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# Experimental infections of Norway rats with avian-derived low-pathogenic influenza A viruses

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Influenza A viruses (IAVs) are a public-health, veterinary, and agricultural concern. Although wild birds are considered the primary reservoir hosts for most IAVs [36], wild-bird IAV strains are known to spill over into poultry, domestic or wild mammals, and humans [9, 17, 29, 34]. Occasionally, spillover events may result in adaptation or reassortment with other strains. Moreover, some IAV strains found in wild waterfowl mutate into highly pathogenic forms in poultry, causing tremendous economic losses [2]. When domestic animals, wildlife, and humans dwell in close proximity to each other, such as may be the case with agricultural operations, wildlife may represent a potential risk for interspecies pathogen transmission [5, 6, 10, 14, 17, 18, 26, 34]. Understanding the pathways through which IAV strains could spillover from waterfowl reservoirs into humans and domestic animals is important for limiting the spread of IAVs, as well as developing biosecurity and containment procedures in livestock and poultry production.

Experimental studies of common wild mammals in the U.S. [19–22, 26], bank voles (*Myodes glareolus*) in Europe and Asia [32], and black rats (*Rattus rattus*) in Japan [11] have shown varying degrees of IAV susceptibility and/or

transmission in these synanthropic species. While Norway rats (*Rattus norvegicus*) are ubiquitous throughout rural and urban areas of the world and have the ability to range between these areas [23, 27], only limited investigations of this species have been conducted [1, 24, 25, 38], and their role in IAV transmission has not been clearly established. The main objective of this study was to further characterize IAV infection in Norway rats using IAV strains derived from poultry and wild water birds.

Norway rats were purchased from two local commercial sources (Animal Attraction, Greeley, CO, USA, and Scales ‘n Tails, Northglenn, CO, USA) and transported to the USDA-APHIS-WS National Wildlife Research Center (NWRC), Fort Collins, CO. The Institutional Animal Care and Use Committee at the NWRC approved all experimental procedures and animal holding conditions prior to and during experimentation (approval no. QA-1620). Animals were group-housed as same-sex cohorts of three with the recommended environmental conditions outlined by the National Research Council [7]. Prior to experimental inoculations, blood samples were collected from each rat to screen for antibodies to IAV using an epitope-blocking enzyme-linked immunosorbent assay (bELISA, [30]), and all animals were confirmed to be seronegative.

One hundred five rats were randomly divided among five treatment groups representing inoculations with one of four viral strains or negative controls. Two of the IAV strains used in the inoculations were derived from chickens, one was collected as part of United States wild-bird surveillance [8] and then passaged through a mallard, and the fourth was collected from the wild-bird surveillance. Each of the viruses was propagated in specific-pathogen free (SPF) chicken eggs prior to experimental infections. Detailed information on these viruses is provided in reference 26.

Twenty-four rats each were inoculated with one of four IAV virus strains: A/CK/CA/S0408793/04(H6N2) [37], A/CK/AL/75(H4N8) [3, 4, 12], A/mallard/CO/

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P66F1-5/08(H4N6) [35], or A/wildbird/CA/187718-26/08(H3N8) [33]. These virus subtypes were selected because they represent two of the most common subtypes in wild birds in North America [13, 15] and have been associated with outbreaks, both in commercial poultry and in a live-bird market [12, 37]. Nine rats were mock inoculated with negative amnio-allantoic fluid (AAF). Each treatment group was kept in a separate animal biosafety level 2 (BSL-2) room to prevent cross-contamination. After isoflurane inhalation anesthesia, rats were inoculated intranasally with approximately  $10^5$  EID<sub>50</sub> of virus delivered in 100  $\mu$ L of AAF or with 100  $\mu$ L of negative AAF by dispensing 50  $\mu$ L of inoculum into each nostril.

Three rats from each virus treatment group were euthanized per day on days 1-7 post-inoculation and then again on day 14. Three negative-control rats were euthanized and sampled on each of days 6, 7, and 14 post-inoculation. Rats were euthanized via carbon dioxide intoxication after anesthesia by isoflurane inhalation. Samples were collected from oral, nasal, and fecal swabs; respiratory tissues from nasal turbinates, tracheas, and cranial and caudal lobes of the lungs; and serum samples. Samples were collected immediately after euthanasia and were stored at  $-80^\circ\text{C}$  until laboratory testing. All samples were homogenized in TRIzol Reagent prior to testing and were then assayed for the presence of IAV RNA by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Viral RNA was extracted from samples using a MagMAX-96 AI/ND Viral RNA Isolation Kit, and qRT-PCR was performed using primers and probes developed by Spackman et al. [28]. The

