

NONSTEROIDAL ANTI-INFLAMMATORY DRUG TREATMENT OF
COCKATIELS (*NYMPHICUS HOLLANDICUS*) EXPERIMENTALLY
INOCULATED WITH PARROT BORNAVIRUS 2

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2015

Major Subject: Biomedical Sciences

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ABSTRACT

This study investigated the effects of nonsteroidal anti-inflammatory drugs on cockatiels experimentally inoculated with Parrot bornavirus-2 (PaBV-2). Twenty-seven cockatiels were randomized into 3 groups of 9 birds each taking into consideration historical PaBV shedding, weight, and sex. Cockatiels were then inoculated with cell culture derived PaBV-2 by the intranasal and intramuscular routes. At 23 days post-inoculation, each group received either oral treatment once daily with celecoxib (10.0 mg/kg), meloxicam (1.0 mg/kg) or placebo. Within 33-79 days post-inoculation; 2 birds were found dead and 6 birds were euthanized based on neurological or gastrointestinal signs consistent with proventricular dilatation disease. At day 173±2 post-inoculation, the remaining birds (21) were euthanized. Necropsy and histopathology results showed lesions characteristic of proventricular dilatation disease in most of the cockatiels; there was no statistical difference in the nature or severity of lesions between the 3 treatment groups. There was no statistical difference between the 3 treatment groups in the detection of PaBV mRNA and PaBV N-protein using RT-PCR and immunohistochemistry, respectively. Two birds from the meloxicam and 2 birds from the celecoxib groups had black intestinal contents consistent with intestinal bleeding. These findings suggest that the use of meloxicam or celecoxib does not alter the clinical presentation, viral shedding, gross lesions, histopathology, or viral distribution in cockatiels experimentally inoculated with PaBV-2.

DEDICATION

This thesis is gratefully dedicated to my family, who have been always there for me and supported me through all my endeavors.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Jeffery Musser, who had belief in me and provided me with the opportunity to pursue my graduate studies under his supervision. He has provided me with amazing opportunities to better my carrier and vouched for me many times, even when I struggled during my research experience. I am thankful for his enduring support and encouragement.

I also want to express gratitude to my committee members, Dr. Tizard and Dr. Heatley for their guidance and support throughout the course of this research and their positive feedback that enhanced my knowledge and understanding.

I would like to also recognize the team at Schubot Exotic Bird Health Center, especially Mrs. Debra Turner, Jenna Kranz, Jianhua Guo, and Dr. Hoppes who since the beginning of my work were there for me and were patient with me throughout all the challenges. They provided me with valuable support for my work, and have vouched for me many times.

None of my accomplishments would have been possible without the love and support of my family, especially my mother. My mother is my rock, my number one supporter, and my strength, without her guidance, care and love I wouldn't be the person that I am today. I am also grateful to my brother, Sebastian for his care and help with whatever he can along the way. I also want to acknowledge my brother Rodrigo, who is always looking out for what is best for me and encouraging me. I am also very thankful for my father for being the reason that I want to be someone great in life. Finally, thanks to my love, who encouraged

me and had faith in my abilities. He was always there supporting me and providing me with encouragement and a shoulder to lean on. Most importantly, he continued to be my strongest supporter and motivator. Thank you for being my companion for life.

NOMENCLATURE

NSAIDs	Nonsteroidal anti-inflammatory drugs
PaBV	Parrot bornavirus
BCS	Body condition score
IHC	Immunohistochemistry
RT-PCR	Reverse transcriptase polymerase chain reaction
N-protein	Nucleocapsid protein
BoDV	Borna disease virus
P	Phosphprotein
M	Matrix protein
PDD	Proventricular dilatation disease
FBS	Fetal bovine serum
DMEM	Dulbecco's modified eagle medium
DEF	Duck embryo fibroblast

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CHAPTER I

INTRODUCTION

Parrot bornaviruses 1-7 (PaBV - 1-7) are associated with Proventricular dilatation disease (PDD), a progressive fatal neurologic syndrome.^{1,2,3} Proventricular dilatation disease is primarily a disease of captive birds of the *Psittacidae* and *Cacatuidae* families, such as cockatoos, cockatiels, lovebirds, parakeets, conures, macaws and parrots, but has been identified in over 80 avian species.^{4,5,6} Proventricular dilatation disease is characterized by infection of the central and peripheral nervous system and other organs with PaBV, leading to lymphocytic, plasmacytic infiltration of those tissues, and culminating in nervous disorders and gastrointestinal malfunctions.⁶⁻¹¹

Based on clinical and pathological signs of PDD, it has been suggested that the disease may result from local inflammatory reactions to the virus within the nerves and other tissues.^{9,12} It has therefore been asserted that administration of nonsteroidal anti-inflammatory drugs (NSAIDs) to birds infected with PaBV may reduce the occurrence and severity of the clinical signs and pathological changes due to the virus.^{6,13} Celecoxib and meloxicam are among the NSAIDs purported to be effective and commonly used by clinicians in the symptomatic treatment of birds diagnosed with PDD.¹⁴⁻¹⁹ However, few studies have examined the use of NSAIDs^{13,14,16} and none have compared the effects of celecoxib and meloxicam in birds infected with PaBV.

Parrot bornavirus administered to cockatiels disseminates into many tissues and causes PDD-like clinical signs, shedding of the virus, and gross pathological and

histological changes.^{10,11,13,20,21} Cockatiels are an acceptable experimental model in studying PDD caused by PaBV.^{10,11,13,20-24}

The objective of this study was to evaluate differences in the clinical signs, viral shedding, and pathology of cockatiels inoculated with PaBV-2 and then treated with either celecoxib, meloxicam, or a placebo. We hypothesized that NSAIDs administered would reduce pathological effects and therefore clinical signs in PaBV-2 inoculated cockatiels.

CHAPTER II

REVIEW OF LITERATURE

Psittaciform 1 Bornavirus

Psittaciform 1 bornavirus, a member of the family *Bornaviridae*, is an enveloped, non-segmented negative strand RNA virus. *Psittaciform 1 bornavirus*, which contains parrot bornaviruses 1-7 (PaBV 1-7) was formerly referred to as avian bornavirus, but recent genomic and protein sequencing and phylogenetic analysis of the *Bornaviridae* family have resulted in renaming and taxonomic reorganization.²⁵ The new nomenclature and taxonomic organization of *Bornaviridae* includes the genus *Bornavirus* with five species: *Mammalian 1 bornavirus*, *Psittaciform 1 bornavirus*, *Passeriform 1 bornavirus*, *Passeriform 2 bornavirus*, and *Waterbird 1 bornavirus*.²⁵ *Bornaviridae*, along with *Filoviridae*, *Nyamiviridae*, *Paramyxoviridae*, and *Rhabdoviridae*, belong to the order *Monoegavirales*. However unlike the other families in that order that replicate in the cytoplasm, viruses within *Bornaviridae* employ intranuclear transcription and replication.²⁶ *Bornaviridae* uses a number of strategies including alternative RNA splicing, overlapping of transcription units and transcriptional signals, read-through of transcriptional initiation sites, and differential use of translational initiation codons.^{27,28,29} For example, *Bornaviridae* employs a unique genome replication strategy that involves trimming of triphosphates from the 5' terminal of the viral RNA to avoid activation of the innate immune response receptors.^{29,30} The bornavirus genome encodes six proteins; nucleocapsid (N), X protein (X), phosphoprotein

(P), matrix (M), enveloped glycoprotein (G), and the RNA- dependent RNA polymerase (L) similar to the other members of the *Mononegavirales* order.^{31,32}

Prior to discovery of bornaviruses in birds, *Mammalian 1 bornavirus*, which contains Borna disease viruses (BoDV), was the only species in *Bornaviridae*. In 2008, novel avian viral strains of *Bornaviridae* were identified in the brain, proventriculus, and adrenal gland of birds with proventricular dilatation disease (PDD).^{1,2} Brain homogenates containing the avian viral strains, which were identified as PaBV, inoculated into cockatiels produced clinical and pathological characteristics of PDD.²¹ Inoculation of Patagonian conures with PaBV isolated from the brain of birds with confirmed PDD, resulted in clinical signs, along with gross and histopathologic lesions, of PDD. PaBV was then subsequently demonstrated in the brains of the infected birds, thus fulfilling Koch's postulates and proving that PaBV is an etiological agent of PDD.³

