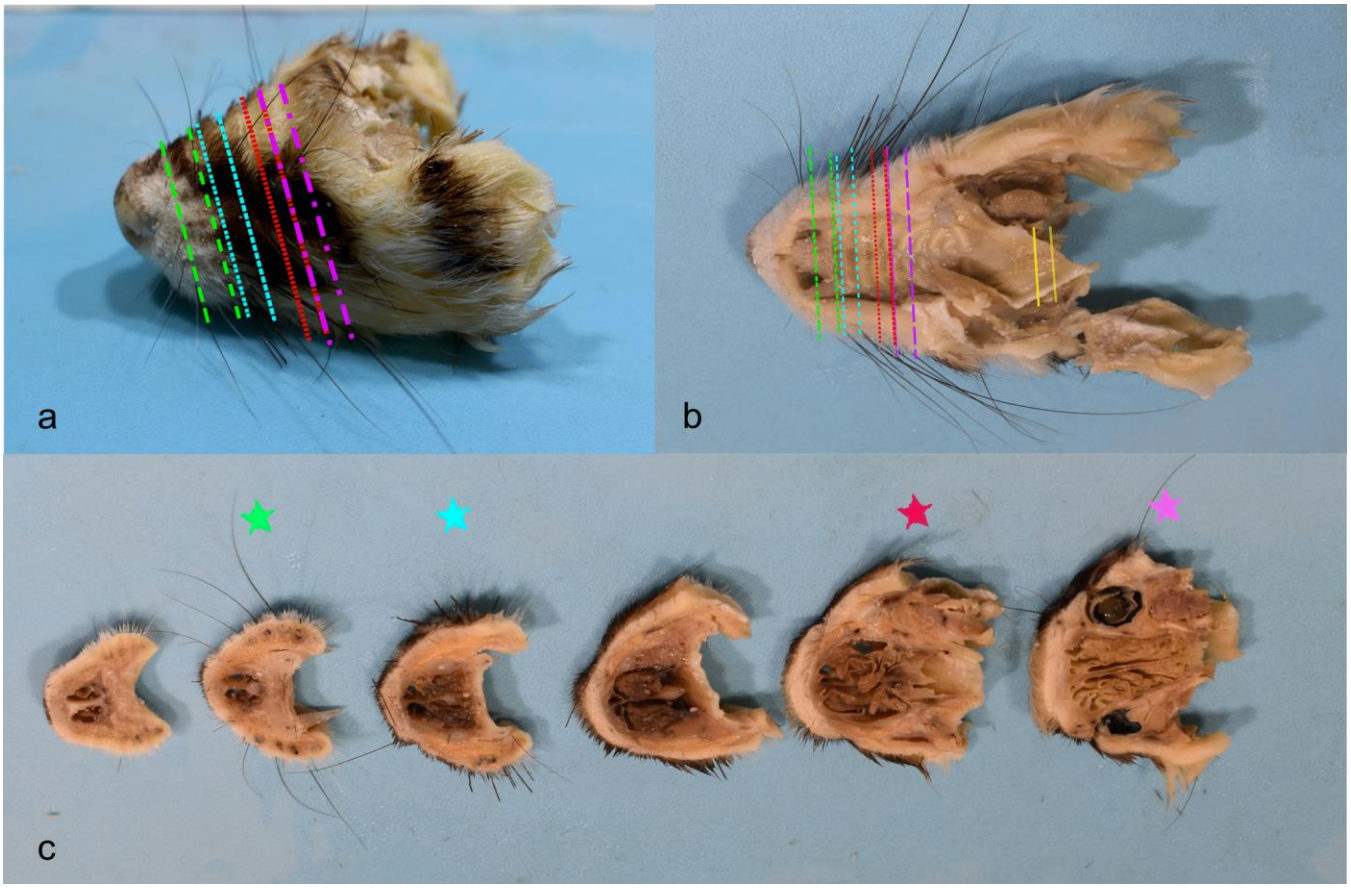
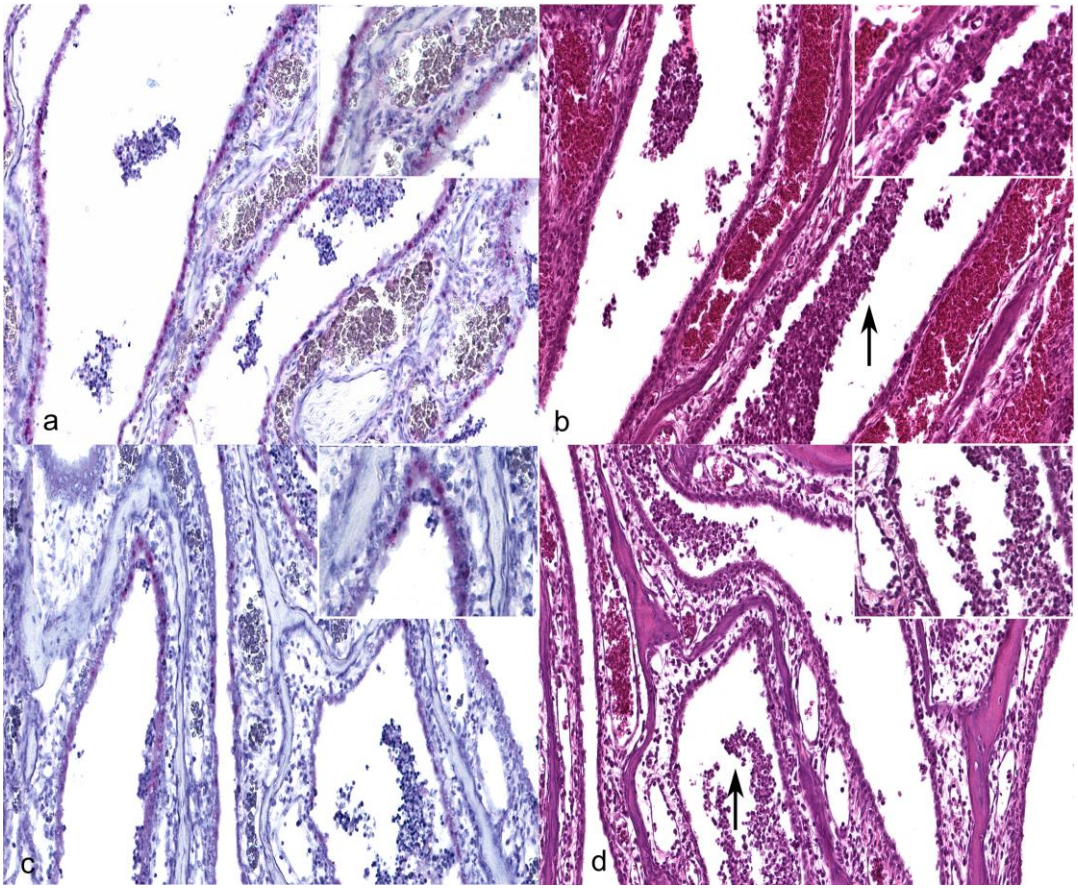
VET-22-BC-0005, Gary et al. Influenza A ocular inoculation in ferrets

