Shedding of a Low Pathogenic Avian Influenza Virus in a Common Synanthropic Mammal – The Cottontail Rabbit

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Introduction

Influenza A viruses are globally important public health and veterinary pathogens infecting numerous avian and mammalian species [1]. These viruses have produced large financial burdens in terms of public health [2] and poultry production [3]. Wild birds of the orders Anseriformes and Charadriiformes are typically considered the primary natural hosts of avian influenza viruses (AIVs) [4]. Despite documented associations of influenza A viruses and wild mammals, the potential role of these species in the ecology of influenza A viruses has received limited attention and is not well understood for select species [5–10].

While infections of mammals with highly pathogenic (HP) Asian strain H5N1 AIV have been most commonly found in species from the mammalian order Carnivora, a few exceptions have been noted. One recent exception occurred in the mammalian order Lagomorpha (e.g., hares, rabbits, and pikas) where 13.4% of 82 wild black-lipped pika (Ochotona curzoniae) had antibodies against HP H5N1 in and around Qinghai Lake, China [10]. In addition, 5 viral isolates of HP H5N1 were obtained from tissues of this species from the same region [10]. While no naturally occurring infections of HP AIV H5N1 have been reported in rabbits [11], the presence of this virus in black-lipped pika represents the first cases of HP H5N1 documented in a lagomorph [10,11]. To our knowledge, no other natural AIV infections have been reported in lagomorphs.

Cottontails (Sylvilagus spp.) occur as multiple species in North America and are broadly distributed throughout the United States [12]. The desert cottontail (Sylvilagus audubonii) has an expansive distribution in western North America, ranging from northern Montana, south to central Mexico, and west to southern California [13]. It is well-adapted for a diversity of habitats [14], is not territorial, and forages primarily on forbs and grasses [15]. Importantly, cottontails are frequently found in peri-domestic situations, often living within farmsteads, commercial properties, parks, and suburban neighborhoods. They are also commonly found in other areas associated with metropolitan landscapes in parts of the United States [16]. Thus, cottontails, to a large extent, are synanthropic. These habits, in the context of biosecurity, may be even more important if one considers an avian-rearing facility, as wild mammals have been documented near bird production areas [17].

Interactions among domestic poultry and other animals have been suggested as a potential pathway of avian pathogen...
introductions for domestic poultry flocks [18]. For example, multiple conduits of exposure of AIVs through wild birds have been verified or alleged in causing outbreaks in poultry [1]. Other species, such as wild mammals, have also been implicated as risk factors associated with the spread of a low pathogenicity (LP) AIV among commercial poultry farms [19]. Given that at least one lagomorph species was naturally susceptible to HP H5N1 [10], this mammalian order warrants more scrutiny for its potential role in AIV ecology. Cottontails, which range throughout much of the U.S., are an obvious choice to further assess the competency of synanthropic lagomorphs to shed AIV. The objective of this study was to assess the shedding potential of cottontails experimentally infected with a LP AIV (H4N6), an AIV frequently found in wild waterfowl in North America [20]. In meeting this objective, we addressed three research questions: what is the magnitude and duration of AIV shedding in cottontails, what are the primary routes of AIV shedding, and how consistent were these characteristics across individuals?

Methods

Ethics Statement
Animal experiments were approved by the Institutional Animal Care and Use Committee of the National Wildlife Research Center (NWRC), Fort Collins, CO, USA (Approval number 1807). Cottontails were captured on state-owned land with facility manager permission under a state collection permit issued by the Colorado Division of Wildlife.

Study animals
Cottontails were live-trapped in box-style traps (15.2 x 15.2 x 48.3 cm; Tomahawk Live Traps, LLC, Hazelhurst, WI, USA) in Larimer County, Colorado. Upon capture, pre-experiment blood and nasal samples were obtained. In addition, all animals were dusted for ecto-parasites and individually marked with microchips. Cottontail species were identified as desert cottontails (Sylvilagus auduboni) using methods described elsewhere [21]. A total of sixteen desert cottontails were used in the experiment outlined below. The sixteen cottontails were randomly assigned to one of two groups: odd or even.

