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Duration of Highly Pathogenic Avian Influenza Virus and Newcastle Disease Virus Infectivity in Dried Ornithologic Study Skins

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ABSTRACT: Ornithologic study skins are specimens of avian skins that have been preserved by drying after removing the viscera and muscle. Because of the high value of study skins for scientific studies, specimens are shared among researchers. There is concern that study skins might be contaminated with high-consequence diseases such as highly pathogenic avian influenza virus (HPAIV) or Newcastle disease virus (NDV). To mitigate risk, thermal or chemical treatment of study skins may be required before transfer; however, such treatments might damage the specimens. Therefore, a study was conducted to evaluate the duration of infectivity of HPAIV and NDV in study skins prepared from infected chickens (*Gallus gallus*). Study skins were prepared from 10 chickens infected with each virus. Skin and feather pulp samples were taken at the time of study skin preparation to establish starting titers. Mean starting titers in the skin was 4.2 $\log_{10}$ and 5.1 $\log_{10}$ 50% egg infectious doses (EID_{50}) for HPAIV and NDV groups respectively, and were 6.7 $\log_{10}$ EID_{50} for HPAIV, and 6.4 $\log_{10}$ EID_{50} for NDV in feather pulp. Samples were collected at 2 and 4 wk of drying to quantify viable virus. At 2 wk, fewer samples had detectable virus and mean titers were 1.8 $\log_{10}$ (skin) and 2.1 $\log_{10}$ (feathers) EID_{50} for HPAIV, and 1.7 $\log_{10}$ (skin) and 3.5 $\log_{10}$ (feathers) EID_{50} for NDV. At 4 wk viable virus could not be detected in either tissue type.

Key words: Avian influenza, avian study skin, disease risk assessment, Newcastle disease, virus inactivation.

Ornithologic study skins are preserved specimens of intact bird skins that are frequently utilized for comparative studies. Similar to taxidermy, the skins are prepared by removing the internal organs, bones, and muscle tissue, except the extremities of the wings and legs (the carpometacarpus, tarsometatarsus, and digits are not removed; Winker 2000). In some cases, the skull also may not be removed.

Because of the high value of study skins for scientific studies, specimens are shared among researchers globally. However, there is concern that they might carry diseases that are important for wildlife and agriculture such as highly pathogenic avian influenza virus (HPAIV) or Newcastle disease virus (NDV). Both HPAIV and NDV can infect a wide range of avian species (Gogoi et al. 2017; Swayne et al. 2020) and there is currently a global epornitic of H5 HPAIV that is affecting numerous domestic and wild avian species that do not normally carry HPAIV (UN-FAO 2023).

The potential for inadvertent disease introduction by contaminated study skins has created barriers to lending study skin collections internationally. Some countries require thermal or chemical treatment of the specimens before importation, or transfer may be entirely restricted. The risk that treatment might damage the specimens is an obstacle to scientific studies. Avian study skins have been used since at least the 1830s (Swainson 1836); therefore specimens can be well over a century old and often have substantial historic value. Regardless of age, study skins are often also fragile and irreplaceable.

Both HPAIV and NDV are enveloped viruses that are labile compared with most microbes (McDonnell 2007). Inactivation data have been produced for both AIV and NDV under numerous conditions using a variety of substrates (Kinde et al. 2004; Benson et al. 2008; Boumart et al. 2016; Mo et al. 2022; Spackman 2023). Virus durability in study skins does not appear to have been evaluated. Because the internal organs of the bird are removed during skin preparation, epithelial cells in the skin and feather pulp are the remaining sites where virus might be found in a study skin; both tissue types have been shown to contain both HPAIV and NDV (Beato et al. 2009, Lee et al. 2016, Dimitrov et al. 2019). Therefore, the objective of our study was to determine how long HPAIV or

NDV might remain infectious in the skin and feather pulp of avian study skins and to inform risk assessments for their handling, transport, and importation.

Specific-pathogen-free white leghorn chickens were obtained from US National Poultry Research Center, US Department of Agriculture–Agricultural Research Service (USNPRC) in-house flocks at 5 wk of age; all procedures involving animals were reviewed and approved by the USNPRC Institutional Animal Care and Use Committee. Chickens were divided into two groups of 10 and housed in in-house-built Horsfall isolators in different rooms. Chickens were inoculated with 10^6 50% egg infectious doses (EID_{50}) in 0.1 mL by the intrachoanal route with either A/turkey/MN/12582/2015 H5N2 HPAIV in one room, or avian orthoavula virus type 1 (AOAV-1) chicken/Pakistan/6/2015 genotype VII NDV in the other room. Virus isolates were obtained from the USNPRC repository and were propagated and titrated in embryonated chicken eggs as described (Spackman and Stephens 2016).

