Pathogenesis of Aquatic Bird Bornavirus in Muscovy Ducks

Melanie Iverson

University of Guelph

Alexander Leacy

University of Guelph

Phuc H. Pham

University of Guelph

Sunoh Che

University of Guelph

Emily Brouwer

University of Guelph

Eva Nagy

University of Guelph

Brandon N. Lillie

University of Guelph

Leonardo Susta (✉ lsusta@uoguelph.ca)

University of Guelph

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Abstract

Aquatic bird bornavirus (ABBV), a type of avian bornavirus, has been associated with inflammation of the central and peripheral nervous systems and neurological disease in wild waterfowl in North America and Europe. The potential of ABBV to infect and cause lesions in commercial waterfowl species is unknown. The aim of this study was to determine the ability of ABBV to infect and cause disease in day-old Muscovy ducks (n = 174), selected as a representative domestic waterfowl. Ducklings became infected with ABBV through both intracranial and intramuscular infection routes: upon intramuscular infection, the virus spread centripetally to the central nervous system (brain and spinal cord), while intracranial infection led to virus spread to the spinal cord, kidneys, proventriculus, and gonads (centrifugal spread). Infected birds developed both encephalitis and myelitis by 4 weeks post infection (wpi), which progressively subsided by 8 and 12 wpi. Despite development of microscopic lesions, clinical signs were not observed. Only five birds had choanal and/or cloacal swabs positive for ABBV, suggesting a moderate potential of Muscovy ducks to shed the virus. This is the first study to document the pathogenesis of ABBV in poultry species, and confirms the ability of ABBV to infect commercial waterfowl.

Introduction

Since the early 20th century, Borna disease virus (BoDV) has been recognized as the causative agent of Borna disease, a chronic neurological disease of horses, sheep, and rodents in central Europe, named after the city of Borna in Saxony, Germany [1]. Classified in the *Bornaviridae* family within the *Mononegavirales* order, BoDV was the only virus in this family until 2008, when the novel parrot bornavirus, a type of avian bornavirus (ABV), was shown to be the causative agent of proventricular dilatation disease (PDD) in psittacines [2–5]. This disease affects the nervous system and is characterized by peripheral polyneuritis and in some cases meningoencephalitis [6]. Birds with PDD typically show gastrointestinal signs including passing of undigested seed in feces, regurgitation, and dilation of the proventriculus due to loss of intestinal tone leading to weight loss and poor body condition. In some cases, neurological signs, including ataxia, paralysis, and seizures are seen [6, 7]. Through a retrospective study of postmortem submissions of wild Canada geese and swans from Southern Ontario, in 2011 researchers from the University of Guelph identified a distinct variant of ABV named aquatic bird bornavirus (ABBV) [8, 9]. This virus appeared associated with lymphoplasmacytic inflammation of the central and peripheral nervous system, as well as gross pathological features similar to those reported for PDD in psittacine birds [9, 10].

Discovery of these new viruses, among others, led to the reorganization of the *Bornaviridae* family into 3 genera encompassing 11 species, with the *Waterbird 1 orthobornavirus* species encompassing the two genotypes aquatic bird bornavirus 1 (ABBV-1) and aquatic bird bornavirus 2 (ABBV-2). ABBV-1 is most commonly identified in Canada geese and swans, and ABBV-2 has been detected in North American ducks (mallards and a wood duck) exclusively [2, 10].

Since its original description, ABBV has been detected or isolated from multiple species of waterfowl [11, 12], both in asymptomatic and neurologically affected birds, and sporadically in birds outside the Anseriformes order, including gulls [13], bald eagles [6], and an emu [14]. In fact, epidemiological studies have shown that wild waterfowl species appear to be the largest reservoir of ABBV in North America, with an estimated prevalence of more than 50% in some populations depending on species, flock, and location [15, 16, 17]. Aquatic bird bornaviruses, therefore, could be considered as primarily waterfowl viruses that may sporadically infect or become established in new avian populations [6], as also supported by phylogenetic analysis, which suggests that horizontal spread is a driving force of interspecies transmission of ABVs [18].

Experimental infection and / or PDD-like disease have been successfully reproduced by inoculating parrot bornaviruses (PaBVs) into cockatiels [19, 20, 21, 22, 23, 24, 25, 26, 27] and conures [28], and canary bornaviruses (CaBVs) into canaries [27, 29]. To date, however, there is no data regarding the ability of ABBV to experimentally infect waterfowl species. Therefore, the goal of the present study was to document ABBV pathogenesis in the Muscovy duck, selected as a representative waterfowl, and to assess the suitability of this species to serve as an animal model of ABBV infection.

Materials And Methods

Cells. Immortalized duck embryo fibroblasts (CCL-141), originated from Pekin duck (*Anas platyrhynchos domesticus*), were purchased from the American Type Culture Collection (ATCC) and passaged as previously described [49, 50]. Primary duck embryo fibroblasts (DEF) were derived from embryonated Pekin duck eggs [40]. Both cells were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % Penicillin-Streptomycin-Amphotericin B (PSA) at 37 °C and 10 % CO₂.

Virus propagation and inoculum preparation. Confluent CCL-141 in 100 - 150 mm dishes (approximately 10⁷ cells / dish) were infected with 10³ to 10⁴ focus forming units (FFU) of ABBV-1, and routinely passaged until approximately 100 dishes of persistently infected cells were produced. To release ABBV-1 from cells, growth medium was replaced with 5–10 mL of deionized sterile water for 30 min (osmotic shock), followed by one cycle of freeze/thaw at -80 °C (ultra-cold freezer). From each dish, cell debris were then dislodged with a cell scraper, the cells/water suspension was pooled, diluted with 10X phosphate-buffered saline (PBS) to reach 1X concentration, vortexed vigorously for 30 seconds, and then centrifuged for 10 min at 3,000 g. The supernatant was collected and incubated overnight at 4 °C with a PEG-8000 (Thermo Fisher) solution (30 % w/v) at a 2:1 ratio (supernatant : PEG), and then centrifuged for 15 min at 3200 g. The pellet was finally resuspended in PBS with 1 % FBS, for a final ratio of supernatant to final resuspended volume of approximately 100:1. The concentrated virus stock was stored at -80 °C. A control inoculum was produced in the same way, using CCL-141 not infected with ABBV-1.

Immunofluorescence and titration of virus stock. The virus stock was titrated in CCL-141 cells by limiting dilutions in 96-well plates. Positive wells were detected by immunofluorescence (IFA) using a rabbit

monospecific antibody against the N-terminus of the ABBV-1 N protein, as described elsewhere [40,50]. The titers were calculated according to the Spearman-Kärber formula as tissue culture infectious dose 50 % / mL (TCID₅₀ / mL), and converted to FFU / mL by multiplying by 0.6951 [51].

Birds and experimental design. A graphical representation of the experimental layout is provided in **Supplementary Figure 1**. A total of 174 one-day-old Muscovy ducks (*Cairina moschata*) was purchased from a local hatchery (Webfoot Hatchery, Elora, ON, Canada). The experiment was designed to include 160 birds, with additional birds purchased to account for unexpected mortality (~9 % attrition rate). Upon arrival, ducklings were randomly divided into 4 rooms (at least 40 birds / room) at the University of Guelph Central Animal Facility Research Isolation Unit (Guelph, ON, Canada), and immediately neck tagged for single bird identification. Ducklings were housed under negative pressure, placed on the floor with 12 hours of light-dark, and provided food and water *ad libitum*. Infrared lamps at floor level were provided until 3 weeks of age.