Psittaciform 1 bornavirus infection, which contains PaBV-1, -2, -3, -4, -7, is found mainly in parrots and macaws,^{1,2,33,34} but is also found in other psittaciformes.³⁵⁻⁴¹ *Bornavirus* is also found in passerines,⁴²⁻⁴⁵ geese,^{46,47,48} mute and trumpeter swans,⁴⁶ and wild ducks.⁴⁹ *Psittaciform 1 bornavirus* is neurotropic, but is found in other tissues including the kidneys, medullary cords of the adrenal gland, heart, spleen, liver, lungs, pancreas, testes and ovaries.^{20,38,50-55} The mechanism of transmission of PaBV is unknown, but a fecal-oral transmission is considered the primary route of infection;^{12,52,56} PaBV mRNA is found uro-fecal droppings, lumen of renal tubules, and urine.⁵⁶ PaBV isolated from urine of naturally infected birds is infectious in cell

culture.⁵⁷ A respiratory component to transmission is proposed as the virus can be aerosolized.^{6,52} It is found in feather calami,⁵⁸ choanal swabs,⁵² and air samples collected up to 150 inches from an PaBV infected and shedding bird;⁶ however, birds in close contact for 4 months with infected birds did not become infected.²³ Vertical transmission is also suggested as a potential route of transmission.⁵⁹⁻⁶³

Proventricular Dilatation Disease

Psittaciform 1 bornavirus infects neurons and other tissues, resulting in an pathologic response in the infected bird to the virus leading to the development of proventricular dilatation disease.⁸ Proventricular dilatation disease was first recognized in North America and Europe in late 1970s and was initially described as Macaw Wasting Disease or Macaw Fading Syndrome because it was first reported in an outbreak involving macaw species.^{64,65} Although the disease is commonly referred to as PDD, it has several synonyms including macaw wasting syndrome, proventricular dilatation syndrome, neuropathic gastric dilatation of psittaci-forms, myenteric ganglioneuritis, proventricular hypertrophys.³⁷ Proventricular dilatation disease is primarily a disease of birds of the *Psittacidae* and *Cacatuidae* families, such as cockatoos, cockatiels, lovebirds, parakeet, conures, macaws and parrots, but also occurs in other avian species.^{2,3,4,5,12,64,65} Proventricular dilatation disease is characterized by inflammation of the central, peripheral, and autonomic nervous systems, affecting two major systems: the neurologic and gastrointestinal systems.^{5,6,8,9,10,12,36,52} The classical signs of PDD involve the gastrointestinal tract and include difficulty in swallowing,

regurgitation, and presence of undigested food in feces. Radiographic evaluation shows a dilated crop, proventriculus, ventriculus and intestines.^{6,12} Upon necropsy, dilation of the crop, proventriculus and ventriculus are frequently noted and the thin walled organs can spontaneously rupture.^{3,12} When clinical signs include gastrointestinal dysfunction, autonomic nerves innervating the esophagus, crop, proventriculus, ventriculus, and duodenum are affected.^{2,6,9,12} Other clinical signs suggestive of proventricular dilatation disease are weight loss over a period of weeks to months, abnormal head movements, motor deficits, abdominal distention, and impaction of the crop.^{5,6,9,12} Birds affected by proventricular dilatation disease may also have signs relating to the central nervous system; blindness, ataxia, aggression, as well as possibly feather plucking, and seizures.^{8,12,66} Peripheral neuritis, myenteric ganglioneuritis, and encephalitis occur in birds with neurological or gastrointestinal tract signs of PDD. Diagnosis of PDD is difficult as it is based upon the presence of clinical signs, radiographic findings and biopsy; however, definitive diagnosis is by histological findings of lymphocytic infiltration of the enteric or central nervous system with corresponding local inflammation within nerves and ganglia.^{5,10,11,12,20,52,64} Similar infiltrates may also be present in the spinal cord, peripheral nerves, conductive tissue of the heart, smooth and cardiac muscle, and adrenal glands.^{2,10,11,20}

Nonsteroidal Anti-Inflammatory Drugs

Presently, there is no proven efficacious treatment for PDD or other *Psittaciform I bornavirus* infections. Experimentally, INF- α inhibits viral infection and viral load in

quail cell culture³⁰ and ribavirin inhibits transcription and reduces viral load in DEF cells.^{67,68} Symptomatic treatment and supportive care are currently the principal therapies for PDD.^{5,12} It is postulated that nonsteroidal anti-inflammatory drugs and immunosuppressant drugs can inhibit or reduce the inflammatory reaction of PaBV infection and thus will lessen the severity of clinical signs of PDD.^{5,12} Celecoxib and meloxicam, both NSAIDs, and cyclosporine, an immunosuppressant, are used clinically and experimentally^{13,14,16} but their use is controversial. In cockatiels experimentally infected with PaBV-4 and treated with meloxicam, severe weight loss and depression resulted in the birds being euthanized between 60 to 118 days after viral inoculation. Gross lesions and histopathology findings were consistent with PDD and blood was found in the proventriculus and intestines.¹³

The principal mechanism of action of nonsteroidal anti-inflammatory drugs is the inhibition cyclooxygenase (COX). Cyclooxygenase catalyzes the production of prostaglandins from arachidonic acid.^{69,70,71} Cyclooxygenase exists as two main isoenzymes, COX-1 and COX-2. COX-1 enzymes are expressed in most tissues and produce prostaglandins and thromboxanes involved in homeostatic physiological processes.^{69,70,71} COX-1 is considered the constitutive or maintenance form of the enzyme, maintaining the integrity of the gastrointestinal mucosa, affecting platelet aggregation, influencing renal blood flow and hemodynamics.^{70,72,73,74} COX-2 is the inducible form of the enzyme, with a low basal expression level and is induced in response to inflammatory stimulus, such as lipopolysaccharides, cytokines, and injury.^{69,70,71,73} Traditional NSAIDs that inhibit both COX-1 and COX-2 are known as

non-selective NSAIDs. With the discovery of COX-2 in the 1990s, selective COX-2 inhibitors were developed to provide anti-inflammatory and analgesic properties without inhibiting the protective mechanisms of COX-1. COX-2 selective inhibitors provide anti-inflammatory and analgesic effects without altering the homeostatic functions of COX-1. Thus the selective COX-2 inhibitors can have fewer toxic effects, such as the gastrointestinal and renal toxicities, than those of the non-selective, traditional NSAIDs.

The toxic effects and pharmacokinetics of NSAIDs such as meloxicam, flunixin meglumine, carprofen, ketoprofen and diclofenac, have been evaluated in birds including bobwhite quail (*Colinus virginianus*),⁷⁵ *Gyps* vultures,⁷⁶⁻⁸⁰ budgerigars (*Melopsittacus undulates*),^{81,82} pigeons (*Columba livia*),⁸³⁻⁸⁶ Japanese quail (*Coturnix japonica*),^{84,87} African grey parrots (*Psittacus erithacus*),^{88,89} cockatiels (*Nymphicus hollandicus*),⁹⁰ American kestrels (*Falco sparverius*),⁹¹ Patagonian conures (*Cyanoliseus patagonus*),⁸² mynah (*Leucopsar rothschildi*),⁸⁴ Hispaniolan amazon parrots (*Amazona ventralis*),^{94,95} and red-tailed hawks (*Buteo jamaicensis*),⁹⁶ domestic fowl,^{97,83,84,92,93} and great horned owls (*Bubo virginianus*).⁹⁶ Possible adverse effects of NSAIDs are a decrease in the secretion of uric acid and ischemia in the kidneys from renal arterial vasoconstriction. NSAID toxicity will be exhibited histopathologically as mild glomerular lesions, renal tubular cell necrosis, gastric ulcers, and/or hepatic lipidosis. Renal and hepatic lesions have been reported in broiler chicks, pigeons, Japanese quail, and mynah birds when administering diclofenac.⁸⁴ Renal lesions are a particular issue in Asian vultures (*Gyps bengalensis*, *Gyps indicus*, and *Gyps tenuirostris*) when administering diclofenac.^{76,80} Renal lesions have been also seen in budgerigar treated with flunixin meglumine.⁸¹

However, the evaluation of meloxicam in budgerigars,⁸¹ African grey parrots,⁸⁹ Hispaniolan amazon parrots,⁹⁵ domestic pigeons,⁸⁶ Japanese quail⁸⁷ and Asian vultures⁷⁸ have found no significant renal, gastrointestinal or hepatic adverse effects. NSAID metabolism, pharmacokinetics and toxicity appear to depend on avian species, NSAID utilized, and route of administration.