extraction methods are described in detail by Shriner et al. [26] and the qRT-PCR methods are described by Van Dalen et al. [35]. Calibrated controls with known viral titers ( $10^2$  EID<sub>50</sub>/mL– $10^5$  EID<sub>50</sub>/mL) were analyzed on each qRT-PCR plate to construct 4-point standard curves. The amount of viral RNA in the samples was extrapolated from the standard curves and expressed as PCR EID<sub>50</sub> equivalents/mL. Selected tissue samples were also tested by virus isolation in SPF embryonated chicken eggs following standard protocols [31] to confirm the presence of infectious virus.

In order to compare infection characteristics, we compared viral replication across viral subtypes (H3N8, H4N6, H4N8, and H6N2), tissue types (caudal lung, cranial lung, nasal turbinates, and trachea), and host sex (male and female) using multivariate linear regression. Prior to statistical analysis, all tissues were standardized by weight. We compared all possible model variable combinations of subtype, tissue type, sex, days post-inoculation, and all two-way and three-way interactions between subtype, tissue type, and sex using Akaike's information criterion (AIC) corrected for small sample sizes (AICc, Table 1). All statistical analyses were conducted in R version 3.4.0 [16]

All control rats remained negative for IAV RNA throughout the study. For the inoculated rats, the rate of viral RNA replication varied by strain and was detected in multiple respiratory tissues across multiple days post-infection, suggesting that these IAV strains were able to replicate in Norway rats. In general, the amount of viral RNA detected in tissue samples was extremely low for the H4N8 virus, minimal for the H6N2 virus, and moderate for the H3N8

**Table 1** Model selection results for regression models testing the relationship between viral RNA concentration as a function of virus subtype, tissue sex, and day post-inoculation (DPI) and two-way interactions between the variables

| Model  | Adj. R <sup>2</sup> | K  | AICc   | delta AICc | AICc weight |
|--|---------------------|----|--------|------------|-------------|
| Subtype + Tissue + Sex + DPI + Subtype*DPI   | 0.34                | 13 | 963.55 | 0.00       | 0.33        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Subtype*Sex                               | 0.34                | 16 | 964.50 | 0.96       | 0.20        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Tissue*Sex                                | 0.34                | 16 | 966.20 | 2.65       | 0.09        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Subtype*Sex + Tissue*DPI                  | 0.35                | 19 | 966.61 | 3.06       | 0.07        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Subtype*Tissue                            | 0.37                | 22 | 966.63 | 3.08       | 0.07        |
| Subtype + Tissue + Sex + DPI + Subtype*Sex + Subtype*DPI + Tissue*Sex                  | 0.34                | 19 | 967.15 | 3.61       | 0.05        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Subtype*Tissue + Tissue*Sex               | 0.36                | 25 | 967.46 | 3.91       | 0.05        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Subtype*Tissue + Subtype*Sex              | 0.37                | 25 | 967.59 | 4.05       | 0.04        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Subtype*Tissue + Subtype*Sex + Tissue*Sex | 0.37                | 28 | 968.38 | 4.84       | 0.03        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Subtype*Tissue + Tissue*DPI               | 0.37                | 25 | 968.75 | 5.21       | 0.02        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Subtype*Tissue + Subtype*Sex + Tissue*DPI | 0.38                | 25 | 968.81 | 5.26       | 0.02        |
| Subtype + Tissue + Sex + DPI + Subtype*DPI + Tissue*Sex + Tissue*DPI                   | 0.34                | 19 | 969.11 | 5.57       | 0.02        |

Only models with a  $\Delta\text{AICc} < 6$  are shown. K is the number of parameters. Adj. R<sup>2</sup> is the R<sup>2</sup> value adjusted for the number of parameters in the model; it indicates the amount of variation explained by the model. AICc is Akaike's information criterion adjusted for small sample size.  $\Delta\text{AICc}$  values indicate the difference between a given model and the best model (the model with the lowest AICc score). The AIC weight shows the relative support for each model