Approximately 24 hours after being received, ducklings were inoculated with ABBV-1 by 1 of 4 routes, each corresponding to a single room: intracranial (IC, 50 µL injected into the subdural space of the right cerebral hemisphere, corresponding to 6.5×10^4 FFU / bird), intramuscular (IM, 100 µL injected into the right gastrocnemius muscle, 1.3×10^5 FFU / bird), or oral (PO, 100 µL delivered into the oral cavity, 1.3×10^5 FFU / bird). Control birds (CO) were sham-inoculated by all three routes using cell lysate from non-infected cells (see inoculum preparation). Intracranial injection was conducted by the same methodology employed for the intracerebral pathogenicity index (ICPI), which is used to test the virulence of avian orthoavulavirus-1 in chickens [52]. Birds were monitored daily, and those that died unexpectedly were sampled as described below. Ten birds at 1, 4, and 8 wpi were randomly selected from each inoculation group, swabbed (oral and cloacal), bled for serum, and euthanized. At 12 wpi, due to the additional birds included in each room, a range of 11–15 birds (total 48) were processed in a similar way.

A complete postmortem analysis was done on all euthanized birds. From each group at each time point, 3 birds were selected for detailed pathology assessment, which entailed collection of over 20 tissues for histopathology (see below); from the remaining birds (range 7–12), 4 tissues only (brain, spinal cord, kidney, and proventriculus) were sampled for both RNA extraction and histopathology. Gonads were collected exclusively from birds at 12 wpi, and used for both RNA extraction and histopathology (**Supplementary Figure 1**).

Birds at 1 wpi were euthanized by carbon dioxide (CO₂) inhalation, after being anesthetized with isoflurane in a 7 L vented induction chamber (VetEquip). Older birds were euthanized by intravenous pentobarbital overdose (100 mg / kg). At 8 and 12 wpi, additional sedation was administered with a mixture of ketamine (30 mg / kg) and dexmedetomidine (0.3 mg / kg) before euthanasia.

Experimental procedures and animal use were approved by the University of Guelph Animal Care and Use Committee (Animal Utilization Protocol 3978). All methods were carried out in accordance with the approved protocol and relevant regulations, and comply with the ARRIVE guidelines.

Histopathology. From the birds undergoing detailed pathology assessment, the following tissues were harvested: brain, spinal cord (3 segments, cervical, thoracic, lumbar), ischiatic nerves, brachial plexuses, kidneys, gonads, proventriculus, ventriculus, heart, pancreas, adrenal glands, thyroid/parathyroid glands, small intestine (at the level of the Merkel's diverticulum), colon, lung, liver, spleen, trachea, esophagus, bursa of Fabricius, thymus, and the right gastrocnemius muscle (site of inoculation, only for IM and CO groups). Tissues were fixed in 10 % neutral buffered formalin for 48–72 hrs and then transferred to 70 % ethanol until processing. Samples of spinal cord at all time points and brain at 1 wpi were collected with intact vertebrae and skull, respectively, to prevent sampling artifact. In these cases, the tissues were decalcified for 24-48 hours after fixation using Cal-Ex II Fixative Decalcifier (Fisher Scientific) prior to trimming. In older birds, the brain was removed from the skull before fixation. For each bird, a coronal section of the cerebrum, optic lobe, brainstem, and cerebellum, and two transverse sections of each segment of the spinal cord were obtained. All remaining tissues were trimmed routinely.

The brain, spinal cord, kidney, proventriculus, and ventriculus from the birds sampled for RNA extraction in the IC and IM groups at 4, 8, and 12 wpi (an additional 48 birds) were also processed for histopathology, to increase the granularity of the scoring. For these birds, only opportunistic samples of the brain could be evaluated as approximately half the tissue was collected for RNA extraction. After trimming, tissues were embedded in paraffin and routinely processed for hematoxylin and eosin (HE).

Immunohistochemistry. IHC for ABBV-1 was carried out from the tissues of 7 and 1 birds, which were sampled from the IC and CO groups at 12 wpi, respectively. The IC group at 12 wpi was chosen because it showed the broadest tissue distribution of ABBV-1 by RT-qPCR.

Immunohistochemistry was performed using a rabbit monospecific antibody against the ABBV-1 N protein (the same used for IFA; 1:6000 dilution) and visualizing the reaction with Nova Red chromogen (Vector Laboratories), as previously described [40]. A formalin-fixed, paraffin-embedded brain from a Canada goose (*Branta canadensis*) naturally infected with ABBV was used as a positive control [9]. For negative reagent controls, non-immune rabbit serum was used instead of the primary antibody.

Presence of T cells (CD3, rabbit polyclonal antibody raised against the human homologue, Dako) and B cells (Pax5, mouse monoclonal antibody raised against the human homologue, clone 24, BD Biosciences) was determined on the brains from the 3 IC birds selected for detailed pathology assessment at 12 wpi, as previously described [53]. All IHC was conducted by the Animal Health Laboratory (Guelph, ON, Canada).

Semi-quantitative scoring of nervous lesions and immunohistochemical reactivity. All histological and IHC slides were examined and subsequently scored by one member of the investigative team (M.I.). A bird was considered positive for ABBV-consistent histopathology if lymphocytic-predominant inflammation was appreciated in any segment of the central, peripheral or autonomic nervous tissue.

A semi-quantitative scoring system was developed to quantify the severity of mononuclear inflammation in the central nervous system (**Table 5**), by recording the thickness of the lymphocytic perivascular cuffs

(intensity score) and the number of vessels affected (distribution score). In the brain, lesions were averaged from up to 10, randomly selected 100X fields for each brain area (i.e., cerebrum, cerebellum, brainstem, optic lobe). In the spinal cord, scoring was done for each transverse section of the spinal cord in its entirety; if more than one transverse section was available (range 1–7), the average was recorded. A sub-score was defined as the compiled intensity and distribution scores for each area of the brain or the spinal cord, ranging from 0 to 6. For the entire brain, a score was calculated by adding the sub-scores and dividing the total by the number of available brain areas. Additional lesions of the nervous system (meningitis, peripheral neuritis, and gliosis) were included in a nominal tally.

For IHC, presence / absence of ABBV immunoreactivity was tallied for all organs of the tested birds. Semi-quantitative assessment of IHC reactivity was assessed by counting the number of positive cells averaged in up to 10, randomly selected 400X fields for each brain areas, or all the complete transverse section of spinal cord (**Table 5**). Only cells with intranuclear or intranuclear and cytoplasmic immunolabeling were considered positive.

Virus titration by RT-qPCR. Samples (choanal and cloacal swabs, brain, lumbar spinal cord, proventriculus, kidneys, and gonads) were collected into sterile screw cap tubes containing 1.0 mL of preserving solution (20 mM ethylenediaminetetraacetic acid [EDTA], 25 mM sodium citrate, and 70 % (w/v) ammonium sulfate with a pH of 5.2), and frozen at -80 °C until RNA extraction. Total RNA was extracted from 300 mg of tissue or, for swabs, 300 µL of preserving solution using E.Z.N.A RNA Kit II (Omega Bio-Tek), following the manufacturer's protocol. Purified RNA was reverse transcribed and amplified using a Luna Universal Probe one-step RT-qPCR kit (NEB) with primers and probes targeting the ABBV-1 N gene (forward, 5'-ATG CAC TTG CAC TCT TAG AC-3'; reverse, 5'-TCC CCA TAA AAC CTC CCA AC-3'; probe, 5'-6-FAM-CCC TGC CCG CAG AGA GAA ATT CCA T-BHQ-3'). The cycling conditions were as follows: 55 °C for 10 min reverse transcription; 95 °C for 1 min initial denaturation, and 40 cycles of 95 °C for 10 s denaturation and 60 °C combined annealing and extension. Samples with cycle threshold (Ct) less than 35 were considered positive.

Genome copy numbers were determined based on a standard curve produced using ten-fold dilutions (3×10^8 to 3×10^1 copies / reaction) of a gene cassette containing a 500 bp fragment of the ABBV-1 N gene (Integrated DNA Technologies), and run in parallel with each plate. The final output of the PCR was reported as genome copy numbers per 150 ng of total extracted RNA (tissues), or per 84 µL of swab fluid.