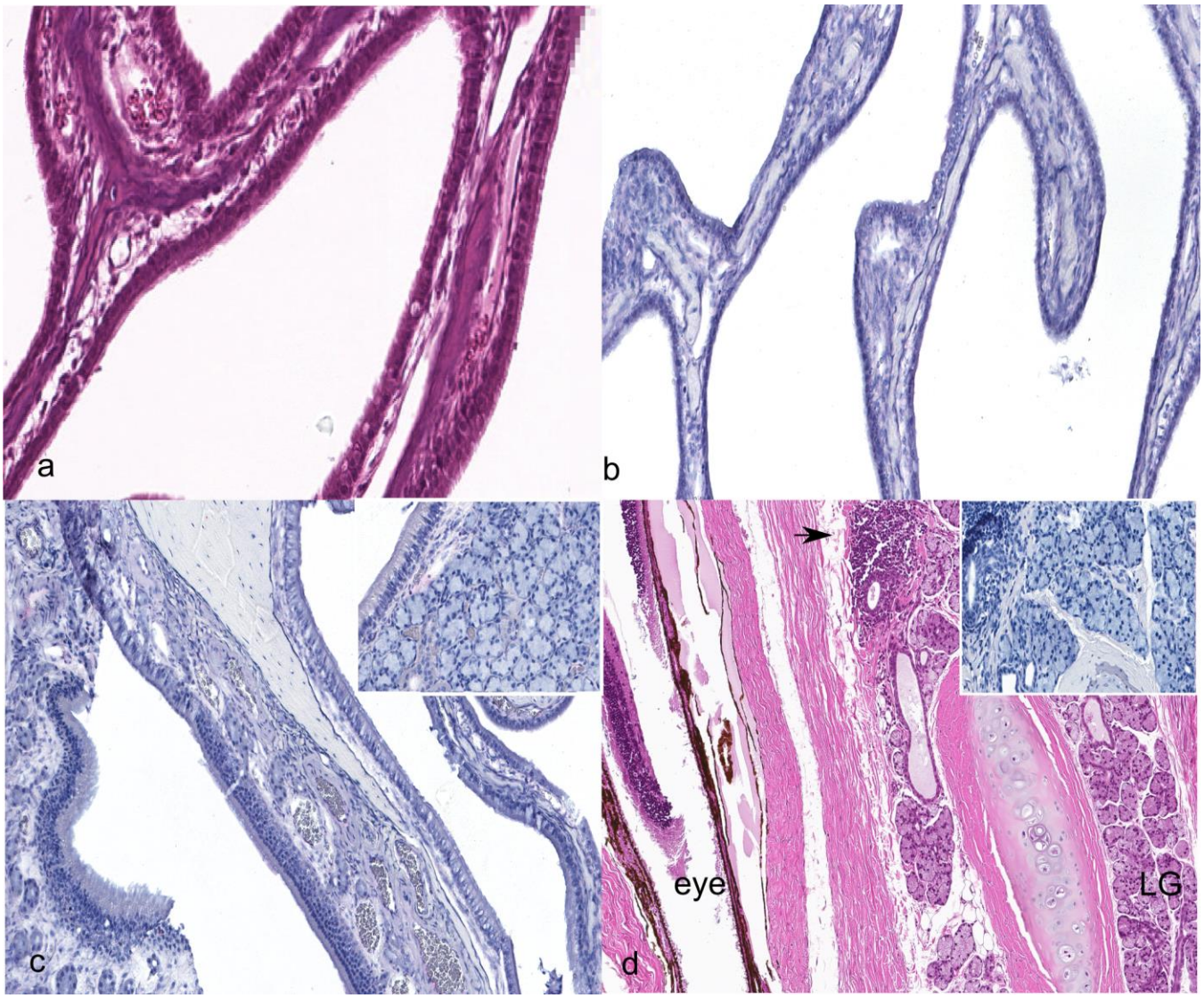
Supplemental Figures



Supplemental Figure 1: Sections through a fully fixed and decalcified ferret nose and proximal calvarium that were taken in order to observe the nasal and periocular structures, shown on the lateral and ventral aspect of the head (**a and b**). Colored lines indicate the different incisions used to create the sections. **c.)** The serial sections through the nose and proximal calvarium resulting from these incisions are shown, and color-coded stars indicate the sections embedded for further evaluation.