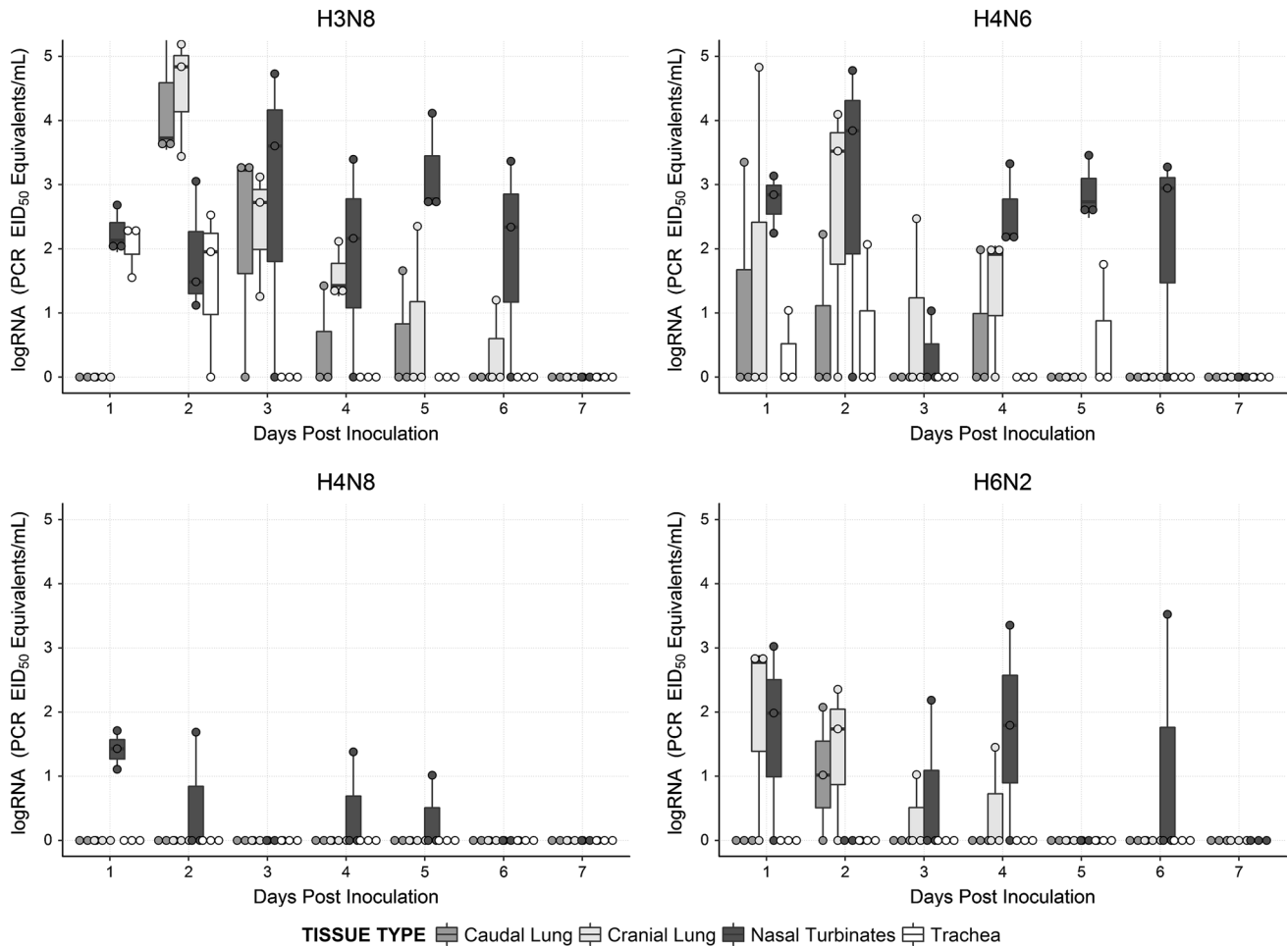
and H4N6 viruses. The amount of viral RNA as monitored by fecal, oral, and nasal swabs was minimal for each of the IAV strains studied. No IAV RNA was detected in fecal or oral swabs, and only 12 of the 94 nasal swabs collected from inoculated rats were positive for viral RNA (H4N8 = 0, H6N2 = 3, H4N6 = 4, H3N8 = 5) with five of the 12 positives identified at 1 day-post inoculation (DPI). The mean quantity of viral RNA detected in the nasal swabs for the different strains was relatively low ( $\leq 3.32 \log_{10}$  PCR EID<sub>50</sub> equivalents/mL). Only two of the 12 rats tested at 14 DPI were seropositive for antibodies to IAV (one inoculated with the H3N8 virus and one inoculated with the H4N6 virus), but this result was expected, since we did not retain the rats for an additional week when antibodies were more likely to be detected.