Western blot. The sera from 6 birds in the per inoculation group at 1 and 12 wpi were selected to test seroconversion by Western blot [50]. Briefly, 30 µg of protein from cells lysate of ABBV-1-infected and non-infected CCL-141 cells were resolved on a 12 % SDS-PAGE (120 V for 1.5 h) and transferred to a PVDF membrane at 25 V for 30 min (semi-dry transfer; Bio Rad). Membranes were blocked with either 5 % skim milk in PBS-T at 4 °C overnight, incubated with sera (1:1000 dilution) overnight at 4 °C, and then incubated 1 h at room temperature with a secondary goat antibody (1:5000; A140-110P-Bethyl Laboratories Inc., Cedarlane) directed against the avian IgG-heavy and light chain, and conjugated with horseradish peroxidase (HRP). Signal was detected by incubating with the SuperSignal™ West Pico PLUS

Chemiluminescent Substrate (ThermoFisher) for at least 5 min before band detection using a ChemiDoc MP Imaging System and Image Lab 6.0.1. software (Bio Rad). As positive control for the technique, the monospecific antibody against the ABBV-1 N protein used in the IFA and IHC assays was used in place of the sera. Furthermore, for each blot, a cell lysate from non-infected cells was also run in parallel.

Virus isolation. To evaluate if the virus detected by RT-qPCR was infectious, virus isolation was conducted from the brain and kidneys of two IC and two CO ducks sampled at 12 wpi. Tissues were minced, resuspended in 10 % FBS/DMEM, and homogenized using the Precellys 24 homogenizer (Bertin Instruments) for 40 seconds (20 s of 5000 rpm bursts with 5 s waiting intervals). Approximately 100 μ L of tissue homogenates were layered on top of confluent CCL-141 monolayers in 6-well plates containing 2 mL of 10 % FBS/DMEM per well. The next day, the medium was replaced, and cells routinely passaged up to three times (20 days post-inoculation), when presence of ABBV-1 in cells was detected by IFA.

Statistical analysis. Differences in proportions of inflammation and infection within and between the IC and IM groups were tested by pairwise comparison for proportions with Hommel's correction for multiple comparisons.

The average magnitude of genome copy numbers in tissues and swabs, as determined by RT-qPCR, were compared between different time points and inoculation routes using a two-way ANOVA test (variables: inoculation route and time point), with multiple comparisons between groups (Tukey's test). Group differences in the semi-quantitative histology and immunohistochemistry scores were compared using the Kruskal-Wallis test followed by multiple comparisons using the Benjamini, Krieger, and Yekutieli procedure for false discovery rate ($q < 0.05$).

Four linear regression models were built using the brain and spinal cord pathology scores in the IC and IM groups as dependent variables, and time post infection (4, 8, 12 wpi), as well as virus titers in select organs (brain, spinal cord, and proventriculus) as explanatory variables. Virus titers were expressed as log 10 copy numbers per 150 ng of total RNA, and zero values were transformed to 0.00001. For each model, 26 observations were present. While the Q-Q plots were approximately normally distributed, the homoscedasticity assumption was not fully met, likely due to the relatively small sample size. Nonetheless, our models had merit and were considered in the analysis [54].

Statistical analysis was carried out using Stata, version 14.0 (Stata Corporation, College Station, Texas, USA) for comparison of proportions and regression analysis, or GraphPad Prism for iOS, version 9 (GraphPad Software, La Jolla, USA) for the other tests. Significance was set at $p < 0.05$.

Results

Clinical disease and gross findings. Groups of Muscovy ducks were inoculated either intracranially (IC), intramuscularly (IM) or orally (*per os*, PO) with ABBV-1. Ducks in the control (CO) group were sham-infected with carrier only through all 3 routes (**Supplementary Figure S1**). Sex distribution, as determined at necropsy, was as follows: 18 males, 26 females, and 2 undetermined in the IC group ($n = 46$); 20 males,

20 females, and 2 undetermined in the IM group (n = 42); 17 males, 24 females, and 1 undetermined in the PO group (n = 42); and 18 males, 23 females, and 1 undetermined in the CO group (n = 42). Throughout the course of the experiment (12 weeks), 4 ducks were found dead. One duckling in each of the IC and CO group died immediately after intracranial inoculation due to peracute cerebral hemorrhage (excluded from the study). One duck in the IM and one in the PO inoculation group were found dead at 3 and 46 days post inoculation (dpi), respectively, with no prior signs of illness and no evidence of gross or histological lesions. Overall, no clinical signs were attributed to ABBV infection.

Microscopic findings.

Descriptive results. Forty-eight ducks underwent detailed pathological assessment (3 birds / group / time point), and lesions attributable to ABBV-1 infection were identified exclusively in the IC and IM ducks. In order to increase the definition of the histopathological assessment in these groups, the brain, spinal cord, proventriculus, ventriculus, and gonads from an additional 22 IM and 26 IC ducks were also evaluated (**Supplementary Figure S1**). The frequency of lesions in the IC and IM groups is reported in **Table 1**. Considering all time points, lesions consisted of mononuclear encephalitis ((92 % in the IC and 47 % in the IM groups), myelitis (67 % IC and 50 % IM), cerebral meningitis (89 % IC and 16 % IM), spinal meningitis (37 % IC and 29 % IM), and less frequently peripheral neuritis (50 % IC and 25 % IM).

In the IC group, inflammation of the central nervous system (CNS) was not present at 1 week post infection (wpi), however by 4 wpi, 100 % and 70 % of birds presented with encephalitis and myelitis, respectively; a frequency that remained approximately constant through the end of the experiment. In this group, inflammation of the peripheral nervous system (PNS; axillary and ischiatic nerves) was detected at 8 and 12 wpi in 100 % of birds. In the IM group, inflammatory lesions were not present at 1 wpi, and at 4 wpi encephalitis and myelitis were present only in 10 % and 30 % of birds. By 12 wpi frequency of inflammation at these sites had increased to 91 % and 64 %, respectively. Peripheral neuritis was observed at 8 and 12 wpi, and it never affected more than 67 % of the birds.

Inflammation did not appear to target specific areas of the nervous tissue, and was characterized by accumulation of a mononuclear infiltrate that expanded the perivascular spaces in the brain and spinal cord, segmentally in the meninges, and in the endoneurium of the axillary or ischiatic nerves (**Figures 1a-e**). Within the brain, both the grey and white matter were affected and in the spinal cord predominantly the grey matter (**Figure 1c, d**). Inflammatory cells were predominately lymphocytes, with fewer macrophages, and rare plasma cells and heterophils (**Figure 1f**). Immunohistochemistry (IHC) conducted on representative duck brains (n = 3) from the IC group at 12 wpi showed that the highest percentage of the inflammatory population was composed of CD3-positive cells (T lymphocytes), with a small percentage of Pax-5-positive cells (B lymphocytes) (**Figures 1g, h**).

Besides inflammation, gliosis characterized by proliferation and hypertrophy of glial cells with formation of glial nodules (**Figure 1i**) was seen in 100 % and 27 % of IC birds at 4 and 12 wpi, respectively, and in 33 % of IM birds at 8 wpi (**Table 1**). Inflammation was not identified in the myenteric nerves or ganglia of the

gastrointestinal tract. No significant microscopic findings were identified in the other tissues from the IC and IM group, and no lesions were identified in the birds from the PO and CO groups.