Chickens were observed twice daily. When a chicken presented with clinical signs characterized by moderate to severe lethargy; hemorrhagic combs, wattles, or shanks; or neurologic signs, it was humanely euthanized by cervical dislocation in accordance with humane guidelines of the American Veterinary Medical Association (AVMA 2020). Immediately after euthanasia, samples were collected to establish starting titers. Skin samples were collected from apteric areas to avoid feather pulp. A 0.5-cm^2 patch of skin from the back and a second patch from the breast or thigh were collected and pooled in one vial per bird with 1 mL of brain–heart infusion (BHI) broth (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) with antibiotics (final concentration of 2 µg/mL amphotericin B, 1,000 units/mL penicillin G, and 100 µg/mL gentamicin). Three feathers 2–4 cm in length containing pulp, including one each from the tail retrices, wing secondaries, and the ventral sternal tract, were also collected and pooled in one vial per bird with 1 mL of BHI broth with antibiotics. Infectious virus titers were determined by titration in eggs as described below.

Study skins were prepared from the chicken carcasses by standard methods for museum specimens. Briefly, all organs, muscle, and bones were removed except the carpometacarpus and surrounding muscles, tarsometatarsus, digits and surrounding muscle, and the skull and skull contents. A total of 10 study skins was prepared for each virus. Study skins were stuffed with polyester batting and sewn closed with cotton thread. The study skins were each pinned to a foam board and were stored in clean Horsfall isolators at ambient temperatures during the drying process. The temperature of the room/isolators was recorded on data loggers and the mean was 22.2 C with a SD of ±0.6 C. The isolators were ventilated with low-velocity (0.1–0.2 m^3/min) supply air from the room that was high-efficiency particulate air (HEPA) filtered, which would help remove moisture; a double HEPA-filtered exhaust was used. We did attempt to measure the rate of drying gravimetrically, but the differences in mass between sample collection times were too small to measure accurately.

Additional feather and skin samples were collected 2, 4, and 6 wk after preparation, identically to the initial samples. Each subsequent skin sample was collected adjacent to a previous sample and feathers were pulled from the same locations. Because neither virus could be detected at 4 wk of drying, samples collected at 6 wk were not processed for quantitative virus isolation. All samples were stored at −80 C until they were processed.

Feather shafts were processed to extract the pulp by crushing the calamus with sterile forceps in a vial with 1 mL of BHI broth as described (Busquets et al. 2010). The feather shafts were discarded, and the remaining BHI was used for virus detection. Skin was processed by homogenizing with quartz beads in a homogenizer (FastPrep-24, MP BioMedicals, Irvine, California, USA); the supernatant was then used for the virus quantification.

Viable virus was quantified in embryonating chicken eggs (ECE), which were selected because they are the most sensitive culture systems for the detection of infectious AIV and AOAV-1 (Moresco et al. 2010, 2012). Supernatant from feather shafts or homogenized skin was

TABLE 1. Log_{10} 50% egg infectious doses per specimen of highly pathogenic avian influenza virus (HPAIV) in skin and feather pulp from chicken carcasses prepared as dried study skins after experimental infection with HPAIV and euthanasia when showing clinical signs.

	Skin			Feather pulp		
Bird	0 wk[a]	2 wk	4 wk	0 wk	2 wk	4 wk
1	5.3	1.8	—	7.8	1.1	—
2	4.3	—[b]	—	7.0	—	—
3	3.0	—	—	6.0	—	—
4	5.0	—	—	7.0	—	—
5	5.0	—	—	8.3	3.3	—
6	3.5	—	—	6.3	—	—
7	3.3	—	—	5.8	—	—
8	5.0	—	—	7.3	—	—
9	3.3	—	—	6.0	—	—
10	4.3	—	—	6.0	—	—
Mean	4.2		—	6.7	2.1	—

[a] wk = number of weeks of drying.
[b] — = not detected.

TABLE 2. Log_{10} 50% egg infectious doses per specimen of Newcastle disease virus (NDV) in skin and feather pulp from chicken carcasses prepared as dried study skins after experimental infection with NDV and euthanasia when showing clinical signs.