The only positive samples from the 23 rats inoculated with the H4N8 virus were from six nasal turbinate samples. Three samples were positive at 1 DPI, with one positive

each on days 2, 4, and 5 post-inoculation. All viral RNA quantities varied between 1.00 and 1.70  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL (Fig. 1).

For the 23 rats inoculated with the H6N2 virus, viral RNA was detected in nasal turbinates, cranial lung tissue, and caudal lung tissue, with no viral RNA detected in trachea. Viral RNA loads were highest in nasal turbinates (n = 6 rats), with a peak viral load of 3.5  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL. RNA was also detected in cranial lung sections (n = 6, peak viral load of 2.89  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL) and in the caudal lung sections of two rats (peak viral load of 2.07  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL).

IAV RNA was detected in all four tissue types tested from the 24 rats inoculated with H4N6. Similar to rats inoculated with other strains, IAV RNA was most often detected in nasal turbinates (n = 14), with a peak viral load of 4.78  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL. Of these 14 rats, six also had IAV RNA detected in cranial lung sections (peak viral load



**Fig. 1** Influenza A viral RNA detection for four respiratory tissues collected from Norway rats inoculated with avian-derived influenza A viruses. The H3N8 and H4N6 viruses were originally collected from

wild birds and the H4N8 and H6N2 viruses were originally collected from poultry. Three rats were sampled per day

of 4.09  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL), three rats had IAV RNA detected in caudal lung sections (peak viral load of 3.35  $\log_{10}$  PCR equivalent EID<sub>50</sub>/mL), and three rats had IAV RNA detected in trachea samples (peak viral load of 2.06  $\log_{10}$  PCR equivalent EID<sub>50</sub>/mL).

Finally, of the 24 rats inoculated with H3N8, 18 individuals had evidence of IAV RNA in tissues. IAV RNA was detected in nasal turbinates of 15 rats (peak viral load of 4.73  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL), caudal lung sections of seven rats (peak viral load of 5.45  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL) and cranial lung sections of 11 rats (peak viral load of 5.19  $\log_{10}$  PCR PCR EID<sub>50</sub> equivalents/mL). Lower quantities of IAV RNA were also detected in the trachea of five individuals (peak viral load of 2.52  $\log_{10}$  PCR EID<sub>50</sub> equivalents/mL).

While we found the highest RNA viral loads in nasal turbinate tissues, the amount of sample remaining after qRT-PCR testing was insufficient for virus isolation. However, we did have adequate tissue available to test four lung samples (two H3N8 and two H4N6) with RNA viral loads suitable for virus isolation. All four samples were positive for infectious virus.

The model-selection results for the regression models testing associations between viral RNA concentrations and viral subtype, tissue type, host sex, DPI, and interactions between these variables showed that the best model by AICc included subtype, tissue, sex, DPI, and an interaction between subtype and DPI (Table 1). This model had an AICc weight of 0.33, indicating that there is a 33% chance that this

model best explains the collected data. All of the models with AICc support included the variables in the top model but also included 1-3 additional interaction variables. The regression coefficients and ANOVA results for the top model (Table 2) show that all of the variables in the top model are statistically significant, indicating that the quantity of viral RNA detected varied by subtype, tissue, sex, and DPI. In general, viral RNA excretion was highest for the H4N6 virus and nasal turbinate tissues. RNA quantities were also slightly higher in females than in males and RNA quantities decreased as DPI increased.

Our results suggest that Norway rats can be infected with multiple strains of IAV, but the efficiency of replication is strain dependent, and only limited shedding of the virus can be measured in fecal, oral, or nasal swabs. On the other hand, moderate levels of replication were observed in respiratory tissues. Interestingly, the two poultry viruses that we tested, H4N8 and H6N2, exhibited significantly lower rates of viral RNA replication when compared with the two wild-bird-origin viruses, H3N8 and H4N6, which replicated without adaptation. Testing of additional virus strains is needed to elucidate whether this difference is associated with general strain and subtype variability or with the origin of these strains in poultry or wild birds. Testing of isolates of the same subtype from both wild birds and poultry could provide further information about the relationship of the origin of the virus to its ability to replicate in rats. One prospect for future study is additional H6N2 testing, since this subtype is common in both wild