Pairwise comparison for proportions of affected areas of the CNS. The frequency of inflammation by anatomical location was compared by pairwise comparison for proportions (**Supplementary Table 1**). In the IC group, inflammation was significantly more frequent in the cerebrum and cerebral meninges compared to the spinal meninges ($p < 0.001$). In the IM group, pairwise comparison did not show significant differences. When groups were compared between each other, inflammation in the cerebrum and cerebral meninges of IC birds was significantly more common than inflammation in the cerebrum, optic lobe, brainstem, cerebellum, meninges (cerebral and spinal), and peripheral nerves of birds in the IM group ($p < 0.042$). Similarly, inflammation in the cerebellum of IC birds was more common compared to cerebral or spinal meningitis in the IM group ($p \leq 0.042$), and myelitis in the IC group was more common than meningitis in the IM group ($p = 0.007$). These results indicate that, while differences in the regional frequency of inflammation in the nervous system are minimal within the same inoculation group, the IC group had a higher frequency of meningoencephalomyelitis compared to the IM group.

Semi-quantitative scoring of nervous lesions. The severity of inflammation in different areas of the brain and spinal cord was also assessed through a semi-quantitative pathological scoring on 38 IC and 34 IM birds (**Figure 2**). In the IC group, the cerebrum, optic lobe, and brainstem had the highest inflammation scores at 4 wpi. The inflammation in the cerebrum at 4 wpi was significantly more severe compared to the cerebrum, spinal cord, and cerebellum at all the other time points. Inflammation in the optic lobe at 4 wpi was significantly more severe compared to the cerebrum at 8 wpi, as well spinal cord and / or cerebellum at all other time points. Encephalitis in the brainstem was more severe compared to the spinal cord at 4 and 8 wpi, as well as the cerebellum at 12 wpi. No significant score differences were seen between areas of the central nervous system at 8 and 12 wpi (**Figure 2a**). In the IM group, low-intensity inflammation was initially recorded only in the cerebrum and spinal cord at 4 wpi. Lesions became more severe at 8 and 12 wpi, with inflammation in the cerebrum at 12 wpi being significantly more severe than at 4 wpi (**Figure 2b**).

When the brain scores (calculated by averaging the inflammation of each available brain area) were considered, birds in the IC group at 4 wpi presented significantly more severe lesions compared to birds in the IM and IC groups at all time points (**Figure 3**). In the IM group, inflammation gradually became more severe as time progressed, with differences being significant between 4 and 12 wpi. These results show that Muscovy ducks inoculated intracranially with ABBV-1 develop more severe lesions at earlier time points compared to birds inoculated through the IM route, and that the peak of inflammation in birds from the IC group occurs at 4 wpi, while a peak of inflammation could not be identified in the IM group.

ABBV titration in tissues.

Frequency of tissues positive for ABBV-1. The genome copy numbers of ABBV were quantified by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), in order to test the magnitude of virus replication in brain, spinal cord, proventriculus, kidneys, and gonads, and shedding through the choanal

and cloacal secretions (**Table 2** and **Figure 4**). In the IC group, at 1 wpi the brain was the only tissue to test positive in 6 of 7 birds. By 4 wpi, and until the end of the experiment, both the brain and the spinal cord were positive in 100 % of tested birds. At 4 wpi, one bird had the proventriculus positive (1/7), and by 8 wpi to the end of the experiment all proventricular tissues tested positive. At both 8 and 12 wpi, 100 % of renal tissues were positive, and at 12 wpi all gonads were also positive (4 testes and 8 ovaries). There was evidence of viral shedding in 27 % and 7 % of choanal and cloacal swabs at 12 wpi. Considering the total tally of positive tissues, pairwise comparison for proportions showed that the brain tested most frequently positive for ABBV compared to the proventriculus ($p = 0.006$) and kidney ($p = 0.003$) (**Supplementary Table 2**).

In the IM group, at 1 wpi the spinal cord was the only tissue to test positive in 2 of 7 birds. At 4 wpi, the spinal cords of 57 % of birds tested positive, and one bird tested positive also in the brain. By 8 wpi all birds tested positive in the spinal cord and brain, and one bird was positive in the proventriculus. At 12 wpi, the proventriculus, kidneys, and gonads (3 testes and 5 ovaries) from all birds were positive, and there was evidence of viral shedding in one cloacal swab only (1/11). Considering the total tally of positive tissues, pairwise comparison did not show significant differences in the proportion of positive tissues (**Supplementary Table 2**).

When both groups were taken into account, the proportion of total positive brains in the IC group was significantly higher than all tested tissues in the IM group ($p < 0.01$). Similarly, the proportion of positive spinal cords in the IC group was significantly higher compared to all the other tissues in the IM group ($p < 0.014$). Rates of shedding (cloacal and choanal swabs) within and between groups were not significantly different.

Magnitude of ABBV-1 replication in tissues. The average concentration of virus genome copies in the brain of IC birds significantly increased from 1 wpi (5.5×10^4 / 150 ng of total tissue RNA) to 4 and 8 wpi (1.0 and 1.6×10^7), after which remained approximately constant (12 wpi). Similarly, the genome copies in the spinal cord significantly increased approximately 2 orders of magnitude from 4 wpi (2.5×10^5) to 8 wpi (1.16×10^7), and remained constant at 12 wpi. Overall, the tissues from brain and / or spinal cord contained significantly more viral genetic material compared to other samples at 4, 8, and 12 wpi (**Figure 4a**).

In the IM group, the average concentration of virus in the spinal cord gradually increased from 1 wpi and peaked at 12 wpi, when it averaged approximately 10^7 genome copy number / 150 ng of tissue RNA, and was significantly higher compared to the other time points. Virus genome copies were first detected in the brain at 4 wpi, and gradually increased to peak at 12 wpi, when the titer was significantly higher compared to all the other time points. The tissues from the brain and / or spinal cord contained significantly more viral genetic material compared to all the other samples at 12 wpi (**Figure 4b**).

Relationship between virus titer in tissues and brain pathology. Multiple linear regression analysis was used to predict brain and spinal cord inflammation scores in both the IC and IM groups, using virus titers

in organs (brain, spinal cord, proventriculus) and time post infection (wpi) as explanatory variables. Only the brain model in the IC group yielded significant results, and it showed that the 4 predictors explained 64.9 % of inflammation variability ($R^2 = 0.649$, $F(4,21) = 9.72$, $p < 0.001$). Only virus titers in the brain significantly predicted pathology scores of the brain (β coefficient = -1.522; $p = 0.001$), indicating that for every log virus titer increase in the brain, the predicted pathology brain score decreased by 1.522 (**Table 3**).

Immunohistochemistry. Seven birds from the IC group at 12 wpi, plus one control from the same time point, were submitted to detect the distribution of ABBV-1 N protein in tissues by IHC. Birds from the IC group at 12 wpi were chosen, as this group had the highest ratio of ABBV-positive organs and magnitude genome copy number, as assessed by RT-qPCR. All infected birds had characteristic histologic lesions in the brain and spinal cord, while controls presented no lesions. The N antigen was detected in all seven infected birds (**Table 4**).

In the CNS, immunolabeling in the neuroparenchyma was multifocal, and not spatially associated with regions with inflammation, as observed histologically (**Figure 5a**). Reactivity of moderate intensity was present in the nuclei and / or nuclei and cytoplasm of predominantly neurons and glial cells, while ependymal cells were also occasionally positive (**Figure 5b-d**). A scoring based on the extent of IHC immunolabeling showed no significant differences between sections of the brain (Kruskal-Wallis test; data not shown).