Supplemental Figure 2: Inoculation with influenza A virus (IAV) intranasally and intraocularly (conjunctival) leads to acute inflammation and edema in the maxillary nasal turbinates with immunolabeling (IHC, in red) for IAV antigen in the respiratory epithelium. **a and b)**. One day after intranasal inoculation with IAV, abundant immunolabeling of IAV antigen in the respiratory epithelium is present in most epithelial cells (a, IHC targeting IAV) with neutrophilic infiltrates in the mucosa and respiratory epithelial cell sloughing into the nasal vestibule, which also contains cell debris, neutrophils, occasional macrophages, and mucus (highlighted an arrow, hematoxylin and eosin, HE, b). **c and d)**. One day after intraocular inoculation, immunolabeling for IAV shows viral antigen in the respiratory epithelium, to a slightly lesser extent than animals that were inoculated intranasally (c, IHC for IAV). The mucosa of the maxillary turbinates is expanded by edema and neutrophilic infiltrates, and sloughed epithelial cells mixed with neutrophils, mucus, and occasional macrophages are present in the nasal vestibule (highlighted by an arrow, d, HE).



Supplemental Figure 3: Sections through the nasal turbinates and periocular structures of a ferret sham inoculated with PBS (control animal), showing normal turbinate structures and periocular structures, and lack of immunolabeling by a IAV IHC assay. **a).** The maxillary turbinates are lined by intact respiratory epithelium and rare mononuclear cells are noted in the submucosa. **b and c.)** No immunolabeling for IAV is seen in the maxillary turbinate respiratory epithelium (b) or in the ethmoid turbinate respiratory, olfactory, or glandular epithelium (c). The inset in figure c shows a lack of immunolabeling for IAV in the maxillary sinus. **d.)** A section through the eye shows the adjacent lacrimal gland (LG) in a control ferret, with normal ducts, a focal duct-associated lymphoid aggregate (arrow), and acini. The inset shows a lack of immunolabeling by an assay targeting IAV in the lacrimal gland.

Supplemental Methods

Virus. A/Nebraska/14/2019 (H1N1) virus was propagated in Madin-Darby canine kidney (MDCK) cells as previously described.[3] Pooled cell supernatant was clarified by centrifugation and frozen in aliquots at -70°C until use. Stock titer was determined by standard plaque assay.[5] Stock was sequenced and tested for exclusivity to rule out the presence of other influenza virus subtypes prior to use.

Ferret challenge. Male 18-month-old ferrets (Triple F Farms, Sayre, PA) were confirmed to be seronegative to currently circulating A (H1N1, H3N2) and B subtypes by hemagglutination inhibition (HI) assay prior to use. Ferrets were housed in a Duo-Flow Bioclean mobile environmental enclosure (Lab Products) for the duration of the experiment. Three ferrets were included for each treatment and time point. Intranasal (i.n.) inoculations were performed under anesthesia (a ketamine cocktail [10-30mg/kg ketamine, 2 mg/kg xylazine] administered i.m.) with 10^6 PFU of virus diluted in PBS in a 1ml volume as previously described.[4] Conjunctival (ocular, i.o.) inoculations were performed under anesthesia with 10^6 PFU of virus diluted in PBS in a 100µl volume (by administering virus dropwise over the surface of the right eye) as previously described.[1] Control ferrets were inoculated only with PBS.

Ferrets were monitored daily post-inoculation, prior to any anesthesia events, for morbidity and clinical signs as previously described.[4] At the specified time points, nasal wash (NW) and conjunctival wash (CW) specimens were collected prior to euthanasia, immediately frozen and stored at -70°C until determination of infectious virus by standard plaque assay, as previously described.[1] Statistical analyses of NW viral titers, weight loss, and temperature were determined by Student's t test. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility.

Tissue preparation. At the time of necropsy, ferret heads were removed at the neck, immediately submerged in 10% neutral buffered formalin, and fixed for 1-2 weeks. After fixation, skin, soft tissue, and

bone caudal to the eyes, as well as the entire mandible, were removed and the remaining rostral portion of the head, including the nose, eyes, hard palate, and a portion of the soft palate, were placed in Versenate decalcification solution (American MasterTech Scientific, CA) for 1.5-2 months and were then placed in Cal-EX-1 decalcification solution (Fisher Chemical, NJ) for 24 hours. Nasal passages and heads were then sectioned into 6, approximately 5.5 mm, coronal sections. Of these, a section directly caudal to nasal planum and the incisors, a section in the middle of the nose, a section directly cranial to the eyes, and a section through the front of the eyes were selected for processing and histopathologic evaluation (Supplemental Figure 1). Sections of soft and hard palate were also collected.

