, 14]. However, to date, few studies have addressed this issue in clade 2.3.4.4 HP H5 viruses.

During recent years, some lagomorph species (e.g., rabbits, hares, and pika) have been shown to be either experimentally susceptible (e.g., cottontail rabbits) or naturally exposed (e.g., plateau pika [*Ochotona curzoniae*]) to various IAVs [12, 19, 21]. Further, a recent study indicated that cottontail rabbits have the capacity to transmit an IAV back to birds through shared resources [13]. Motivated by these IAV susceptibilities in wild lagomorphs, the objective of this study was to characterize the infection dynamics in a common mammalian farm-side resident, the cottontail rabbit (*Sylvilagus* sp.), experimentally inoculated with three HP H5Nx clade 2.3.4.4 IAVs that have been recently detected in North America.

Three viruses were used in the experimental infections. These included A/Northern pintail/Washington/40964/2014 (H5N2) (hereinafter = Northern pintail), A/turkey/Minnesota/9845-4/2015 (H5N2) (hereinafter = turkey), and A/gyrfalcon/Washington/41088-6/2014 (H5N8) (hereinafter = gyrfalcon). All viruses were kindly provided by the National Veterinary Services Laboratory, Ames, IA. Viruses were propagated in embryonated chicken eggs and subsequently titrated with plaque assays (see methods below).

Twelve cottontail rabbits *(Sylvilagus* sp.) were wild-caught in Larimer County, CO with box traps. The twelve cottontail rabbits were dusted for ectoparasites and bled to

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J. Jeffrey Root and Angela M. Bosco-Lauth contributed equally to this work.

✉ J. Jeffrey Root
Jeff.root@aphis.usda.gov

[1] United States Department of Agriculture, National Wildlife Research Center, Fort Collins, CO, USA

[2] Colorado State University, Fort Collins, CO, USA

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assess if they had pre-exiting IAV antibodies (see below). In addition, a subcutaneous transponder (IPPT-300 transponders, Bio Medic Data Systems) that provided an individual identification number and temperature readings was implanted into each cottontail. Following brief quarantine periods, animals were transferred to an enhanced animal biosafety level-three (ABSL-3) facility at Colorado State University. All cottontails were housed in rabbit cages placed within HEPA-filtered isolator cages outfitted with a nest box. Cottontail rabbits were maintained with rabbit chow, alfalfa, and fruit. Food and water were replenished daily. Animal care and use protocols were approved by the National Wildlife Research Center and Colorado State University.

The twelve cottontail rabbits, all assessed to be negative to IAV antibodies, were divided into three experimental groups of four rabbits for each of the three viruses listed above and were inoculated with approximately $10^{5.3}$ plaque forming units (PFU) (turkey virus) or $10^{5.9}$ PFU (Northern pintail and gyrfalcon viruses) in 200 µl BA-1 [16] diluent vehicles by the nasal route. Each nostril received approximately 100 µl. Following inoculations, one-half of the cottontail rabbits were sampled on odd days (n = 2 for each virus) and the other one-half were sampled on even days (n = 2 for each virus) over 1-8 days post-infection (DPI). This sampling time period was chosen based on previous studies indicating that cottontail rabbits typically cease shedding or shed other IAVs at low levels by the end of this time-frame [12]. Daily sampling consisted of an oral swab (stored in 1 mL of BA-1 viral transport media) and a nasal flush (1 mL of BA-1) for sampled animals, as well as general health observations of all animals. In addition, body temperature data was recorded each day an animal was processed, when possible. All swab and nasal flush samples were stored at − 80º C prior to testing. Animals were euthanized on 18 DPI. To accomplish nasal inoculations and animal processing, cottontails were anesthetized with 10mg/kg ketamine and 1 mg/kg xylazine in combination intramuscularly.