Meloxicam and celecoxib are frequently prescribed NSAIDs by veterinarians for companion birds.^{14,16} Celecoxib (Celebrex®), a coxib class of NSAID, was the first COX-2 selective inhibitor introduced for the treatment of humans with active osteoarthritis, ankylosing spondylitis or rheumatoid arthritis in 1998.⁷² Meloxicam is a member of the enolic class of NSAIDs, which is also a COX-2 selective inhibitor used for its analgesic, anti-inflammatory, and antipyretic properties. Celecoxib selectivity for COX-2 is believed to be 30-fold more than for COX-1, and meloxicam is believed to have an 18-fold greater selectivity for COX-2 than for COX-1.⁹⁸

Experimental Models

Experimentally infection of birds with PaBV has confirmed the virus's etiological role in the development of PDD.^{3,10,11,20,21} PDD can be experimentally induced in healthy birds by inoculation of PaBV infected-brain tissue²¹ or PaBV obtained from cell culture.^{3,10,11,20} Gray et al. infected Patagonian conures with PaBV, grown in DEF cell culture, by intramuscular injection; the birds developed clinical signs, gross abnormalities, and histological lesions consistent with PDD within 66 days post-inoculation.³

A cockatiel model is routinely used for the investigation of PaBV infections and PDD.^{10,11,13,20-24,62} Gancz et al. first demonstrated that clinical and pathological changes characteristic of PDD could be induced in cockatiels using intramuscular, intraocular, intranasal, and oral inoculation with PaBV positive brain homogenate from birds with PDD. Clinical signs, gross changes, and histopathological lesions consistent with PDD were found within 60-95 days post-inoculation.²¹

Virus isolated from infected birds and grown in DEF cell culture can also cause infection and PDD-like lesions when inoculated into cockatiels.¹⁰ Cockatiels inoculated by the oral and intramuscular route developed clinical signs and histopathological lesions of PDD 30-45 days post-inoculation.¹⁰ Payne et al. showed unusually severe gross and histopathology lesions in cockatiels 92 and 110 days post-inoculation.²⁰ Hoppes et al. used DEF cell culture derived PaBV-4 to evaluate the use of meloxicam in experimentally induce PDD; challenged birds developed PDD-like lesions.¹²

The growth of infectious PaBV is not restricted to DEF cells. Piepenbring et al. infected cockatiels by the intracerebral and intravenous routes with PaBV-4 grown in a quail cell line; birds develop clinical signs, gross necropsy findings, and histopathological changes characteristic of PDD.¹¹ Rubbenstroth et al. inoculated cockatiels by a combination of intramuscular, subcutaneous, and oral routes with PaBV-4 grown and isolated from quail fibroblast cells. PaBV-4 inoculated cockatiels showed clinical signs of PDD within 24 weeks post-inoculation.²³

These studies confirm cockatiels are an accepted experimental model for PDD research, since PaBV isolated directly from the brain of infected birds or grown in cell

culture is inoculated by the intramuscular, oral, subcutaneous, intracerebral or intravenous route results in clinical signs, gross lesions, and histological changes characteristic of PDD.

CHAPTER III

MATERIALS AND METHODS

Parrot Bornavirus

Virus was obtained from a supply cultured and isolated from the brain of experimentally infected cockatiels' (*Nymphicus hollandicus*) that developed PDD.¹⁰ Virus for inoculation was grown as previously described.⁹⁹ Briefly, duck embryo fibroblast (DEF) cell cultures were inoculated with an aliquot of stock virus and maintained in Dulbecco's modified eagle medium (DMEM) (Gibco®, Life Technologies Co) with 10% fetal bovine serum (FBS) (Gibco®, Life Technologies Co) at 37°C in an atmosphere of 5% CO₂. After 3 days of incubation, DEF cells were harvested three times by divided into 1.0mL of stock virus aliquots, and stored at -80°C. Virus was confirmed as PaBV-2 by RNA extraction and RT-PCR analysis followed by sequence analysis of the PCR product.⁹⁹

Birds were inoculated by intranasal and intramuscular administration with DEF cells containing 8×10^4 focus forming units (FFU) of PaBV-2. A similar method is effective in inducing proventricular dilatation disease in cockatiels.^{10,13}

Nonsteroidal Anti-Inflammatory Drugs

Meloxicam, 15mg tablets, was used (Lupin, Pharmaceuticals, Inc. Baltimore, MD, USA) A meloxicam liquid suspension, 1.0 mg/mL, was made by crushing 3 tablets, using a mortar and pestle, dissolving in 1.0mL deionized water, adding 10.0 mL of Ora-

Plus (Perrigro® Co., Dublin, Ireland) suspending vehicle, and adding Ora-Sweet (Perrigro® Co., Dublin, Ireland) to obtain a final volume of 45.0 mL.

Celecoxib, 50.0 mg capsules, was used (Pfizer Inc., Mission, KS, USA). A celecoxib liquid suspension, 10.0 mg/mL, was made by combining 9 capsules with 1.0mL deionized water and 10.0 mL of Ora-Plus suspending vehicle, and adding Ora-Sweet to obtain a final volume of 45.0mL.

Nonsteroidal anti-inflammatory drugs for this study were compounded in the laboratory from commercially available formulations to facilitate drug delivery to study subjects. All solutions were stored at 4°C and recompounded every 30 days. Prior to administration, drug solutions and control solution were warmed to room temperature.

Animals

Twenty-seven male and female cockatiels (*Nymphicus hollandicus*), ranging in weight from 79 to 145 g, mean of 101 g were used. The birds were assessed as healthy based on physical examination and medical history. The cockatiels were quarantined for 60 days, during which time each bird was tested thrice for Parrot bornavirus (PaBV), psittacid herpesvirus (genotype1-4), *Chlamydophila spp* and *Macrorhabdus ornithogaster* (avian gastric yeast) via cloacal swab. The cockatiels were housed in flight cages, 14 or 13 birds per cage, at the Schubot Exotic Bird Health Center. Birds were fed a 1/6 cup per bird of premium daily FruitBlend with natural fruit flavors (ZuPreem®, Shawnee, KS, USA) bird food, and had access to water ad libitum. This research study was approved by the Texas A&M University Office of Research Compliance.

Experimental Protocol

The procedure protocol timeline is illustrated in Figure 1. Approximately, one month prior to beginning experimental inoculation, birds were randomized, matched with respect to historical shedding, weight and sex, into three groups of nine birds each: Group 1 - experimentally inoculated and then 23 days following inoculation, treated orally, once daily with the delivery solution (water, Ora-plus, and Ora-Sweet) without any NSAID; Group 2 - experimentally inoculated and then 23 days following inoculation, treated orally, once daily with 1.0 mg/kg meloxicam; and, Group 3 - experimentally inoculated and then 23 days following inoculation, treated orally, once daily with 10.0 mg/kg celecoxib. Treatments were done prior to morning feeding. Drug treatment of each group was kept blinded to the person treating and assessing the birds until study completion; this was a randomized placebo-controlled blinded study.

Birds were observed daily for any abnormal clinical signs and deviations from normal behavior; including feather fluffing, bowed head, lack of vocalization, almond shape eye, over eating, lethargy, and staying on bottom of cage. Cloacal swabs were collected twice and the weights and body condition scores (BCS) assessed once before inoculation. After viral inoculation, birds were weighed and BCS was assessed weekly; cloacal swabs were collected every third week and stored at -80°C until assayed by RT-PCR.

Body condition scores were determined by assessing the shape of the keel and pectoral muscle. Body condition scores ranged from 1 to 5, lowest to highest score,

respectively. Criteria for determining BCS was adapted from Heatley et al. and is described in Table 1.¹⁰⁰

A bird was withdrawn from the study and euthanized if it met the following pre-established criteria: weight loss >20% of initial weight; BCS of 1, and/or, recommendation by attending veterinarian. The procedure for euthanasia, sample collection, and necropsy was as described for birds that completed the treatment period.

On day 173 (± 2) following experimental inoculation and day 150 (± 2) of NSAID or placebo treatment, birds were euthanized: Birds were anesthetized with 5% isoflurane followed by 100% carbon dioxide. Prior to euthanasia, weight, BCS, urine, cloacal swab, and blood were collected. Urine was collected as previously described by Heatley et al.⁵⁶ Immediately following death, a complete necropsy was performed and gross lesions were recorded and photographed. Samples, 2 per organ, of heart, liver, feather follicle, spleen, crop, proventriculus, ventriculus, intestine, gonad, pancreas, adrenal gland, kidney, lung, spinal cord, brain, eye, aqueous humor, optic nerve, brachial plexus, and sciatic nerve were collected and stored at -80°C freezer for later analyses by RT-PCR. The remainders of the organs were placed in 10% buffered formalin for histopathology and immunohistochemistry (IHC) testing.

Figure 1. Experimental timeline of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib.