Outside of the central nervous system, immunoreactivity was found in peripheral nerves (**Figure 5e**), ganglia and plexuses of the proventriculus, ventriculus, esophagus, small intestine, colon (**Figures 5f, g**), lung, and pancreas (**Figure 5h**). In addition, scattered epithelial cells of the proventricular glands, acinar cells of the pancreas, and cortical and medullary cells of the adrenal gland (**Figure 5i**) exhibited positive intranuclear and occasionally intracytoplasmic immunoreactivity. In all three tested males, reactivity was seen in the interstitial cells (**Figure 5j**) and the epithelium of the epididymis. In the three tested females, reactivity was identified in scattered cells of the theca interna and externa, rare granulosa cells, and the ovarian interstitium (**Figure 5k,l**), besides neurons in adjacent ganglia. Immunoreactivity was not observed in kidney (despite high RT-qPCR signal), heart, liver, spleen, trachea, bursa of Fabricius, or thymus in any of the examined birds. The control duck exhibited no immunolabeling in any of the examined tissues.

Serology. Western blot assays were conducted on lysates from persistently infected cells, using serum from infected ducks at 1 and 12 wpi as primary antibodies. Using sera from birds sampled at 12 wpi, all tested ducks from the IC group ($n = 6$) and 2/6 ducks from the IM group reacted strongly with a 35-40 kDa band in the lysate from persistently infected cells. This molecular weight is compatible with the predicted size of ABBV N-protein (**Figure 6**). None of the sera from birds sampled at 1 wpi from the IC and IM groups reacted with a protein of the expected molecular size, suggesting seroconversion at 12 wpi. No reactivity was observed in birds from the PO and CO groups at either time points.

Virus isolation from brains and kidneys. The brains from 2 IC inoculated ducks at 12 wpi were used for virus isolation. Both brains yielded ABBV-1 in CCI-141 cells after three passages, as shown by nuclear and weak cytoplasmic signal in scattered cells by IFA (**Supplementary Figure S2**). Similarly, the kidneys from 2 ducks in the IC group at 12 wpi, which tested positive for RT-qPCR but were negative by IHC, yielded ABBV-1 in CCI-141 cells after three passages, as shown by nuclear and weak cytoplasmic signal in scattered cells by IFA (**Supplementary Figure S2**). The control brains and kidney from the CO group yielded no virus. These data indicate that RT-qPCR signal corresponds to infectious virus in tissues.

Discussion

This study describes for the first time the successful experimental infection of an avian species with ABBV-1, and confirms the ability of this virus to infect commercial waterfowl. Intracranial and intramuscular infection of Muscovy ducklings with ABBV-1 led to viral spread and replication in multiple tissues and development of nervous lesions analogous to those observed in natural cases, indicating that Muscovy ducks are a suitable model for experimental infection with ABBV-1.

To determine viral replication upon infection, ABBV-1 genome copy number was evaluated in multiple tissues at 1, 4, 8, and 12 wpi. ABBV-1 RNA was detected in all tissues from birds in the IC and IM groups by 12 wpi. In the IC infected ducks, viral RNA was identified in the brain as soon as 1 wpi. By 4 wpi until the end of the experiment all tested brains were positive. These findings indicate intracranial inoculation is a very effective method of ABBV-1 infection and is in agreement with other studies, which documented the intracranial route as a successful delivery method to infect rodents and psittacine birds with BoDV and PaBV [25, 26], respectively. By 4 wpi, ducks in the IC group showed viral replication in the spinal cord, and by 12 wpi all tested peripheral organs (kidney and proventriculus) were also positive. This time-wise spread of the virus from the brain to the spinal cord and to the visceral organs is suggestive of a centrifugal spread from the central nervous tissue to the periphery. Conversely, in the IM group, ABBV-1 RNA was first identified in the spinal cord at 1 wpi. By 8 wpi, all tested spinal cords and brains were positive, and by 12 wpi all peripheral tissues were positive. Time-wise progression of virus spread suggests a centripetal spread from the muscle to the spinal cord and brain, as well as a centrifugal spread from the central nervous tissue to visceral organs. Our findings are consistent with reports describing experimental intramuscular inoculation of PaBV in psittacine birds [22, 30]. Additionally, infectious virus was successfully re-isolated from the brains and kidneys of 2 IC ducks at 12 wpi, indicating that RNA signal corresponds to infectious virus.

Ducks inoculated orally could not become infected, as shown by lack of ABBV-1 RNA in any collected tissues. Oral administration of PaBV [21] and BoDV [31] in cockatiels and rats, respectively, did not yield infection or clinical signs, in agreement with our findings. Difficulty in reproducing successful infection through oral administration has led to questioning fecal-oral transmission, which had been postulated as the natural route of infection for avian bornaviruses [32]. A recent study successfully reproduced PaBV infection and clinical signs in cockatiels by applying the infectious inoculum through defects in the skin of the footpad, suggesting that wound contamination may be a natural way for PaBV infection to occur

[33]; in this case infection would depend on the levels of environmental contamination, rather than bird-to-bird transmission. In our study, only a few birds (n = 6) tested positive for ABBV-1 RNA in the choanal and / or cloacal swabs at 12 wpi, indicating the potential for virus shedding and environmental contamination. Moreover, all tested kidneys from ducks in both the IC and IM groups at 12 wpi were positive for ABBV RNA, suggesting that shedding through the urine is plausible. This is consistent with successful demonstration of PaBV RNA in the urine of experimentally and naturally infected psittacine birds [34]. Despite establishment of persistent infection in all ducks in the IC and IM groups (including renal tissues), however, only a few swabs tested positive with low virus titers, consistent with intermittent and low-intensity viral shedding, similar to what described for other ABVs [35, 36]. While presence of ABBV-1 RNA in the swabs indicates potential for horizontal transmission, presence of genetic material does not necessarily correlate with presence of infectious viral particles, and even if shedding of infectious virus particle were to be conclusively demonstrated, it still remains to be explained why the oral route is ineffective in establishing experimental infection.

Vertical transmission is another potential route of ABBV-1 infection. In our Muscovy ducks, immunoreactive ABBV antigen was present in the interstitial cells of the testis, epithelium of the epididymis, ovarian granulosa cells, and interstitial stromal cells. Presence of virus protein in cells that can be in contact with the sperm or ovum suggests that vertical transmission may be possible. Similarly, PaBV and CnBV-2 antigen has been demonstrated in testes, ovaries, and shell gland of psittacine and canary birds, both naturally and experimentally infected [24, 29, 37]. In conures, PaBV-2 was even detected in the embryos from persistently infected breeders and in the blood of one hatchling [38], and another study reported the presence of ABBV-1 RNA in the egg yolk of one out of 53 non embryonated Canada goose eggs [39]. While in our study female ducks did not reach sexual maturity and eggs could not be tested for ABBV-1, it should be noted that even if the virus can reach the egg, actual infection of the embryo may not necessarily follow. In our experience, experimental inoculation of fertile Pekin duck eggs with purified ABBV-1 through the yolk sac and allantoic fluid was not able to successfully infect the embryos [40].

Viral distribution was also evaluated by IHC. Consistent intranuclear with or without cytoplasmic immunoreactivity was present in neurons, glial cells, and ependymal cells of the central nervous system. Immunolabeling for ABBV was also identified in numerous visceral organs, in agreement with detection of ABBV-1 RNA in proventriculus, testes, and ovaries by RT-qPCR. Virus distribution outside of the CNS and in visceral organs is consistent with natural reports of ABBV infections [9], and multiple accounts of experimental PaBV infection in psittacine birds [37, 41, 42]. None of the visceral tissue with IHC signal showed inflammatory lesions, as well, inflammation in the CNS did not overlap with areas of IHC reactivity. In an experimental infection study of parrot bornavirus 2 (PaBV-2) in cockatiels, immunolabeling for PaBV N-protein preceded the development of inflammation throughout all time points [30]. It is possible that a longer duration of our study may have been needed for inflammatory lesions to overlap with areas of ABBV replication. Notably, while kidneys had large amounts of ABBV-1 genome copy number and virus was re-isolated from the two tested kidneys, no IHC reactivity was observed in the sections from renal tissue. Reports of natural and experimental PaBV infection indicate

that bornavirus immunoreactivity is often observed in the renal tubular epithelium [24, 37]. The reason for this discrepancy is unclear; and may be caused by low-level expression of virus protein in renal tissues of the ducks in our study [28, 43], which may be below the sensitivity threshold of IHC. Low virus replication in the renal tubules may affect the amount of virus shed in the urine in our infection model.