For quarantine purposes, cottontails were housed in an outdoor animal research building in customized 58.4 x 66.0 x 91.4 cm dog crates (Precision Pet Products, Costa Mesa, CA, USA). Each crate was outfitted with a hide made from PVC tube with a 20 cm inner diameter, a water bowl, a food dish, a hay bowl, and an enrichment toy. Food (Purina Rabbit Chow [Purina Mills, St. Louis, MO, USA], alfalfa, and apples or carrots) and water were replenished daily. Following a minimum of a 14-day quarantine period, all rabbits were transferred to a BSL-2 animal facility and housed individually in 39.7 x 40.6 x 45.7 cm rabbit racks outfitted with the same materials as the dog crates. The control animals were maintained in a separate rack within the same animal room.

Experimental Infection
On day 0 of this experiment, fourteen animals were anesthetized with isoflurane vaporizers and nasally inoculated with approximately $10^{3.2}$ EID$_{50}$ of a LP AIV H4N6 diluted in 250 μL of BA-1 viral transport medium (see [8] for formula). Details of the virus have been presented elsewhere [22]. The control cottontails (n = 2) received 250 μL mock inoculations of BA-1 containing no virus.

For sampling from 1–8 days post infection (DPI), eight cottontails were sampled on odd days and eight cottontails were sampled on even days so that each animal was processed every other day to limit handling stress. All animals were also sampled on 16 and 22 DPI. Prior to sampling, all animals were anesthetized with isoflurane. Daily processing consisted of a nasal wash, an oral swab, and the collection of a fecal pellet from each individual sampled. Swabs and fecal pellets were stored in 1 mL of BA-1 medium and nasal cavities were washed using 1 mL of BA-1. All samples were stored on ice packs in animal rooms and were transferred to −80°C freezers immediately following the conclusion of daily processing. On 22 DPI, blood was collected and all animals were humanely euthanized.

Necropsy and Tissue Processing
Following euthanasia, animals were examined. Gross lesions were not observed in any of the cottontails. Tissues from major organs were collected for histopathology and preserved in 10% neutral buffered formalin. Fixed tissues were trimmed, placed in cassettes, processed overnight (Sakura Tissue-Tek VIP 6), embedded in paraffin, sectioned at 5 um and stained with hematoxylin and eosin. In addition, approximately 75 mg of nasal turbinates, trachea, upper lung lobe, lower lung lobe, and colon were collected into vials with 1 mL BA1 and homogenized and centrifuged as previously described [8] for testing by real-time reverse-transcription polymerase chain reaction (RRT-PCR). Animal carcasses were incinerated following necropsy procedures.

Laboratory Testing
Fecal pellets, oral swabs, and nasal washes were tested in duplicate by RRT-PCR for viral RNA detection and quantification. Primers and protocols [23,24], along with protocol modifications have been described in detail elsewhere [25]. Consistent with a previous study, positive samples were defined as those yielding a two-well positive amplification with a Ct value of ≤38 and suspect positive samples were defined as those yielding a two-well positive amplification with a Ct value of >38 [25]. Negative samples were defined as those yielding no Ct value or one that amplified a single well. Calibrated control samples were also analyzed with RRT-PCR to construct standard curves for each run. Viral RNA quantities from samples were extrapolated from the standard curves and are presented as PCR EID$_{50}$ equivalents/mL. Details about this procedure have been published elsewhere [25]. In addition, select nasal wash (2 and 7 DPI) and oral swab samples (2 and 5 DPI) were tested for live virus by virus isolation in embryonated chicken eggs following published protocols [26].

Serum samples collected pre- and post-inoculation were tested for anti-influenza virus A antibodies via the FlockCheck Avian Influenza Multi-Screen Antibody Test Kit (IDEXX Laboratories, Inc., Westbrook, ME) and Agar Gel Immunodiffusion (AGID) [27,28].

Results
Nasal Shedding
All inoculated animals yielded a minimum of $10^{4.5}$ PCR EID$_{50}$ equivalent/mL from nasal washes during the first DPI they were sampled (1 or 2 DPI; Figure 1). Nasal shedding peaked on 1 DPI yielding an average of $10^{6.47}$ PCR EID$_{50}$ equivalent/mL (range $10^{4.92}$ to $10^{6.99}$; Figure 1). With one exception, all treatment cottontails yielded their highest nasal wash on the first day they were sampled. As expected, a declining trend in viral RNA was noted during 1–8 DPI. By 16 DPI, eight of fourteen (57.1%) test animals were suspect positive. At 22 DPI, a single cottontail still had evidence of nasal shedding of viral RNA. The nasal washes from the two control cottontails remained negative throughout the study. All nasal washes tested positive for live virus.
during 2 DPI; however, this figure was greatly reduced by 7 DPI, as a single nasal wash tested positive for live virus at this time point.