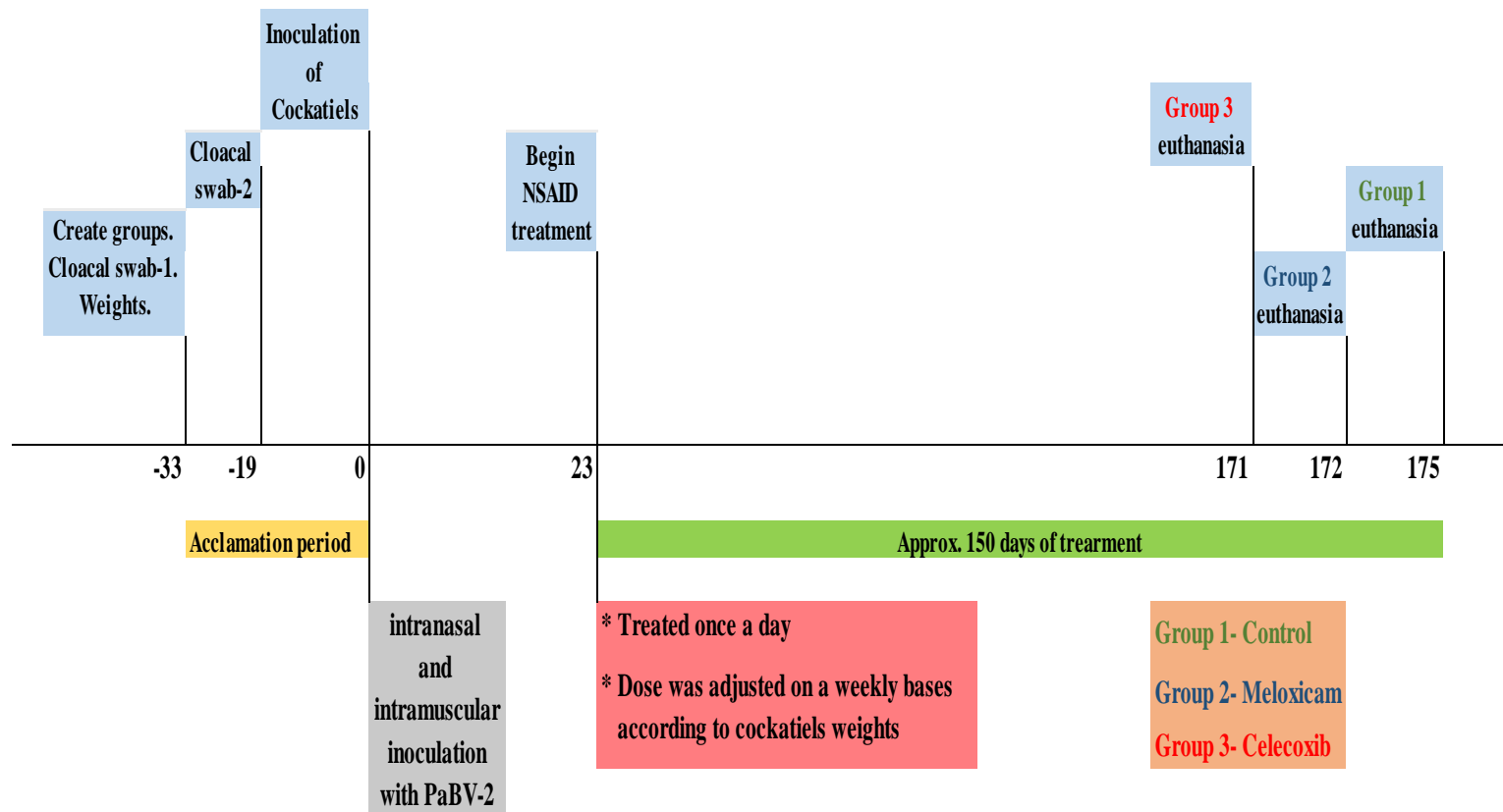


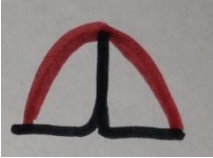




Table 1. Criteria for body condition score used on cockatiels experimentally inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib. In the schematic representations, the bird is in a ventrodorsal orientation. The keel is illustrated in black and pectoral muscles are illustrated in red. Adapted from Heatley et al.¹⁰⁰

BCS	Criteria	Schematic representation
1	pectoral muscles are concave around the keel	
2	pectoral muscles forms a flat silhouette line around the keel	
3	pectoral muscles are convex around the keel (ellipse)	
4	pectoral muscles form a semi-circular arch over and around the keel (1/2 circle)	
5	pectoral muscles extend beyond the keel bone (McDonald's M)	

Detection of PaBV mRNA by RT-PCR

Tissue, urine, and cloacal swabs were analyzed by TaqMan-based one-step reverse transcriptase real-time PCR (TaqMan® Fast Virus 1-Step, Life Technologies Co.). Total viral RNA was isolated from each sample using the QIAamp viral RNA mini kit (Qiagen, Valencia, Ca). Purified RNA was eluted in 60.0 uL elution buffer and stored at -80°C until use. PaBV phosphoprotein (P) gene mRNA was detected using Taqman RT-PCR assay performed with TaqMan® Fast Virus 1-Step Master Mix (Invitrogen, Carlsbad, Ca) and PaBV P primers (5' AAGAAGAA[Y]CC[Y]TCCATGATCTC 3' and 5'-AA[Y]TGCCGAAT[B]A[R]GTCATC- 3') and Taqman probe (5'-FAM-TCGATAACTG [Y]TCCCTTCCGGTC-BHQ-3'). PaBV matrix (M) gene mRNA was detected using PaBV M primers (5'- GGTAATTGTTCCCTGGATGG-3') and (5'-ACACCAATGTTCCGAAGACG-3'), and Taqman probe. (5'-FAM-TCGATAACTG [Y]TCCCTTCCGGTC-BHQ-3'). Each reaction was carried out using 6.0 µL of Taqman fast virus master mix, 5.0 µL of mRNA of sample, 0.4µL ultrapure H₂O, 0.3 µM primers and 0.3 µM probe in a 12.0 µL final reaction volume. All reactions were carried out using: initial denaturing for 5 minutes at 48°C and 20 seconds at 95°C, then 45 amplification cycling parameters, and then reverse transcriptase inactivation and polymerase activation for 3 seconds at 95°C and 30 seconds at 56°C. Results were analyzed using the Sequence Detection System, Version 2.4.1 (SDS 2.4) software (Life Technologies Co.). All samples were tested in duplicate for both the M and P gene. Samples were considered negative when cycle threshold (C_T) ≥37.0. If a sample was

positive for the M gene and negative for the P gene or vice versa, samples were retested with the Taqman RT-PCR assay.

Immunohistochemistry

Immunohistochemistry was performed on tissues obtained at necropsy. The assay was performed according to previously described methods.⁴² Presence of viral protein and assignment of a semi-quantified category the pathologist as: none detected, mild amount detected, moderated amount detected, severe amount detected.

Statistical Analysis

The Gehan-Breslow method was used to analyze differences in survival between the treatment groups. Two Way Repeated Measures Analysis of Variance (ANOVA) was used to compare weight changes between the treatment groups and day over the experimental time course. The Kruskal-Wallis One Way ANOVA on Ranks was used to ascertain differences in histological and immunohistochemistry findings. A $P \leq 0.05$ was considered statistically significant. SigmaPlot version 10.0.1 was used for performing all statistical analyses (Systat Software, Inc., San Jose, CA).

CHAPTER IV

RESULTS

Clinical Observations

No significant difference in survival rate occurred between the 3 treatment groups (Figure 2). In the control group, 2 of 9 birds were euthanized prior to the end of the study; birds number's 237 and 302 were euthanized on day 54 and 61 after inoculation (day 31 and 38 of treatment), respectively. In the meloxicam treatment group, 2 of 9 birds were euthanized or found dead prior to the end of the study; birds number's 10 and 2725 were euthanized or found dead on day 33 and 79 after inoculation (day 10 and 56 of treatment), respectively. In the celecoxib treatment group, 4 of 9 birds were euthanized or found prior to the end of the study; birds number's 204, 2441, 241, and 196 were euthanized or found dead on day 37, 43, 45, and 74 after inoculation (day 14, 20, 22, and 51 of treatment), respectively. Of these 8 birds, two were found dead and the remaining 6 showed neurological or gastrointestinal signs characteristic to PDD (Table 2).

No significant differences in weight occurred between the groups over the study period within each treatment group, the weights at the end of the study, day 173 \pm 2 post-inoculation was not significantly different from the weight prior to inoculation nor prior to treatment (Figure 3). Within each treatment group, weight varied significantly based on day. In the control group, the weight on day -31 and 55 after inoculation was significantly different, 99.0 and 89.7 grams, respectively. In the meloxicam treated

group, the weights on days 26, 33, 41, 48, 55, 63, and 69 were significantly less than on day -31 after inoculation, 94.2, 87.0, 88.9, 88.6, 89.4, 92.2, 93.0 and 102.9 grams, respectively. In the celecoxib treated group, the weights on days 33, 41, 48, 55, 63, 69, and 76 were significantly less than on day -31 after inoculation, 87.9, 89.8, 90.7, 89.6, 91.5, 91.6, 91.2, and 102.6 grams, respectively.

BCS did not differ significantly based on treatment group or day over the study period (Figure 4).

However, birds that were euthanized early from the treatment trial, tended to have lost weight and BCS over the course of the study. An exception was bird number 2725 from the meloxicam group, that at the time of euthanasia had a greatly increased weight compared to other birds at that time period but had a low BCS; at necropsy this bird had a profoundly dilated crop and a dilated proventriculus that were full of feed (Figure 5B). This accumulated seed may have contributed to the observed increase in weight.

Figure 2. Survival analysis of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib. The Gehan-Breslow statistic for survival curve was used to generate the survival analysis. Birds were experimentally inoculated with PaBV-2 on day 0. On day 23 post-inoculation (dashed arrow), birds were treated orally, once daily with the following: placebo control group (solid black line), meloxicam (dotted grey line) and celecoxib (dashed grey line).