Oral swab and nasal flush samples were tested by plaque assay as described in a previous study [1]. The limit of detection for both sample types was 10 PFU/mL. Pre-experiment sero-negativity was determined with agar gel immunodiffusion (AGID) tests [10], a procedure that has been successfully employed during previous studies with cottontail rabbit sera [12, 15]. Post-experiment serology was conducted with standard hemagglutination-inhibition (HI) assays using equine erythrocytes and an H5 virus with cottontail rabbit sera collected on 18 DPI. Serological responses were delineated as HI titers of ≥ 10.

Cottontail rabbits were infected by the three tested viruses, as all cottontail rabbits in each virus group (n = 4) shed virus on at least one DPI (Tables 1 and 2) but did not exhibit any overt signs of disease. The highest virus titers were noted from nasal samples from rabbits infected with the H5N2 turkey virus, as two rabbits shed at levels of ≥ $10^{5.1}$ PFU/mL (Table 1). Oral shedding of this virus was also relatively high, as one rabbit shed a titer of $10^{4.3}$ PFU/mL, while three of four rabbits shed at levels of > $10^{3.0}$ PFU/mL (Table 2). Of interest, the rabbit that shed the highest titer in this treatment group was the only animal that shed

Table 1 Nasal shedding and serological responses of cottontail rabbits (*Sylvilagus* sp.) experimentally infected with clade 2.3.4.4 highly pathogenic H5N2 and H5N8 avian influenza A viruses

		Days post infection								
Rabbit number	Virus	1	2	3	4	5	6	7	8	HI status[f]
1	Turkey[a]	5.1[d]		4.5		1.6		<1		+
2	Turkey		5.3		2.4		4.6		2.2	+
3	Turkey	3.9		4.2		ND[e]		ND		ND
4	Turkey		3.4		1.3		<1		<1	+
5	Northern Pintail[b]	3.8		2.3		<1		<1		+
6	Northern Pintail		2.1		<1		ND[e]		ND	ND
7	Northern Pintail	3.9		2.8		<1		<1		+
8	Northern Pintail		3.6		<1		<1		<1	+
9	Gyrfalcon[c]	3.5		3.0		2.3		<1		+
10	Gyrfalcon		2.5		2.2		<1		<1	+
11	Gyrfalcon	2.5		1.0		<1		<1		+
12	Gyrfalcon		2.9		1.3		<1		<1	+

[a] A/turkey/Minnesota/9845-4/2015 (H5N2)

[b] A/Northern pintail/Washington/40964/2014 (H5N2)

[c] A/gyrfalcon/Washington/41088-6/2014 (H5N8)

[d] Values represent $\log_{10}$ PFU/mL

[e] ND = not done. Animal died from complications with anesthesia during a previous sampling period

[f] HI conducted with sera collected on 18 DPI. A "+" indicates a positive serological response (see methods)

Table 2 Oral shedding of cottontail rabbits (*Sylvilagus* sp.) experimentally infected with clade 2.3.4.4 highly pathogenic H5N2 and H5N8 avian influenza A viruses

Rabbit number	Virus	Days post infection							
		1	2	3	4	5	6	7	8
1	Turkey[a]	3.1[d]		3.3		<1		<1	
2	Turkey		4.3		1.8		3.4		1.8
3	Turkey	2.3		3.4		ND[e]		ND	
4	Turkey		2.1		<1		<1		<1
5	Northern Pintail[b]	3.0		1.3		<1		<1	
6	Northern Pintail		2.1		<1		ND[e]		ND
7	Northern Pintail	2.4		2.3		<1		<1	
8	Northern Pintail		2.5		<1		<1		<1
9	Gyrfalcon[c]	2.0		1.8		2.1		<1	
10	Gyrfalcon		<1		1.0		<1		<1
11	Gyrfalcon	1.7		<1		<1		<1	
12	Gyrfalcon		C[f]		C		C		C

[a]A/turkey/Minnesota/9845-4/2015 (H5N2)

[b]A/Northern pintail/Washington/40964/2014 (H5N2)

[c]A/gyrfalcon/Washington/41088-6/2014 (H5N8)

[d]Values represent $\log_{10}$ PFU/mL

[e]ND = not done. Animal died from complications with anesthesia during a previous sampling period

[f]Bacterial contamination noted in assays from oral swab samples, likely due to colonization of the oral cavity of this particular rabbit

each day it was sampled (i.e., 2, 4, 6, and 8 DPI) by both the oral and nasal routes. Overall, the turkey virus produced the highest shedding levels for cottontail rabbits on most DPI as measured by daily maximums for both nasal ($10^{5.3}$ PFU/mL) and oral ($10^{4.3}$ PFU/mL) samples (Figure 1). One rabbit shed virus through at least 8 DPI (the last day it was sampled), which is consistent with a previous study [12].