	Skin			Feather pulp		
Bird	0 wk[a]	2 wk	4 wk	0 wk	2 wk	4 wk
1	5.5	2.8	—	7.3	5.5	—
2	6.3	—[b]	—	6.0	3.0	—
3	5.3	—	—	6.0	3.3	—
4	5.8	1.0	—	6.8	4.3	—
5	6.3	—	—	6.0	2.8	—
6	3.8	—	—	5.8	3.5	—
7	5.3	—	—	6.8	3.3	—
8	6.0	1.3	—	8.3	4.8	—
9	3.0	—	—	5.8	2.5	—
10	4.3	—	—	5.0	2.0	—
Mean	5.1	1.7	—	6.4	3.5	—

[a] wk = number of weeks of drying.
[b] — = not detected.

diluted 10-fold in BHI broth with antibiotics and 0.1 mL of each dilution was inoculated into three ECE as described (Spackman and Stephens 2016). Eggs were incubated for 4 d for HPAIV and 5 d for NDV. Fluid was collected from each egg and individually tested for hemagglutination using standard methods (Killian 2014). The limit of detection for this assay is approximately 0.5 log_{10} EID_{50}/mL. Titers were calculated with the Reed–Muench method (Reed and Muench 1938).

The titers of HPAIV in skin and feather pulp are shown Table 1. At the time of skin preparation, 10/10 (100%) skin and feather samples were positive for infectious HPAIV, with means of 4.2 and 6.7 log_{10} EID_{50} respectively. After 2 wk of drying, virus was detected in the skin from 1/10 (10%) study skins with a titer of 1.8 log_{10} EID_{50} and in feather pulp samples from 2/10 (20%) study skins with a mean of 2.1 log_{10} EID_{50}. Virus was not detected in any samples after 4 wk of drying.

Titers of NDV in skin and feather pulp are shown Table 2. All skin and feather pulp samples were positive for infectious NDV at the time of study skin preparation and mean titers in skin and feather pulp were 5.1 and 6.4 log_{10} EID_{50} respectively. By 2 wk, virus was detectable from 4/10 (40%) of the study skins with a mean titer of 1.7 log_{10} EID_{50} and virus was detected in 10/10 (100%) of the feather pulp with a mean titer of 3.5 log_{10} EID_{50}. Infectious NDV could not be detected in either skin or feather pulp after 4 wk of drying.

Under the conditions evaluated, both viruses exhibited a titer loss of up to 8 log_{10} from feather pulp and up to 6 log_{10} from skin tissue. This is consistent with previously reported data on AIV durability at similar temperatures (around 22 C) in feather pulp (Busquets et al. 2010; Yamamoto et al. 2010) and AIV and NDV durability in a variety of substrates (Guan et al. 2016; Stephens et al. 2020; Mo et al. 2022). However, HPAIV has been shown to remain infectious much longer in water and on surfaces (Spackman 2023). Scant data are available for NDV; studies at similar temperatures have evaluated stabilized nonvirulent strains of AOAV type-1 for use as live vaccine (Frerichs and Hebert 1974; Boumart et al. 2016), so are not comparable. Numerous studies for both AIV and NDV are not equivocal because 1) virus needed to be artificially inoculated into the target substrate and 2)

other substrates are very different environments for the virus than desiccating skin or feather pulp. Our test material was tissue from infected birds, which is a simulation of conditions that might be encountered in the field with an infected bird.

There are several limitations of our study. First, because these evaluations are very resource intensive, it was only possible to evaluate at one temperature. Second, the times and temperatures used for the study skin drying process are variable; virus infectivity might persist longer at lower temperatures. However, if there were concerns that study skins had only been held at lower temperatures, they could be held at 20–25 C for 4 wk or more to mitigate the risk. Third, virus replication may not be distributed evenly within tissues, and titers can vary among species. To account for this, the study was designed to achieve the highest titers possible in the skin and feather pulp: Chickens were used because both HPAIV and NDV replicate systemically to high titers in this species, and the time of euthanasia was selected to achieve the highest level of skin and feather contamination.

Our finding of decline in infectious virus to undetectable levels within 4 wk suggests that the risk of transporting study skins with HPAIV or NDV contamination is probably very low for fresh specimens after complete drying and is probably negligible for older study skins. Risk assessments also should consider that the way study skins are used does not provide an interface with live birds. The potential for a given species to be infected with either virus at the time of death is another important component of determining risk. Finally, considering data from other reports will help inform approaches to evaluating and mitigating risk. Future studies evaluating additional environmental conditions of storage would be beneficial.

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