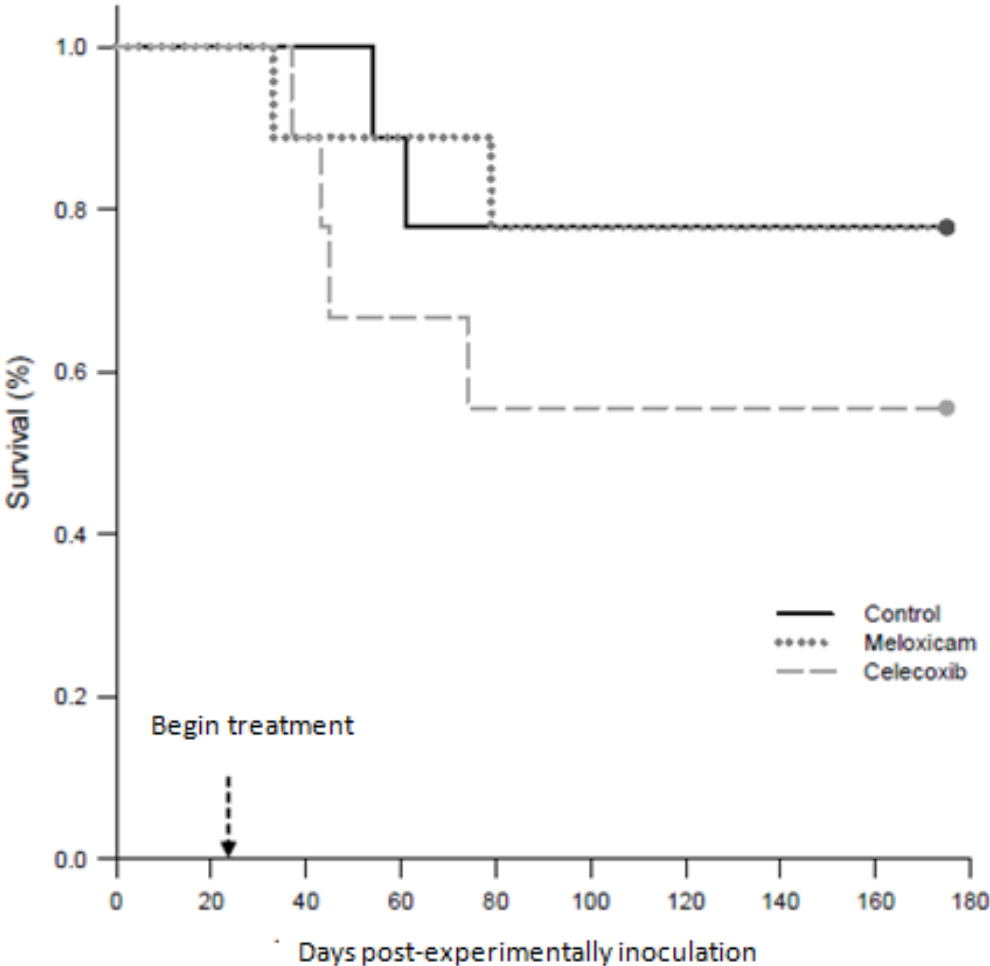


Table 2. Cockatiels experimentally inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib which were removed early from treatment study. Eight of the 27 birds were euthanized or died prior to the end of the study. BCS and weights were collected prior to necropsy.

Treatment	Bird ID	Euthanized day	BCS	Weights (gms)	Observations
Control	237	54	3	95	Loss of balance and flying into cage wall
	302	61	2	74.8	Regurgitating with a distended crop
Meloxicam	10	33	1	76	Found dead
	2725	79	2	117.6	Regurgitation, enlarged crops, feather fluffing, head drooping and tilted, off balanced, lethargic, spends most of the time on the cage floor
Celecoxib	204	37	2	97	Found dead
	2441	43	2	74.5	Eyes closed, unstable, head tilt, lethargic.
	241	45	3	83	Eyes closed, unstable, head tilt, lethargic
	196	74	1	64	Enlarged crop, eyes closed, unstable, head tilt, lethargic, feces dark in color

Figure 3. Mean weights of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib. Birds were experimentally inoculated with PaBV-2 on day 0. On day 23 post-inoculation (solid arrow), birds were treated orally, once daily with the following: placebo control group (green triangle, solid black line), meloxicam (blue circle, dotted black line) and celecoxib (red square, dashed black line) The final weight of the birds terminated early is represented by green triangles (control), blue circles (meloxicam), or red squares (celecoxib). ^aFor control group, weight significantly different from weight at day -33 post-inoculation . ^bFor meloxicam treated group, weight significantly different from weight at day -33 post-inoculation. ^cFor celecoxib treated group, weight significantly different from weight at day -33 post-inoculation.

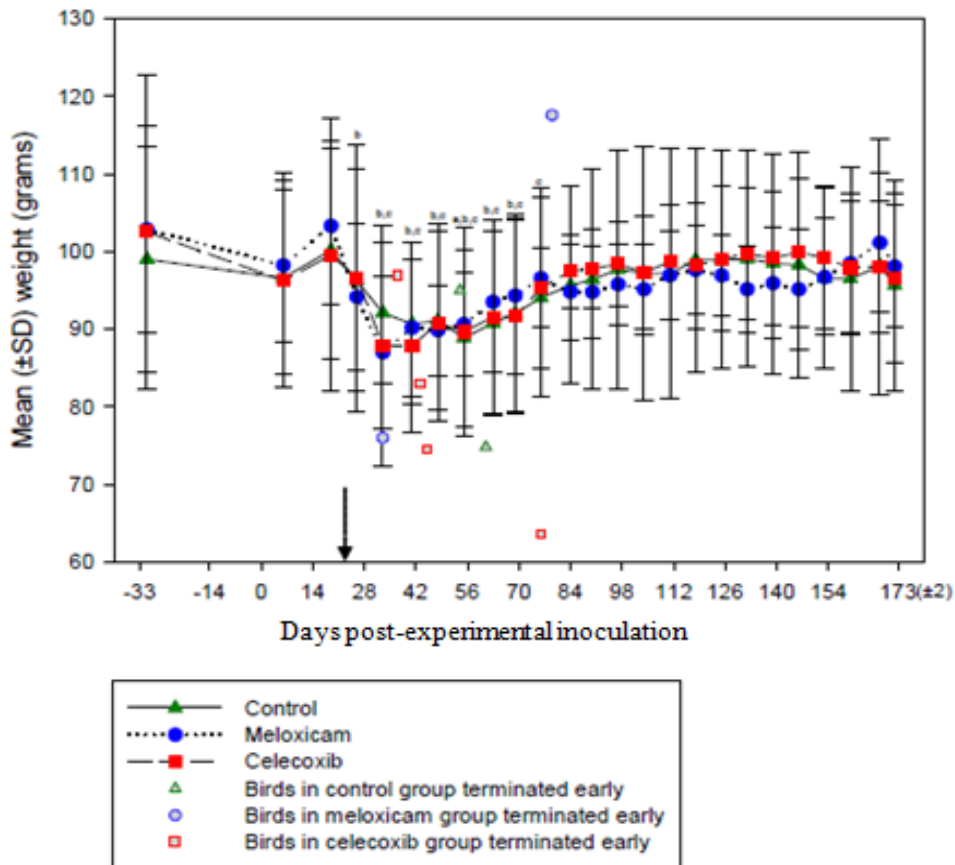


Figure 4. Mean body condition score of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib. Birds were experimentally inoculated with PaBV-2 on day 0. On day 23 post-inoculation (solid arrow), birds were treated orally, once daily with the following: placebo control group – (green triangle, solid black line), meloxicam (blue circle, dotted black line) and celecoxib (red square, dashed black line). Final body condition score of birds that died or were removed early are represented by green triangles (control), blue circles (meloxicam), or red squares (celecoxib).