Viral shedding of the H5N2 Northern pintail virus tended to be of a lower titer and of a shorter duration when compared to the turkey virus. All rabbits shed this virus by the nasal route, and three of four rabbits shed at levels of $\geq 10^{3.6}$ PFU/mL (Table 1). In addition, all animals shed virus by the oral route on at least one DPI. The highest titer shed by the oral route was $10^{3.0}$ PFU/mL (Table 2). Two of four cottontails associated with this virus only shed virus at detectable levels the first day they were sampled (2 DPI). The H5N2 Northern pintail virus was shed up to 3 DPI by the oral and nasal routes (Tables 1 and 2).

Overall, the H5N8 gyrfalcon virus produced the lowest levels of viral shedding in cottontail rabbits in terms of peak viral titers. The highest titer from a nasal flush sample was $10^{3.5}$ PFU/mL, while the highest titer from an oral swab sample was $10^{2.1}$ PFU/mL, of the three animals evaluated for oral shedding for this virus (Tables 1 and 2). In one of four individuals, bacterial contamination was noted in assays from oral swab samples, likely due to colonization of the oral cavity of this particular rabbit. Although peak levels of shedding of this virus were lower than the Northern pintail virus, the duration of shedding was longer for the gyrfalcon virus (up to 5 DPI by one rabbit by both shedding routes; Tables 1 and 2).

Overall, 10 of 10 cottontail rabbits seroconverted for the three viruses. Serology tests indicated 3/3 animals seroconverted following inoculations with the turkey virus, 3/3 animals seroconverted following inoculations with the Northern pintail virus, and 4/4 animals seroconverted following inoculations with the gyrfalcon virus (Table 1; see table footnotes for denominator explanations).

Recent literature suggests that cottontail rabbits are susceptible to multiple LP IAV strains, and can be susceptible to low doses of virus [12, 14, 15]. However, to our knowledge, this is the first evaluation of HP viruses in cottontail rabbits. Consistent with other studies evaluating cottontail rabbits, the current study also indicates that this species can shed relatively high viral titers by multiple routes. Of interest, the virus that produced the highest levels of viral shedding in cottontails during the current study was isolated from poultry, thereby suggesting that this species may pose more of a threat for viral movement between poultry barns once the virus is established at a farm, as compared to the rabbits acquiring a viral infection from a wild bird on the farm premises. Nonetheless, the higher levels of oral and nasal shedding observed in cottontail rabbits inoculated with a poultry virus (Figure 1) were largely the result of high titers of this virus from two of four rabbits. Thus, individual heterogeneity in shedding responses to the three viruses likely accounts for at least some the observed differences in the viral titers that were shed.