**Table 2** Regression coefficients and ANOVA table for the best model as selected by Akaike's information criteria (AIC)

| Variable                | Parameter estimate | Std. error | t-value | Pr(> t ) |         |
|-------------------------|--------------------|------------|---------|----------|---------|
| (Intercept)             | 2.379              | 0.272      | 8.751   | < 0.001  |         |
| Subtype H4N6            | -0.863             | 0.352      | -2.454  | 0.002    |         |
| Subtype H4N8            | -2.341             | 0.354      | -6.621  | < 0.001  |         |
| Subtype H6N2            | -1.635             | 0.351      | -4.655  | < 0.001  |         |
| Tissue cranial lung     | 0.322              | 0.155      | 2.075   | 0.0388   |         |
| Tissue nasal turbinates | 0.872              | 0.155      | 5.620   | < 0.001  |         |
| Tissue trachea          | -0.230             | 0.157      | -1.466  | 0.014    |         |
| Sex male                | -0.224             | 0.118      | -1.891  | 0.060    |         |
| DPI                     | -0.323             | 0.055      | -5.87   | < 0.001  |         |
| Subtype H4N6:DPI        | 0.124              | 0.079      | 1.568   | 0.118    |         |
| Subtype H4N8:DPI        | 0.294              | 0.079      | 3.741   | 0.001    |         |
| Subtype H6N2:DPI        | 0.196              | 0.080      | 2.447   | 0.015    |         |
| Variable                | Df                 | Sum Sq     | Mean Sq | F-value  | Pr(>F)  |
| Subtype                 | 3                  | 63.100     | 21.030  | 20.790   | < 0.001 |
| Tissue                  | 3                  | 55.460     | 18.490  | 18.274   | < 0.001 |
| Sex                     | 1                  | 6.910      | 6.909   | 6.830    | 0.009   |
| DPI                     | 1                  | 38.100     | 38.100  | 37.666   | < 0.001 |
| Subtype:DPI             | 3                  | 15.060     | 5.021   | 4.963    | 0.002   |

DPI is days post-inoculation. Subtype comparisons are to subtype H3N8, tissue comparisons are to caudal lung, and subtype:DPI comparisons are to subtype H3N8

birds and outbreaks in live-bird markets. Other possibilities worth investigating are receptor characterization in Norway rat tissues and molecular adaptations associated with replication in this species. A difference between the wild-bird and poultry viruses that should be noted is their differing passage histories. Both of the poultry viruses were laboratory adapted, as they had been passaged in eggs many times, while the two wild-bird viruses were first passages.

We detected little to no IAV shedding in oral swabs or feces, but we did detect moderate quantities of IAV RNA in nasal secretions ( $\leq 3.32 \log_{10}$  PCR EID<sub>50</sub> equivalents/mL) and upper respiratory tissues. These results are similar to those obtained with wild house mice infected with multiple IAVs where nasal shedding of viral RNA was moderate (mean  $\leq 4.49 \log_{10}$  PCR EID<sub>50</sub> equivalents/mL) but oral shedding and fecal shedding was minimal or undetectable [26]. Likewise, oropharyngeal swabs of Sprague-Dawley rats inoculated with H7N3 or H5N2 viruses were all negative, although 80–100% of inoculated rats developed antibodies to IAV [1]. These results differ from those obtained with other common mammals, such as striped skunks and cottontails inoculated with H4N6, which shed greater quantities of IAV RNA orally and nasally (maximum concentrations were between 5.09 and 6.94  $\log_{10}$  PCR equivalent EID<sub>50</sub>/mL) [20, 21], and may differ from those obtained with rodents experimentally infected with highly pathogenic avian influenza (HPAI) strains [24, 32].

While shedding of viral RNA in Norway rats was minimal to moderate for all of the virus strains tested, these results demonstrate that IAV RNA can be detected in respiratory tract tissues at higher quantities than in oral or nasal swabs. Preventing the potential spread of IAV from reservoir waterfowl to humans, domestic animals, and livestock requires an understanding of viral transmission in human-occupied areas and agricultural operations. The higher levels of IAV RNA detected in respiratory tissues of Norway rats (versus external shedding) suggests that the main risk of IAV transmission from rats would be due to consumption of infected respiratory tissues by predators and/or scavengers. Many domesticated animals, such as swine, barnyard chickens, dogs, and cats prey on or scavenge infected Norway rats, and humans may be at risk when handling infected carcasses. While Norway rats are not likely to play a large role in IAV transmission, infection may be strain dependent, and further investigation of the infection dynamics of additional strains and other small mammals will aid in directing risk mitigation, biosafety practices, and rodent control on farms and in urban areas.

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