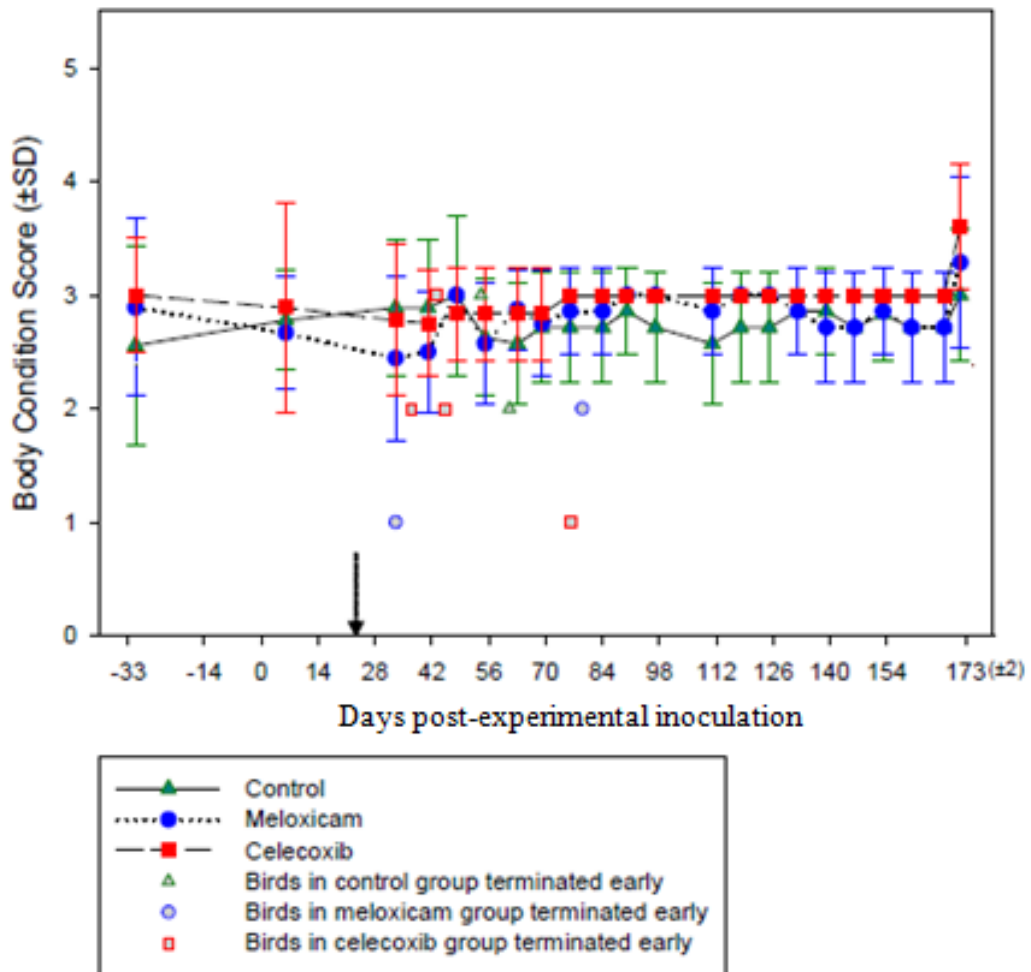
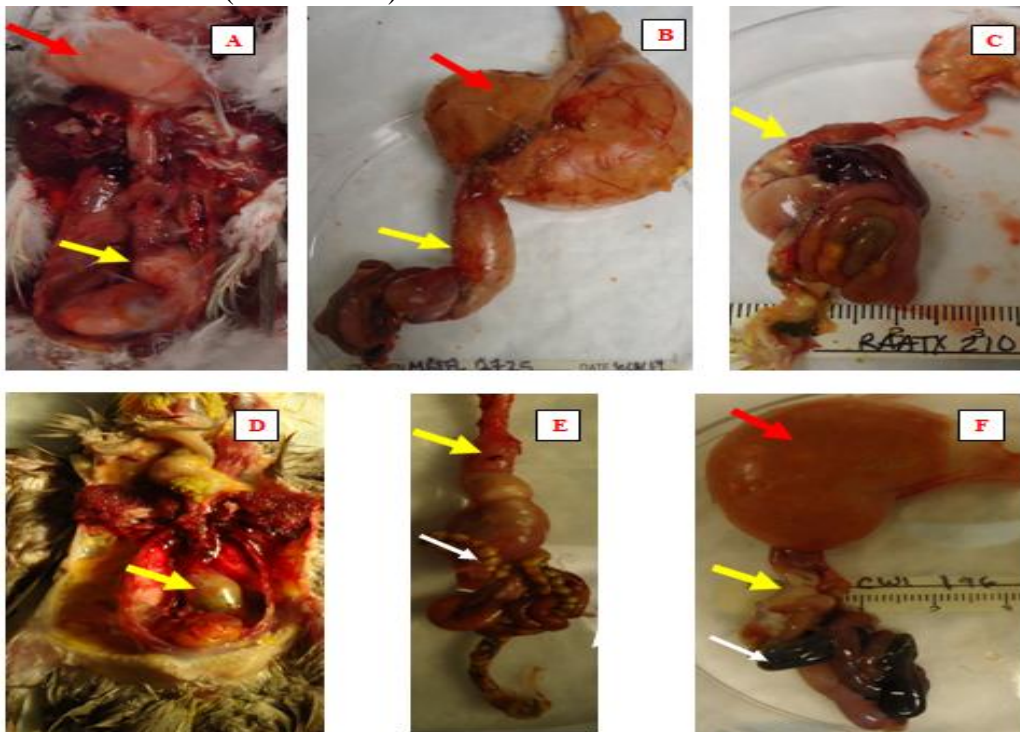


Figure 5. Gross findings at necropsy of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib.

- A. Bird number 302 control group, euthanized on day 61 post-inoculation. Severe dilation of proventriculus (yellow arrow) and crop (red arrow)
- B. Bird number 2725 meloxicam group, euthanized on day 79 post-inoculation. Severe dilation of proventriculus (yellow arrow) and crop (red arrow)
- C. Bird number 210 meloxicam group, euthanized on day 173 post-inoculation. Moderate dilation of proventriculus (yellow arrow)
- D. Bird number 2441 celecoxib group, euthanized on day 43 post-inoculation. Moderate dilation of proventriculus (yellow arrow)
- E. Bird number 241 celecoxib group, euthanized on day 45 post-inoculation. Mild dilation of proventriculus (yellow arrow) with undigested seed present in the intestines (white arrow).
- F. Bird number 196 celecoxib group, euthanized on day 74 post-inoculation. Mild dilation of proventriculus (yellow arrow), severe dilation of crop (red arrow) and blacken intestinal content (white arrow)



Detection of PaBV mRNA on Cloacal Swabs

Prior to experimental inoculation, cloacal swab testing for viral mRNA were negative for all birds, with the exception of one; bird number 221 had a positive cloacal swab on day -17 of inoculation; however, this bird's was negative on days -31, 13, and 20 post-inoculation (Table 3). PaBV shedding was first detected 42 days post-inoculation in the meloxicam and celecoxib groups. At the termination of the study (173 ± 2 days), all birds that were not removed early from the study had positive cloacal swabs, with the exception of bird number 261 in the meloxicam group. The cumulative numbers of birds with positive cloacal swabs by the end of the study were 8 of 9, 6 of 9, and 7 of 9 in the control, meloxicam, and celecoxib groups, respectively.

Necropsy and Histopathology

Gross abnormalities at necropsy were dilated crop, dilated proventriculus and dilated heart. However these abnormalities were not consistently present nor of consistent severity (Table 4 and Figure 5). Birds that were removed or died early had more pronounced crop and proventriculus dilatation than birds that survived until day 173 ± 2 post-inoculation. Gross abnormalities were less commonly noted in the air sacs, intestines, pancreas, kidneys, liver and spleen (APPENDIX B). Additionally, black intestinal contents were present in 2 birds of meloxicam and 2 birds of celecoxib group. These four birds numbers, 10 and 2725 in the meloxicam and 196 and 204 in the celebrex group were euthanized early in the study.

Histopathological changes were noted in a variety of tissues but were not significantly different between the three groups (Table 5). The lesions observed included lymphoplasmacytic myenteric ganglioneuritis in the crop, proventriculus, ventriculus and intestines; lymph nodules scattered, multifocal dilation of tubules/ interstitial inflammation, fibrosis and/or mineralization in the kidney; lymphoplasmacytic infiltration within the epicardial, myocardial, and/or Purkinje cells of the heart; lymphoplasmacytic perivascular cuffing in the central nervous system. Histopathological changes were seen in the, liver, pancreas, lung, spleen, optic nerve and adrenals but in lesser frequency (APPENDIX C).

Immunohistochemistry

There was no significant difference in the viral distribution or amount between the three groups and tissues. The virus was predominantly detected in the brain, heart, gastrointestinal tract and kidneys (Table 6). A lower prevalence of virus was also detected in liver, pancreas, lung, spleen, adrenal optic nerve, uropygial gland, cloaca, gonads, and skin/ feather follicles (APPENDIX C). Skeletal muscle was the only tissue consistently negative for the virus. Virus was not detected in any tissues of 4 birds, 1, 2, and 1 in the control, meloxicam, and celecoxib, respectively.

Detection of Viral mRNA in Necropsy

There was no significant difference in the detection of viral mRNA between groups. Viral mRNA was detected in midbrain, hindbrain, cerebellum, forebrain, kidney,

urine and cloacal swab (Table 7). In the control and meloxicam group, no viral mRNA could be detected in 1 and 2 birds, respectively. In the celecoxib group, no viral mRNA could be detected in the kidney of two birds (numbers 204 and 2441), but was detected in the brain. However in bird number 2441, viral mRNA was detected in the urine sample. Birds with negative cloacal swab results, except bird number 261 from the meloxicam group, were removed from the study early.