Oral Shedding

All treatment cottontails showed evidence of oral shedding of AIV RNA by one or two DPI (Figure 1). Similarly to nasal washes, oral shedding peaked on the first day sampled with an average of $10^{4.68}$ PCR EID$_{50}$ equivalent/mL (range = $10^{3.48}$ to $10^{5.09}$; Figure 1). By 8 DPI, all but one treatment animals were negative. By 16 DPI, only one individual was suspect positive, which was a different individual than that sampled during 8 DPI. At 22 DPI, all oral swabs were negative. Both control animals yielded negative results throughout the study. All oral swabs tested positive for live virus during 2 DPI, while five of seven tested positive for live virus on 5 DPI.

Fecal Shedding

Viral RNA was rarely detected in or on the fecal pellets of cottontails during 2 to 4 DPI. Positive results ($n = 2$) of approximately $10^{2.0}$ PCR EID$_{50}$ equivalent/mL were noted on 2 DPI, while suspect positive results were noted on 2, 3, and 4 DPI. The control animals remained negative throughout the experiment. It should be noted, however, that the positive results we observed may simply be a result of contamination by oral or nasal secretions.

Individual Variation

Although all inoculated cottontails shed during this study, as expected, individual heterogeneity (variation among individuals) was commonly observed from both the nasal and oral routes of shedding (Figure 1). However, no clear pattern associated with DPI was noted.

RNA Detection in Tissues

Select tissues (nasal turbinates, trachea, lung [lower and upper lobes], and colon) were collected during necropsy on 22 DPI for RRT-PCR analyses. Two trachea samples yielded the only positive results. However, all other samples types yielded at least one suspect positive result, which suggests that in general this virus primarily cleared in this species within or earlier than three weeks of infection.

Serology

Although the FlockCheck Avian Influenza MultiS-Screen Antibody Test Kit has been evaluated for some mammal species [29], this test did not appear to work with our cottontail sera. However, all inoculated cottontails yielded evidence of a serological response in their convalescent sera at 22 DPI based on AGID. Serum samples from the 14 treatment cottontails were scored as strong positive (78.6%; $n = 11$) or positive (21.4%; $n = 3$). The 22 DPI serum samples from the control animals were scored as negative.

Figure 1. Mean nasal and oral shedding of avian influenza virus RNA of desert cottontails experimentally infected with a low-pathogenic avian influenza virus. Shedding was assessed from nasal washes and oral swabs by RRT-PCR. Results are presented as log$_{10}$ PCR EID$_{50}$ equivalents/mL. Vertical bars represent the maximum and minimum quantities detected on a given day.

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Similar scenario might be plausible for cottontails, as cottontails black-lipped pika, a close relative of rabbits, were exposed to HP infections in mammals. For example, it has been hypothesized that species [32]. These factors are also likely linked to HP cross-species transmission of LP AIV to various mammalian reservoirs are likely important elements that may have facilitated detection AIVs in different mammal species as early as 1 DPI [31]. 

A high levels of nasal shedding of AIV RNA by cottontails in this study by 1 DPI was somewhat surprising and raises the question of the potential of residual virus from nasal inoculations affecting the titers we observed. However, other studies have detected AIVs in different mammal species as early as 1 DPI [31]. 

It has been suggested that proximity to and contact with avian reservoirs are likely important elements that may have facilitated cross-species transmission of LP AIV to various mammalian species [32]. These factors are also likely linked to HP AIV infections in mammals. For example, it has been hypothesized that black-lipped pika, a close relative of rabbits, were exposed to HP H5N1 through shared vegetative foraging sites with birds [10]. A similar scenario might be plausible for cottontails, as cottontails could utilize the same foraging sites as wild birds and acquire an AIV infection via environmental contamination. Similarly, this scenario is possible in poultry rearing facilities with insufficient biosecurity, as a cottontail could acquire an AIV infection in contaminated bird pens or potentially transmit AIV through shared feed. Notably, bird to mammal transmission of AIV, likely through environmental contamination, has recently been documented in an experimental setting [9].