In this study, the experimentally infected ducks in the IC and IM group developed histologic lesions exclusively in the nervous system, such as of lymphocytic encephalitis, myelitis, meningitis, and peripheral neuritis, which are consistent with descriptions of natural ABBV-1 infection in waterfowl [9]. Other features described in natural infection [9], including Wallerian degeneration, malacia, cerebral edema, and inflammation of the autonomic nervous system, were not appreciated. While ganglioneuritis is the characteristic histological lesion in cases of proventricular dilation disease, it appears as a less common finding in cases of ABBV-1 infection, although still described [6, 9].

In the IC group, the overall brain inflammation score peaked at 4 wpi and decreased at later time points, despite the virus titers in the brain remaining constant or even slightly increasing. This is confirmed by regression analysis, which showed that increasing virus titers in the brain corresponded to a decrease in brain pathology scores. In the IM ducks the overall brain inflammation score gradually increased over time, as titers increased, but an inflammatory peak was not observed for the duration of the study. In this group, birds showed inflammation in the spinal cord first at 4wpi, and the peak of inflammation was seen at 8 wpi, while the highest virus titers in this organ were detected at 12 wpi. Therefore, encephalitis and myelitis follow the pattern of initial ABBV-1 RNA infection, but the magnitude of the inflammation does not appear to be related to the virus titer in tissues.

Despite development of histologic lesions characteristic of ABBV-1 infection and demonstrating viral replication in multiple tissues, ABBV-1 experimentally infected Muscovy ducks did not show macroscopic lesions (e.g. proventricular dilation) or develop clinical signs at any point during the 12-week duration of this study. While clinical signs attributed to natural ABBV-1 infection in wild waterfowl include poor body condition, gastrointestinal signs, and neurologic deficits [9], ABBV-1 has been identified in tissues of apparently healthy waterfowl [17, 44]. In two different studies of canaries experimentally infected with CnBV-1 and 2, regardless of route of administration (IM, PO, subcutaneous, nasal), birds did not develop any clinical signs for the 154-day and 161-day duration of the studies, respectively, despite having high loads of viral genome in multiple tissues (e.g. brain, proventriculus, lung, heart, liver, and duodenum, kidney) as assessed by RT-qPCR [27, 29]. Experimental infection of PaBV-4 intramuscularly in adult cockatiels did not yield clinical signs until 92 dpi [24]. Furthermore, mallard ducklings inoculated through intraocular, IM, or PO administration with PaBV-4 failed to develop clinical signs or lesions (gross or microscopic) throughout the 42-day duration of the study, but all were shedding the virus (feces positive by RT-PCR) and had seroconverted by 3 wpi [45]. It is unclear what triggers may promote development of clinical disease in persistently infected birds. The pathogenesis of the inflammation in psittacine birds infected with PaBV has been proposed to be associated with an immune mechanism, rather than by direct virus damage to the infected cells [46, 47]. Therefore, loss of immune-tolerance and activation of the immune system against normal tissue elements may lead to severe tissue damage and clinical signs.

This is supported, for instance, by the fact that birds treated with cyclosporin become infected but do not develop clinical signs [46]. A recent article has shown that hatchling cockatiels infected with PaBV-4 became readily infected and developed encephalitis but no clinical signs, as opposed to older birds which developed both severe encephalitis and clinical signs [20]. These findings are very similar to what is reported in our study, and suggest that infection of ducklings with an immune system that is not fully developed [48] may promote immune-tolerance and a carrier state [20]. Nonetheless, despite the ducklings being infected at a very young age, birds from the IC and IM groups seroconverted against ABBV-1, indicating that a humoral immune response against the virus developed, as also suggested by the inflammatory lesions in the CNS.

The Muscovy ducks used in this study were obtained from a local hatchery in Southern Ontario. As ABBV is known to be widespread in free-ranging waterfowl in the area [9, 15], we considered the possibility that these commercial ducklings could already have been infected when obtained at 1-day old. However, all birds in both the CO and PO group, at no point throughout the duration of the study demonstrated viral RNA in any tested tissues or swabs, nor did they develop histologic lesions in contrast to the IC and IM inoculated birds, making us confident these ducks were not already infected, prior to inoculation. Additionally, as birds were received in the isolation facilities at day of age from a hatchery, the potential for infection with ABBV-1 would have been minimal.

Conclusions

Muscovy ducklings could become infected with ABBV-1 by intracranial and intramuscular, but not oral, inoculation routes. Despite virus replication in multiple tissues, development of inflammatory lesions in the central nervous system, and seroconversion, infected birds did not develop clinical signs. Shedding of virus was low and likely intermittent, suggesting that low-rate environmental contamination by infected birds may be possible. This is the first documented experimental infection with ABBV-1, and suggests that the Muscovy duck, and Anseriformes, is a suitable model to study ABBV-1 pathogenesis.

Declarations

Animal use and experimental procedures involving animals were approved by the University of Guelph Animal Care and Use Committee (Animal Utilization Protocol #3978).

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Author Contributions. MI wrote the manuscript and had critical input in the study design. LS devised and designed the experiment, received funding and regulatory approval to conduct the study. MI, AL, PP, and LS carried out the live animal study. MI conducted microscopic analysis of sampled tissues, and devised and implemented a semi-quantitative score for histology and immunohistochemistry. AL and PP prepared the ABBV virus stock, and conducted RNA extraction, RT-qPCR, Western blot analysis, and virus isolation from brain. EN provided the virus and primary cell culture systems, critically reviewed the experimental protocol, and edited the manuscript. MI, SC, LS participated in data analysis, figure and graph production, and statistical analysis. BL and EB contributed to finalize the study design, data analysis, and critically reviewed the manuscript.

Competing interests. The authors declare no competing interests.

Consent for publication. All authors reviewed the manuscript and approved it in its final form.

Availability of data and materials. Any additional data is submitted as Supplementary Material.

Supplementary materials. Supplementary Tables 1 and 2, and Supplementary Figures 1 and 2.

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Tables

Table 1. Frequency of inflammation and gliosis in different areas of the nervous system of Muscovy ducks inoculated with ABBV-1 by the intracranial (IC) and intramuscular (IM) routes at 1, 4, 8, and 12 weeks post infection (wpi).

Affected Area or Disease Category	IC					IM				
	1 wpi	4 wpi	8 wpi	12 wpi	Total	1 wpi	4 wpi	8 wpi	12 wpi	Total
Cerebrum ^a	0/3 ^d	10/10	10/10	15/15	35/38 ^e	0/3	1/10	4/10	10/11	15/34 ^e
Optic lobe	0/3	3/3	3/3	3/3	9/12	0/3	0/3	1/3	2/3	3/12
Brainstem	0/3	4/4	2/2	3/3	9/12	0/3	0/3	2/3	2/3	4/12
Cerebellum	0/3	7/8	7/7	9/12	23/30	0/3	0/10	3/7	6/9	9/29
Spinal cord	0/3	7/10	7/10	10/13	24/36	0/3	3/10	6/8	7/11	16/32
Brain meninges	0/3	10/10	9/10	14/14	33/37	0/3	0/10	0/10	5/9	5/32
Spinal meninges	0/3	3/10	3/10	7/12	13/35	0/3	2/10	4/10	4/11	10/34
Peripheral nerves	0/3	0/3	3/3	3/3	6/12	0/3	0/3	2/3	1/3	3/12
Encephalitis ^b	0/3	10/10	10/10	15/15	35/38	0/3	1/10	5/10	10/11	16/34
Gliosis in brain ^c	0/3	10/10	0/10	4/15	14/38	0/3	0/10	3/10	0/11	3/34

^aIndicates anatomical areas affected by inflammation, with the exception of “gliosis” category. ^bEncephalitis, as tallied in birds with inflammatory lesions in any brain section (cerebrum, optic lobe, brainstem, cerebellum). ^cGliosis, as tallied in birds with proliferation / hypertrophy of glial cells or glial nodules in any brain section (cerebrum, optic lobe, brainstem, cerebellum). ^dNumber of birds with disease in that anatomical location / number of birds tested at that time point. ^eNumber of birds with disease in that anatomical location / total number of birds tested over all the time points.