Table 3. Detection of viral mRNA in cloaca of cockatiels inoculated with PaBV-2

and treated with placebo, meloxicam, or celecoxib. PaBV m-RNA detection in

cloacal swabs of uro-feces from PaBV-2 inoculated cockatiels. RT-PCR testing

identified PaBV matrix and phosphoprotein mRNA. (-) negative-cycle thresholds >37.0;

(+) positive detection for viral mRNA; (N)-no sample collected; (*) euthanized prior to

the end of treatment.

Treatment	Bird ID	-31	-17	13	20	33	37	42	43	45	54	61	63	74	79	84	103	125	146	173±2	
Control	*237	-	-	-	-	N	N	-	N	N	-	N	N	N	N	N	N	N	N	N	N
	*302	-	-	-	-	N	N	-	N	N	N	+	N	N	N	N	N	N	N	N	N
	236	-	-	-	-	N	N	-	N	N	N	N	-	N	N	+	+	+	+	+	+
	239	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	+	+
	791	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	+	+
	9491	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	+	+
	92	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	+	+
	2820	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	-	+
	1460	-	-	-	-	N	N	-	N	N	N	N	-	N	N	+	+	+	+	+	+
Meloxicam	*10	-	-	-	-	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	*2725	-	-	-	-	N	N	-	N	N	N	N	-	N	-	N	N	N	N	N	N
	1662	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	+	+
	254	-	-	-	-	N	N	-	N	N	N	N	-	N	N	+	+	+	+	+	+
	691	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	+	+
	210	-	-	-	-	N	N	-	N	N	N	N	-	N	N	-	-	+	-	-	+
	229	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	+	+
	221	-	+	-	-	N	N	+	N	N	N	N	+	N	N	+	+	+	+	+	+
	261	-	-	-	-	N	N	-	N	N	N	N	-	N	N	-	-	-	-	-	-
Celecoxib	*204	-	-	-	-	N	-	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	*2441	-	-	-	-	N	N	-	-	N	N	N	N	N	N	N	N	N	N	N	N
	*241	-	-	-	-	N	N	+	N	-	N	N	N	N	N	N	N	N	N	N	N
	*196	-	-	-	-	N	N	+	N	N	N	N	-	+	N	N	N	N	N	N	N
	265	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	-	+
	215	-	-	-	-	N	N	+	N	N	N	N	+	N	N	+	+	+	+	+	+
	247	-	-	-	-	N	N	-	N	N	N	N	-	N	N	+	+	+	+	+	+
	297	-	-	-	-	N	N	+	N	N	N	N	+	N	N	+	+	+	+	+	+
	279	-	-	-	-	N	N	-	N	N	N	N	+	N	N	+	+	+	+	+	+

Table 4. Gross necropsy results of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib. For heart: (-) no dilatation; (+) dilatation. For crop and proventriculus: (-) no dilatation; (+) mild dilatation; (++) moderate dilatation; (+++) severe dilatation. Bird ID proceeded with (*) euthanized prior to the end of treatment.

Treatment	Bird ID	Heart dilatation	Crop dilataion	Proventriculus dilatation
Control	*237	-	-	-
	*302	-	+++	+++
	236	+	-	-
	239	-	-	-
	791	+	-	+
	9491	+	-	+
	92	-	-	-
	2820	+	-	-
	1460	+	-	+
	Meloxicam	*10	-	++
*2725		-	+++	+++
1662		+	+	+
254		+	+	+
691		-	-	-
210		+	-	++
229		-	+	+
221		-	+	+
261		-	-	+
Celecoxib		*204	-	+++
	*2441	-	-	++
	*241	-	-	+
	*196	-	+++	+
	265	+	-	-
	215	+	-	+
	247	+	-	+
	297	+	-	-
	279	+	-	+

Table 5. Histopathological lesions of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib. Distribution of histopathologic lesions attributable to PaBV infection. Presence (+) or absence (-) of lesions. No statistical difference between groups. P<0.05, Kruskal-Walli One Way Analysis of Variance on Ranks. (N) tissue not examined histopathologically; (*) euthanized prior to the end of study.

Treatment	Bird ID	Brain	Spinal cord	Heart	Liver	Intestine	Crop	Proventriculus	Ventriculus	Pancreas	Spleen	Kidney	Lung	Optic nerve	Adrenal
Control	*237	-	+	-	-	-	-	-	-	-	-	+	-	N	N
	*302	-	-	-	+	+	+	+	+	-	-	+	-	-	N
	236	-	-	+	-	+	+	+	+	+	+	+	+	-	N
	239	+	-	+	-	+	-	+	+	-	+	+	-	-	N
	791	-	-	+	-	+	-	+	+	-	-	+	-	-	N
	9491	+	+	+	-	-	+	+	+	-	+	+	-	-	+
	92	+	+	+	+	-	+	+	+	-	N	+	+	+	N
	2820	-	-	+	-	+	-	+	+	-	N	+	-	-	N
	1460	+	-	+	+	+	+	+	+	+	+	+	-	-	N
Meloxicam	*10	-	-	-	+	-	-	+	-	-	-	+	-	-	N
	*2725	+	+	+	+	+	+	+	+	-	N	+	+	-	N
	1662	-	-	-	-	+	+	+	+	-	N	+	+	-	N
	254	-	-	+	+	+	-	+	+	+	-	+	-	-	+
	691	-	-	-	+	-	-	-	-	-	+	+	-	+	+
	210	+	+	+	-	-	-	+	+	-	-	+	+	+	+
	229	-	+	-	+	-	-	+	+	-	-	-	-	-	+
	221	+	+	+	-	+	+	+	+	-	-	+	+	+	+
261	+	-	-	+	-	-	+	+	-	-	+	-	-	N	
Celecoxib	*204	+	+	+	+	+	+	+	+	N	N	+	-	+	+
	*2441	+	-	+	+	+	+	+	+	-	-	+	-	N	N
	*241	+	+	+	-	+	+	+	+	-	-	+	-	N	N
	*196	+	+	+	+	+	+	+	+	+	N	+	N	+	N
	265	+	-	+	-	+	-	+	+	-	+	+	-	-	+
	215	-	-	+	+	+	+	+	+	-	+	+	+	-	+
	247	+	-	+	-	+	+	+	+	-	+	+	-	-	N
	297	-	-	+	-	-	-	-	-	-	-	+	-	N	-
	279	-	-	-	+	-	-	-	-	-	+	+	-	N	N
p-value	0.565	N	0.236	0.343	0.147	0.368	0.538	0.538	N	N	0.343	0.754	N	N	

Table 6. IHC detection of PaBV-2 in tissues of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib. Tissue distribution of PaBV-2 N-protein antigen by IHC. Immunohistochemistry staining scores: (-) no PaBV-2 N-protein antigen detection; (+) mild PaBV-2 N-protein antigen detection; (++) moderate PaBV-2 N-protein antigen detection; (+++) severe PaBV-2 N-protein antigen detection; (N) tissue not examined; (*) euthanized prior to the end of treatment.

Treatment	Bird ID	Brain	Heart	Liver	Intestine	Crop	Proventriculus	Ventriculus	Pancreas	Kidney	Lung
Control	*237	-	N	N	N	N	-	N	N	N	N
	*302	+++	N	N	N	N	-	N	N	N	N
	236	+++	++	-	+++	++	+++	+++	+++	++	+
	239	+++	N	N	++	++	+++	+++	+++	++	N
	791	++	N	N	++	++	++	++	N	N	+
	9491	+++	+++	N	++	++	++	++	-	+	N
	92	+++	+++	+	++	++	++	++	+	+++	+
	2820	+	+	-	++	++	+++	+++	+++	++	+
	1460	+++	++	+	+++	+	+++	+++	+++	-	+
	*10	-	N	N	N	N	-	N	N	N	N
Meloxicam	*2725	+++	N	N	N	N	-	N	N	N	N
	1662	++	N	-	++	+	++	++	N	N	-
	254	++	N	-	+++	+	++	++	++	++	-
	691	+++	++	++	++	+	++	++	++	+++	++
	210	+++	N	N	-	-	+	+	-	N	N
	229	+++	N	N	++	+	++	++	-	-	N
	221	+++	++	++	++	+	++	++	N	++	++
	261	-	N	N	-	-	-	-	-	N	N
Celecoxib	*204	+	N	N	N	N	-	N	N	N	N
	*2441	-	N	N	N	N	-	N	N	N	N
	*241	++	N	N	N	N	-	N	N	N	N
	*196	+++	N	N	N	N	++	N	N	N	N
	265	+++	N	-	++	+	++	++	++	+++	+
	215	++	N	++	++	++	+++	+++	++	+++	+
	247	+++	++	N	++	++	+++	+++	++	++	++
	297	+++	N	N	++	++	++	++	N	N	++
279	+++	N	++	++	+	+	+	++	+++	N	

Table 7. Viral mRNA detected at necropsy of cockatiels inoculated with PaBV-2 and treated with placebo, meloxicam, or celecoxib. PaBV matrix and phosphoprotein mRNA reverse transcription polymerase chain reaction (RT-PCR) on samples collected at necropsy. (-) No viral mRNA detected; (+) viral mRNA detected; (N) sample not examined or no sample collected; (*) euthanized prior to the end of treatment.