Histochemical staining and immunohistochemical analysis. All sections were routinely processed for paraffin histology, sectioned at 4 µm, and stained by hematoxylin and eosin (H&E). An immunohistochemical (IHC) assay was also performed on these sections using a mouse monoclonal antibody raised against the influenza A nucleoprotein (A3) at 1:10,000 dilution (CDC biological products ATCC) and using a Mach 4 Universal AP Polymer Kit (Biocare Medical, Pacheco, CA) with Permanent Red Chromogen (Cell Marque/Millipore Sigma, Burlington, MA). Tissue sections were digested with 0.1 mg/mL proteinase K (Roche Diagnostics, Indianapolis, IN) in proteinase K buffer (0.6 M Tris (pH 7.5)/ 0.1% $CaCl_2$) for 15 min and then blocked with 20% normal sheep serum in Tris-saline-tween-20 prior to staining. The positive control consisted of formalin-fixed, paraffin-embedded MDCK cells infected with a 2009 H1N1 influenza A virus. Appropriate negative control serum was run in parallel. Slides were counterstained with Mayer's hematoxylin (Poly Scientific, https://www.polyrnd.com) and blued in lithium carbonate (Poly Scientific). Validation of the antibody was performed as previously described.[2]

H&E-stained sections were reviewed and scored semi-quantitatively by a boarded veterinary pathologist, who was not blinded as to the experimental treatment groups. Scoring was performed for several criteria, including acute and chronic inflammatory infiltrates, edema, and inflammatory exudates in the nasal passages, and necrosis, epithelial hyperplasia or metaplasia, and re-epithelialization in different tissues (maxilloturbiantes, ethmoturbinates, submucosal glands, conjunctiva, maxillary sinus,

lacrimal gland, and hard and soft palate). Scores ranged from 0 (no change), 1 (minimal, with only scattered inflammatory cells or rare associated changes such as edema, 2 (mild, with an noticeable, but limited inflammatory infiltrate and occasional associated tissue response, without destruction or disruption of tissue structure), 3 (moderate, with a notable inflammatory infiltrate and multifocal tissue response with only occasional disruption of tissue structure), 4 (marked, with an abundant inflammatory infiltrate and associated tissue response, leading to disruption of normal tissue structure), to 5 (severe, with all tissue effaced by inflammatory infiltrates and the associated tissue response). Inflammatory scores for each animal were comprised of combined scores for acute and chronic infiltrates, exudates, edema, and necrosis (with a total of 25 possible). Reparative scores represented the combined scores for hyperplasia, metaplasia, and re-epithelialization (15 possible). Immunolabeling for influenza A virus antigen was also scored semi-quantitatively by a non-blinded, boarded veterinary pathologist on a 0-5 scale, as follows: 0 (no staining), 1 (rare staining), 2 (occasional staining), 3 (multifocal staining), 4 (abundant staining), and 5 (diffuse staining). The mean of each of these scores from the three animals per inoculation group are reported in Table 1. Localization of immunolabeling in the tissue was also noted.

References

1. Belser JA, Gustin KM, Maines TR, Pantin-Jackwood MJ, Katz JM, Tumpey TM. Influenza virus respiratory infection and transmission following ocular inoculation in ferrets. *PLoS Pathog*. 2012;8**:** e1002569.
2. Guarner J, Shieh WJ, Dawson J, et al. Immunohistochemical and in situ hybridization studies of influenza A virus infection in human lungs. *Am J Clin Pathol*. 2000;114**:** 227-233.
3. Maines TR, Jayaraman A, Belser JA, et al. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. *Science*. 2009;325**:** 484-487.
4. Maines TR, Lu XH, Erb SM, et al. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. *J Virol*. 2005;79**:** 11788-11800.
5. Szretter KJ, Balish AL, Katz JM. Influenza: propagation, quantification, and storage. *Curr Protoc Microbiol*. 2006;Chapter 15**:** Unit 15G 11.