Table 2. Number of birds in the intracranial (IC) and intramuscular (IM) group, which tested positive for the ABBV-1 N gene in a cohort of select tissues at 1, 4, 8, and 12 weeks post infection (wpi).

Samples	IC					IM				
	1 wpi	4 wpi	8 wpi	12 wpi	Total	1 wpi	4 wpi	8 wpi	12 wpi	Total
Brain	6/7 ^a	7/7	7/7	12/12	32/33 ^e	0/7	1/7	7/7	8/8	16/29 ^e
	(6.42 x 10 ⁴) ^b	(1.00 x 10 ⁷)	(1.66 x 10 ⁷)	(1.33 x 10 ⁷)		-	(4.21 x 10 ⁴)	(1.24 x 10 ⁵)	(1.66 x 10 ⁷)	
Spinal cord	0/7	7/7	7/7	12/12	26/33	2/7	4/7	7/7	8/8	21/29
	-	(2.46 x 10 ⁵)	(1.16 x 10 ⁷)	(1.11 x 10 ⁷)		(7.86 x 10 ²)	(5.76 x 10 ⁵)	(3.69 x 10 ⁶)	(9.63 x 10 ⁶)	
PV ^c	0/7	1/7	7/7	12/12	20/33	0/7	0/7	1/7	8/8	9/29
	-	(3.23 x 10 ¹)	(7.14 x 10 ³)	(3.25 x 10 ⁵)		-	-	(6.77 X 10 ¹)	(5.04 x 10 ³)	
Kidney	0/7	0/7	7/7	12/12	19/33	0/7	0/7	0/7	8/8	8/29
	-	-	(3.84 x 10 ¹)	(9.28 x 10 ³)		-	-		(2.23 x 10 ³)	
Gonad	NA ^d	NA	NA	12/12	12/12	NA	NA	NA	8/8	8/8
	NA	NA	NA	(2.10 x 10 ⁶)		NA	NA	NA	(3.85 x 10 ⁵)	
Cloacal swab	0/10	0/10	0/10	1/15	1/45	0/10	0/10	0/10	1/11	1/41
	-	-	-	(8.96 x 10 ²)		-	-	-	(8.67 x 10 ¹)	
Choanal swab	0/10	0/10	0/10	4/15	4/45	0/10	0/10	0/10	0/11	0/41
	-	-	-	(6.14 x 10 ¹)		-	-	-	-	

^aIndicates number of birds positive for that tissue / total number of birds tested for that tissue at that time point.

^bIndicates parenthesis is the average virus titer calculated from birds that tested positive.

^cPV = proventriculus. ^dNA = the tissue was not available for analysis. ^eNumber of birds with disease in that anatomical location / total number of birds tested over all the time points.

Table 3. Multiple linear regression analysis using the brain pathology score as the dependent variable, and virus titer in selected tissues and week post infection as independent variables, as assessed in a cohort of Muscovy ducks infected with aquatic bird bornavirus (ABBV)-1 delivered intracranially.

Predictor	Coefficient	Standard Error	P value	95 % confidence intervals	
<i>Week post infection</i> ^a	0.129	0.219	0.561	-0.326	0.584
<i>Brain titer</i> ^b	-1.522	0.406	0.001	-2.367	-0.677
<i>Spinal cord titer</i>	-0.107	0.159	0.506	-0.438	0.223
<i>Proventriculus titer</i>	-0.107	0.064	0.111	-0.241	0.027

^aOnly 4, 8 and 12 week post infection were evaluated in the analysis

^bTiter in tissues is expressed as log10 of virus copy number per 150 ng of tissue RNA

Table 4. Distribution of tissue reactivity for aquatic bird bornavirus N protein in seven ducks infected with ABBV-1 through the intracranial route 12 weeks post infection (wpi).

Tissues	#1	#2	#3	#4	#5	#6	#7	Total	Cells positive for ABBV antigen ^e
Cerebrum	3 ^a	1	3	2	2	3	3	7/7 ^b	Neurons, glial cells, Purkinje cells, ependymal cells
Optic lobe	2	2	1	NA	NA	NA	NA	3/3	
Brainstem	1	1	2	NA	NA	NA	NA	3/3	
Cerebellum	2	2	3	2	NA	2	3	6/6	
Spinal cord	1	2	1	NA	3	2	2	6/6	Neurons, glial cells, ependymal cells
Peripheral nerves	+ ^c	+	-	NA	NA	NA	NA	2/3	Ganglia, nerve fibers, glial cells
Kidney	NA	-	-	-	-	-	-	0/6	-
Gonad	+	NA	+	+	+	+	+	6/6	Interstitial of testes and ovaries, theca, and granulosa cells
	(M) ^d		(M)	(F)	(M)	(F)	(F)		
Proventriculus	+	+	+	+	+	+	+	7/7	Glands, myenteric plexuses
Ventriculus	+	+	NA	NA	+	+	+	5/5	Myenteric plexuses
Heart	-	-	-	NA	NA	NA	NA	0/3	-
Pancreas	+	-	+	NA	NA	NA	NA	2/3	Acinar cells, ganglia
Adrenal	+	NA	+	NA	NA	NA	NA	2/2	Cortical cells, medullary cells
Thyroid/ parathyroid	-	NA	-	NA	NA	NA	NA	0/2	-
Small intestine	+	+	+	NA	NA	NA	NA	3/3	Submucosal and myenteric plexuses
Colon	+	+	+	NA	NA	NA	NA	3/3	Submucosal and myenteric plexuses
Lung	+	-	+	NA	NA	NA	NA	2/3	Ganglia
Liver	-	-	-	NA	NA	NA	NA	0/3	-
Spleen	-	-	-	NA	NA	NA	NA	0/3	-
Trachea	-	-	-	NA	NA	NA	NA	0/3	-
Esophagus	NA	+	-	NA	NA	NA	NA	1/2	Submucosal and myenteric plexuses
Bursa	-	NA	-	NA	NA	NA	NA	0/2	-
Thymus	-	NA	-	NA	NA	NA	NA	0/2	-

^aIndicates IHC distribution score for cerebrum, optic lobe, brainstem, cerebellum, and

spinal cord. ^bIndicates the total number of birds with immunohistochemical reactivity in

that tissue / total number of birds with available tissue for testing. ^cIndicates presence (+) or absence (-) of immunohistochemical reactivity in the tissue (without distribution score). ^dIn parenthesis, male (M) or female (F) sex of the bird is indicated. ^eDescription of the type of positive cells. NA = tissue was not available for analysis.

Table 5. Semi-quantitative scoring of inflammation and immunohistochemical reactivity for aquatic bird bornavirus nucleoprotein in the brain and spinal cord of Muscovy ducks experi-mentally infected with ABBV-1.