Host species barriers can limit the cross-species transmission of AIV to mammals [32,33]. As such, a myriad of factors such as virus-host interactions (e.g., within host barriers) and host-host interactions (e.g., cross-species contacts) are thought to be important requisites for cross-species transmission [33]. We have established that cottontails are effectively infected with AIV via the nasal route and subsequently shed viral RNA for several days. In addition, the histological lesions found in five of fourteen infected cottontails suggested that the virus we studied did invade pulmonary tissues but caused minimal tissue damage that was likely reversible. However, effective natural exposures of these animals to AIVs along with their ability to transmit the virus to a new host are undetermined at this time. Thus, multiple barriers may limit the effective host range of AIV in the mammal species we studied [32]. However, in the case of wild peridomestic mammals, a single infection that does not spread to conspecifics could pose some risk, as the mammal could transfer the virus to more susceptible avian species in operational settings.

Overall, this study suggests that at least one species of cottontail is susceptible to AIV infection and sheds relatively large quantities of viral RNA through both the nasal and oral routes. The shedding potential of cottontails, coupled with their synanthropic habits and their ability, in some instances, to move from pen-to-pen in production facilities with limited biosecurity suggests that this species could pose a threat to poultry operations. Additional studies are needed to assess the natural exposure routes of AIVs in cottontails to assess if and how frequently cottontails are exposed to these viruses in natural settings.

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Author Contributions

Conceived and designed the experiments: JR SAS KKV ABF. Performed the experiments: JR KTB TG NLM. Analyzed the data: JR SAS KTB TG NLM KKV HJS TRS. Contributed reagents/materials/analysis tools: HJS. Wrote the paper: JR SAS KKV TRS ABF.