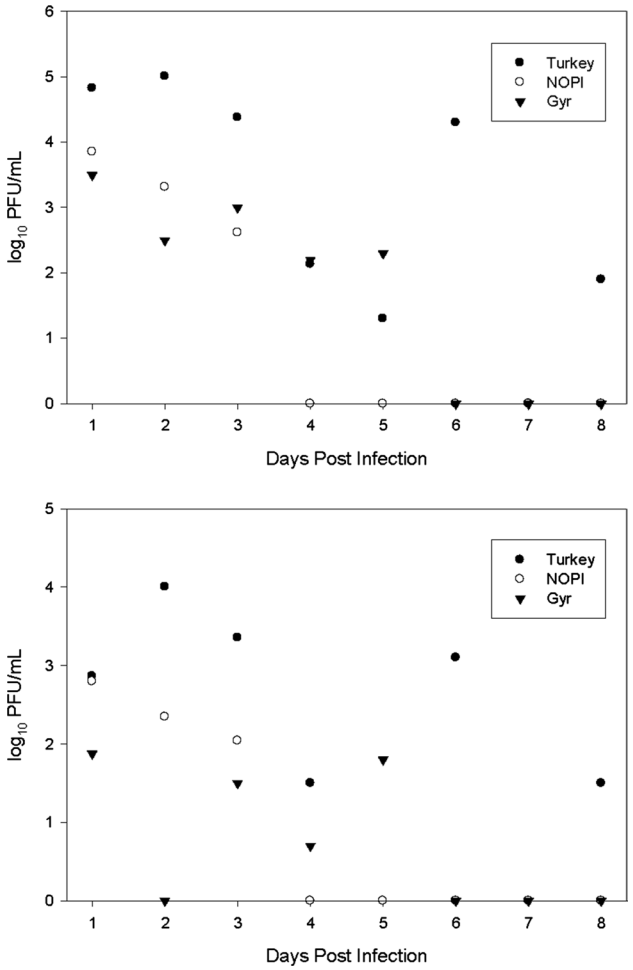

Fig. 1 Average nasal (top) and oral (bottom) shedding of three different highly pathogenic avian influenza A viruses in experimentally infected cottontail rabbits. Samples with values of < 10 PFU/mL or those that were not taken (see Table 1) are evaluated as zero

Influenza viral isolates from wild lagomorphs have been reported from the Qinghai Lake region in China, where intensive wildlife influenza investigations have been undertaken. For example, H5N1, H9N2, and H7N2 IAVs have been isolated from plateau pika in Qinghai Province [18, 19, 21]. Further, antibodies reactive with H5 and H9 subtypes have also been reported in this species from this region [20, 21]. This suggests that a comparable AI hotspot in North America, one where wild lagomorphs and waterfowl are in close proximity to each other, could facilitate natural IAV infections in cottontails. At this point in time, no large-scale IAV surveillance studies have been conducted in cottontail rabbits; however, this could represent a useful investigation if an appropriate location, harboring large numbers of cottontail rabbits and waterfowl in an area with known IAV activity, is identified.

Ferrets that were experimentally inoculated with the same Northern pintail and gyrfalcon viruses as used in this study showed minimal nasal shedding, thereby leading these authors to conclude that this species has limited capacity for replication of these viruses [7]. However, others have suggested efficient replication of these viruses in the respiratory tracts of this species [11]. No viral RNA was detected from nasal swabs from swine infected with the two viruses mentioned above when sampled on 1, 3, or 5 DPI; however, broncho-alveolar lavage fluids did show evidence of viral RNA on 3 and 5 DPI [8]. Consistent with the viral titers in the lungs of mice infected with two of the viruses used in the current study [7], cottontail rabbits tended to shed higher levels of the Northern pintail virus as compared to the gyrfalcon virus during early DPI.

Similar to previous studies involving relatively high inoculation doses [12], the viral shedding dynamics in the current study were fairly brief, with most animals ceasing viral shedding by 6-7 DPI. Nonetheless, one animal shed virus through 8 DPI (the last day samples were taken) and shed relatively high titers out to 6 DPI ($10^{4.6}$ PFU/mL by the nasal route and $10^{3.4}$ PFU/mL by the oral route). For obvious reasons, cottontail rabbits that shed higher titers and shed for longer periods of time likely pose a greater epidemiological risk in most situations.

As has been reported previously for multiple LP IAV strains [12, 14], the current study indicates that cottontail rabbits can shed relatively high levels of some HP IAVs along with a lack of any obvious signs of disease. As a symptomless carrier of IAVs, if infected, cottontail rabbits are unlikely to provide any type of obvious warning of the presence of a virus on poultry facilities without the use of active surveillance. Thus, reducing wildlife attractants and preventing wildlife access to facility premises, such as poultry houses, are key to mitigating the potential risk posed by this and certain other wildlife species [17].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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