Inflammation^a			Immunohistochemistry^b
<i>Score</i>	<i>Intensity</i>	<i>Distribution</i>	<i>Distribution</i>
+0	No PVCs ^c observed	No PVCs observed	No reactivity observed
+1	PVCs are 1-2 layers wide	1-10 vessels per 100X field are affected	1-7 positive cells per 400X field
+2	PVCs are 3-4 layers wide	11-20 vessels per 100X field are affected	8-14 positive cells per 400X field
+3	PVCs are > 5 layers wide	>21 vessels per 100X field are affected	>15 positive cells per 400X field
Sub-score ^d = intensity score + distribution score (range, 0-6) BRAIN SCORE = (sum of sub-scores from each available anatomical area of brain) / number of areas (range, 0-6)			Sub-score = distribution score (range, 0-3) FINAL SCORE (brain) = sum of sub-score from each anatomical segment of brain (range, 0-12)

^aThe inflammation score accounts for both intensity and distribution.

^bThe immunohistochemistry score accounts for distribution only.

^cPVCs = perivascular cuffs.

^dSub-scores are defined as scores for each segment of the central nervous system (cerebrum, optic lobe, brainstem, cerebellum, spinal cord)

Figures

Figure 1

Figure 1

Histology of representative lesions in the nervous tissue of Muscovy ducks experimentally infected with aquatic bird bornavirus 1 (ABBV-1). **(a)** Cerebrum, duck #519 IC group, sampled at 4 weeks post infection (wpi). The leptomeninges (black arrowhead) and Virchow-Robin spaces (empty arrowhead) are segmentally and multifocally expanded by an inflammatory infiltrate. HE, original magnification 100X. **(b)** Cerebrum, duck #519 IC group, 4 wpi. The perivascular spaces are expanded by a severe mononuclear inflammatory infiltrate. HE, original magnification 40X. **(c, d)** Spinal cord, duck #612 IM group, 8 wpi. Mainly affecting the grey matter (circle), there are multifocal areas of inflammation composed of mononuclear cells. Empty arrowhead indicates the central canal. HE, original magnification 40X **(c)** and 100X **(d)**. **(e)** Peripheral nerve (ischiatric or axillary), duck #621 IM group, 8 wpi. There are scattered inflammatory perivascular cuffs in the endoneurium of affected nerves. HE, original magnification 200X. **(f)** Cerebrum, duck #502 IC group, 12 wpi. The perivascular cuffs are composed of mononuclear cells, predominately lymphocytes with scattered plasma cells. HE, original magnification 400X. **(g, h)**. Optic

lobe, duck #502 Immunohistochemistry for CD3 (g) and Pax-5 (h) antigens on consecutive sections. The large majority of inflammatory cells displays strong cytoplasmic reactivity for CD3 antigen (T lymphocytes); while scattered cells display intranuclear reactivity for Pax-5 (B lymphocytes). Reactivity for CD3 clearly shows spilling of T lymphocytes from the perivascular cuffs into the adjacent neuroparenchyma. IHC, DAB chromogen with hematoxylin counterstain, original magnification 200X. (i) Cerebrum, duck #534 IC group, 4 wpi. There are rare, randomly distributed, accumulations of glial cells arranged in small clusters (glial nodules). These cells are round with moderate amounts of eosinophilic cytoplasm and open chromatin. HE, original magnification 400X.

Figure 2

Graphs showing the pathology inflammation subscores in different areas of the brain of Muscovy ducks experimentally infected with ABBV-1 through the intracranial (a) or intramuscular (b) routes of infection, assessed at 1, 4, 8, and 12 weeks post infection (wpi). Significant differences between terms are identified with simple binary connectors. Flat lines at the end of a connector indicate differences with multiple underlying terms (in that case, significance level is reported for the higher value). Multiple comparisons against a single term are indicated by a line with multiple prongs and the common term indicated by a short horizontal segment. Pairwise comparisons with data points scoring 0 (no inflammation) are not represented. Data columns represent median with data range. Kruskal-Wallis test with Benjamini, Krieger, and Yekutieli procedure for false discovery rate ($* < 0.05$, $** < 0.01$, $*** < 0.001$).

Figure 3

Graph showing the overall brain scores of inflammation in Muscovy ducks experimentally infected with aquatic bird bornavirus 1 (ABBV-1) through the intracranial (IC) or intra-muscular (IM) routes of infection, assessed at 1, 4, 8, and 12 weeks post infection (wpi). Significant differences between terms are identified with simple binary connectors. Multiple comparisons against a single term are indicated by a line with multiple prongs, and the common term is indicated by a short horizontal segment. Pairwise comparisons with data points scoring 0 (no in-flammation) are not represented. Data columns represent median with data range. Kruskal-Wallis test with Benjamini, Krieger, and Yekutieli procedure for false discovery rate (* < 0.05, ** < 0.01, *** < 0.001).

Figure 4

Figure 4

Magnitude of virus replication, represented as log10 genome copy number, in multiple tissues and swabs from Muscovy ducks experimentally infected with ABBV-1 through the intracranial (a) or intramuscular (b) routes of infection, assessed at 1, 4, 8, and 12 weeks post infection (wpi). Negative data points are represented as round-shaped placeholders. Significant differences between terms are identified with simple binary connectors. Flat lines at the end of a connector indicate differences with multiple underlying terms (in that case, significance level is reported for the higher value). Multiple comparisons against a single term are indicated by a line with multiple prongs and the common term is indicated by a short horizontal segment. Pairwise comparisons between different organs at different time points (i.e., brain at 4 wpi vs. kidney at 8 wpi) are not represented. Data columns represent mean log10 virus copy number / 150 total tissue RNA (tis-sues), or 84 µl of swab liquid. Two-way ANOVA with Tukey's test for multiple comparisons (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001). Data points for gonads, as available only at 12 wpi, were not included in the analysis.

Figure 5

Figure 5

Representative immunohistochemical (IHC) reactivity for ABBV-1 nucleoprotein (N) in tissues of Muscovy ducks experimentally infected intracranially with ABBV-1 and sampled at 12 weeks post infection (wpi). **(a–d)** Immunoreactivity for ABBV-1 N is observed in scattered neurons and glial cells in the cortex of the forebrain (**a**, duck #502), periventricular neuroparenchyma and rare ependymal cells (**b**, duck #537), in clusters of large neurons in the medulla oblongata (**c**, duck #543), and in scattered Purkinje and granular cell in the cerebellum (**d**, duck #502). Reactivity is most often intensely nuclear, with rare light

cytoplasmic signal. (e-l) In the peripheral nervous tissue, immunoreactivity is observed in the axons of peripheral nerves (e, duck #502), myenteric plexuses of the small intestine (f, duck #542), and ventriculus (g, duck #502), ganglia and acinar cells of the pancreas (h, duck #502), and medullary and cortical cells of the adrenal gland (i, duck #502). (j-l) In the testis, reactivity is observed in the interstitial cells between tubules (j, duck #502), and in the ovary, reactivity is present in the interstitial cells between follicles, theca cells, and granulosa cells (k-l, duck #542).

Figure 6

Representative Western blots used to identify presence of immunoglobulin against aquatic bird bornavirus 1 (ABBV-1) nucleoprotein (N), in the serum of Muscovy ducks experimentally infected with ABBV-1. Depicted are blots done using the sera from ducks infected through the intracranial (top row, panels a and b) or intramuscular (bottom row, panels c and d) routes, both at 1 (panels a and c) and 12 (panels b and d) weeks post infection (wpi). (+) positive control consisting of lysate of CCL-141 cells persistently infected with ABBV-1, and blotted using a monospecific rabbit antibody against the ABBV-1 N protein. (L) ladder. (C) Lysate of CCL-141 cells not infected with ABBV-1 and blotted using Muscovy duck serum as primary antibody. (V) Lysate of CCL-141 cells persistently infected with ABBV-1, and blotted using Muscovy duck serum as primary antibody. Each bird is identified by its number at the bottom of each blot. The ABBV-1 N protein migrates between 40 and 30 kDa; birds with serum identifying a band of this molecular size are considered seropositive.

Supplementary Files

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- [SUPPLEMENTARYMATERIALMuscovy.pdf](#)