References

1. Suarez DL (2008) Influenza A virus. In: Swanye DE, editor. Avian Influenza. Oxford: Blackwell Publishing. pp. 3–22.
2. Kumar S, Haven PL, Chusid MJ, Willoughby Jr RE, Simpson P, et al. (2010) Clinical and epidemiologic characteristics of children hospitalized with 2009 pandemic H1N1 influenza a infection. Pediatric Infectious Disease Journal 29: 591–594.
3. Swanye DE: (2008) The global nature of avian influenza. In: Swanye DE, editor. Avian Influenza. Oxford: Blackwell Publishing. pp. 123–123.
4. Halvorson (2008) Control of Low Pathogenicity Avian Influenza. In: Swanye DE, editor. Avian Influenza. Oxford: Blackwell Publishing. pp. 513–536.
5. Hall JS, Bentler KT, Lendahl G, Elmore SA, Minnis RB, et al. (2008) Influenza infection in wild raptors. Emerging Infectious Diseases 14: 1842–1848.
6. Hall JS, Minnis RB, Campbell TA, Barra S, DeYoung RW, et al. (2008) Influenza exposure in United States feral swine populations. Journal of Wildlife Diseases 44: 362–368.
7. Britton AP, Soejosoet K, Sowarno AP, Bidholka JJ (2010) Pandemic (H1N1) 2009 in skunks, Canada. Emerging Infectious Diseases 16: 1043–1045.
8. Shriram SA, VanDalen KK, Mosers NL, Ellis JW, Sullivan HJ, et al. (2012) Low-pathogenic avian influenza viruses in wild house mice. PLoS ONE 7: e9206.
9. Achenbach JE, Bowen RA (2011) Transmission of avian influenza A viruses among species in an artificial barnyard. PLoS ONE 6: e17643.
10. Zhou J, Sun W, Wang J, Guo J, Yin W, et al. (2009) Characterization of the H5N1 highly pathogenic avian influenza virus derived from wild pikas in China. Journal of Virology 83: 3957–3964.
11. Cardona CJ, Xing Z, Sandrock CE, Davis CE (2009) Avian influenza in birds and mammals. Comparative Immunology, Microbiology and Infectious Diseases 32: 253–273.
12. Chapman JA, Livaisa JA (2003) Eastern Cottontail. In: Feldhamer GA, Thompson BC, Chapman JA, editors. Wild Mammals of North America Biology, Management, and Conservation. 2 ed. Baltimore: The Johns Hopkins University Press. pp. 101–125.
13. Chapman JA, Willner GR (1976) Sylvilagus audubonii. Mammalian Species 10c: 1–4.
14. Schmidt DJ (2004) The Mammals of Texas. Austin: University of Texas Press. 501 p.
15. Armstrong, DM, Fitzgerald JP, Mcaney CA (2011) Mammals of Colorado. Boulder: University Press of Colorado. 620 p.
16. Adams LA (1994) Urban wildlife habitats: a landscape perspective. Minneapolis: University of Minnesota Press. 194 p.
17. Slota KE, Hill AE, Keeffe TJ, Bowen RA, Miller RS, et al. (2011) Human-bird interactions in the United States upland gamebird industry and the potential for zoonotic disease transmission. Vector-Borne and Zoonotic Diseases 11: 1115–1123.
18. Slota KE, Hill AE, Keeffe TJ, Bowen RA, Pabilonia KL (2011) Biosecurity and bird movement practices in upland game bird facilities in the United States. Avian Diseases 55: 190–196.
19. McQuiston JH, Garber LP, Porter-Spalding BA, Hahn JW, Pierson FW, et al. (2005) Evaluation of risk factors for the spread of low pathogenicity H7N2 avian influenza virus among commercial poultry farms. Journal of the American Veterinary Medical Association 226: 767–772.
20. Pedersen K, Swafford SR, Deliberto TJ (2010) Low pathogenicity avian influenza subtypes isolated from wild birds in the United States, 2006–2008. Avian Diseases 54: 405–410.
21. Root JJ, Hopken MW, Gidlewski T, Plaggio AJ (2013) Cottontail rabbit papillomavirus infection in a desert cottontail (Sylvilagus audubonii) from Colorado. Journal of Wildlife Diseases 49: 1060–1062.
22. Root JJ, Bender KT, Shriner SA, Mooers NL, VanDalen KK, et al. (2014) Ecological routes of avian influenza virus transmission to a common mesopredator: an experimental evaluation of alternatives. PLoS ONE In press.
23. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, et al. (2002) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology 40: 3256–3260.
24. NVSL (2008) Real-time RT-PCR for the detection of avian influenza virus and identification of H5 or H7 subtypes in clinical samples. NVSL document AVPRO1510042008.
25. Root JJ, Shriner SA, Bender KT, Gidlewski T, Mooers NL, et al. (2014) Extended viral shedding of a low pathogenic avian influenza virus by striped skunks (Mephitis mephitis) PLoS ONE e070639.
26. Szretter KJ, Balish AL, Katz JM (2006) Influenza: propagation, quantification, and storage. Current protocols in microbiology: John Wiley & Sons, Inc. pp. 15G.11.11–15G.11.22.
27. Beard CW (1970) Demonstration of type-specific antibody in mammalian and avian sera by immunodiffusion. Bulletin of the World Health Organization 42: 779–785.
28. CVB, NVSL (2003) Center for Veterinary Biologics and National Veterinary Services Laboratories Testing Protocol. ‘Avian Influenza Agar Gel Immunodiffusion Test to Detect Serum Antibodies to Type A Influenza Viruses’. Protocol #: AVPRO 0100.05, 2003.: USDA.
29. Chavez-Zamella JR, Vincent AL, Prickett JR, Zimmerman SM, Zimmerman JJ (2010) Detection of anti-influenza A nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked immunosorbent assay developed for avian species. Journal of Veterinary Diagnostic Investigation 22: 3–9.
30. Perkins LEL, Swayne DE (2003) Comparative susceptibility of selected avian and mammalian species to a Hong Kong-origin H5N1 high-pathogenicity avian influenza virus. Avian Diseases 47: 956–967.
31. Hinshaw VS, Webster RG, Easterday BC, Bean Jr WJ (1981) Replication of avian influenza A viruses in mammals. Infection and Immunity 34: 354–361.
32. Reperant LA, Rimmelzaan GF, Kuiken T (2009) Avian influenza viruses in mammals. OIE Revue Scientifique et Technique 28: 137–159.
33. Kuiken T, Holmes EC, McCallum J, Rimmelzaan GF, Williams CS, et al. (2006) Host species barriers to influenza virus infections. Science 312: 394–397.