Treatment	Bird ID	Midbrain	Hindbrain	Cerebellum	Forebrain	Kidney	Urine	Cloacal swab
Control	*237	-	-	-	-	-	-	-
	*302	+	+	+	+	+	N	+
	236	+	+	+	+	+	+	+
	239	+	+	+	+	+	+	+
	791	+	+	+	+	+	+	+
	9491	+	+	+	+	+	+	+
	92	+	+	+	+	+	+	+
	2820	+	+	+	+	+	+	+
	1460	+	+	+	+	+	+	+
Meloxicam	*10	-	-	-	-	-	N	-
	*2725	+	+	+	+	+	N	-
	1662	+	+	+	+	+	+	+
	254	+	+	+	+	+	+	+
	691	+	+	+	+	+	N	+
	210	+	+	+	+	+	N	+
	229	+	+	+	+	+	+	+
	221	+	+	+	+	+	+	+
	261	-	-	-	-	-	-	-
Celecoxib	*204	+	+	+	+	-	N	-
	*2441	-	+	-	+	-	+	-
	*241	+	+	+	+	+	N	-
	*196	+	+	+	+	+	N	+
	265	+	+	+	+	+	N	+
	215	+	+	+	+	+	+	+
	247	+	+	+	+	+	+	+
	297	+	+	+	+	+	+	+
	279	+	+	+	+	+	N	+

CHAPTER V

DISCUSSION AND CONCLUSION

Oral administration of meloxicam, 1.0 mg/kg, and celecoxib, 10.0 mg/kg, once daily for 150 (± 2) days yielded no difference in the clinical presentation, viral shedding, gross lesions, viral distribution, nor histopathology in cockatiels experimentally inoculated with PaBV-2 compared with birds not treated with a NSAID. Our results are in agreement with some reports,¹³ but conflicts with reports on the treatment of birds with clinical PDD.^{14,16} Differences in the species of birds treated, cockatiels experimentally inoculated with PaBV versus birds with PDD, may account for these disparate outcomes. Nevertheless, cockatiels experimentally inoculated with PaBV, whether or not treated with meloxicam and celecoxib, develop characteristics of PDD.

In our study, black intestinal contents and pathological lesions consistent with PDD were observed in birds treated with meloxicam or celecoxib. In the studies of Hoppes et al., cockatiels inoculated with PaBV and treated with meloxicam exhibited gastrointestinal hemorrhage, while birds not infected with PaBV nor treated with meloxicam had no evidence of similar hemorrhage.¹³ In mammals, gastrointestinal abnormalities, such as ulcers and bleeding, and renal necrosis due to ischemia are major aspects of NSAID toxicity; however, most studies of NSAIDs in normal birds failed to report toxic effects.^{78,81,86,88-91,94,95,96} Cockatiels inoculated with PaBV and treated with NSAIDs may be at an increased risk for gastrointestinal abnormalities. A possible explanation of gastrointestinal bleeding and dark intestinal contents (presumably blood)

is that the administration of NSAIDs to a bird with gastrointestinal dysfunction, such as found in infections with PaBV, could exacerbate the potential for gastrointestinal irritation and toxicity. The birds in our study that had black intestinal contents had gross and histopathological lesions of the proventriculus and intestines consistent with PDD.

The administration of NSAID to birds infected with PaBV did not alter weight loss or BCS. Birds that displayed severe signs of PDD or infection with PaBV, and were removed early or died, had decreased weight and BCS. Weight loss and poor BCS are commonly reported in the literature and is consistent with the gastrointestinal dysfunction that leads to starvation of birds.^{3,10,13,20} While most birds infected with PaBV decrease in weight, one bird in our study, number 2725, gained weight at the time of early removal from the study. Upon necropsy, the crop and proventriculus were distended with feed; this was most likely based on the gastrointestinal dysfunction caused by PaBV infection. Weight should not be used as the sole parameter to assess the progression of infection or disease, as the bird in question additionally had a decreased BCS, consistent with starvation but illness marked by enlarged crop, was lethargic and regurgitating prior to necropsy. (Table 2 and Figure 5B).

Neither survival nor early removal from the study were different between the groups; however, the number of birds with clinical state warranting early removal was twice as high in the celecoxib group as compared with the control and meloxicam groups (Figure 2). However, this difference was not statistically significant, sample sizes were small, and this is the first study on celecoxib treatment of birds infected with PaBV. Even though the NSAIDs had no effect on mortality, the early removal of cockatiels

occurred between 33-79 days, consistent to the time period seen in other birds experimentally inoculated with PaBV.^{3, 10, 11, 13, 20, 21} Thirty to one hundred days is the common time period when birds experimentally inoculated with virus begin to develop clinical disease; however, Piepenbring et al. showed that clinical signs may take over 150 days to occur in some cockatiels.¹¹ These time frame differences in the appearance of clinical signs may be based on variations in viral inoculum, viral distribution, and the birds' immune response.

Gross findings showed no significant difference between birds in the three treatment groups. All groups had clinical presentations consistent with the progression of PDD described in literature.^{3,10,11,13,20,21} Clinical presentations most commonly seen were dilation of proventriculus, dilated crop, and undigested seed within the intestines (Figure 5). These changes are a result of decrease in motility caused by the lymphocytic infiltration against the viral infection.^{2,3,6,9,32,52}

Contrary to our hypothesis, NSAIDs treatments in cockatiels inoculated with PaBV-2 did not significantly decrease lymphoplasmacytic distribution and severity. Histopathology showed similar lymphoplasmacytic infiltration among the three groups despite NSAID treatment, related to PaBV inoculation manifesting as PDD.^{3,10,11,13,21,52} Thus, the administration of meloxicam and celecoxib were not effective in reducing the inflammatory response seen in experimentally PaBV-2 inoculated cockatiels.

No difference was seen in the shedding of PaBV as determined by mRNA detection on cloacal swab samples. Viral shedding was first detected on day 42 post-inoculation and became more consistent on day 63 post-inoculation (Table 3). Five of

the eight birds that were euthanized or died early did not have mRNA detected in the cloaca at necropsy. Even though clinical signs are accompanied by histopathological changes in the brain of infected animals¹⁰¹ and these eight birds had histopathological lesions in the kidneys, virus may not be detected in the cloaca. The reasons for this are uncertain. Pathological changes may occur prior to virus levels in the tissue being at detectable levels. Though all eight birds had histopathological lesion in the kidney, four birds did not have viral mRNA detected in the kidney. Thus virus may not have been shed into the urine and secondarily being in the cloaca; these four birds were also negative for mRNA on cloaca swabs. An additional possibility is that the amount of virus in the cloaca, and collected on the cloacal swab, may have been lower than the limit of detection for the RT-PCR. For one of the five birds negative on the cloacal swab, viral mRNA was detected in the kidney. It would have been useful to have done an IHC assay for PaBV on the kidneys of these eight birds. Unfortunately, IHC was done only on the brain and proventriculus of the birds that were removed early from the study.

By day 84 post-inoculation all but two surviving birds were shedding virus and at the end of the study, all the surviving birds, except for one, were shedding virus. In our study, detection of PaBV on cloacal swabs was inconsistent in 3 of 19 birds (Table 3), which was similar to the intermittent shedding of the virus as noted by other investigators^{20,23,52,56} As stated earlier, the question arises whether or not this is intermittent shedding or a factor of low levels of the virus present at collection. Birds may be PCR-negative on fecal swabs and PCR-positive for PaBV in urine.⁵⁶ Collecting multiple samples per bird during the day and pooling them for testing alleviate the

negative cloacal results but pooling of five uro-fecal samples per day for 5 consecutive days but may still result in inconsistent PCR findings.⁵²

One bird, number 261, was negative for the presence of PaBV by all tests throughout the study; however at necropsy, the proventriculus was dilated and contained undigested seed. Histopathology showed perivascular cuffing with increased glial cells in the brain and lymph nodules in the mucosa, ganglia, and serosa of the ventriculus of this bird. *Cryptosporidium* identified in the proventriculus may have contributed to the dilation and histopathological lesions in the ventriculus.¹⁰²

The administration of NSAIDs did not affect distribution or viral load of the tissues. No differences between treated and untreated birds were expected as NSAIDs are anti-inflammatory in action but not antiviral. Virus was detected most often in nervous, gastrointestinal, heart and kidney tissues but was also found in many other organs: lungs, mucosa of the trachea, eye, liver, gallbladder, skin, feather follicles, oviduct, pancreas, and gonads. Parrot bornavirus is neurotropic but also has a broad tissue tropism, which does not always correlate with degeneration, necrosis, and/or inflammation of these tissues^{11,20,33,51,52,54,56}

Conclusion

The administration of meloxicam or celecoxib did not alter the progression or severity of clinical signs, pathological changes, viral shedding, or viral RNA distribution in cockatiels experimentally infected with PaBV-2. There is therefore no justification for continuing the practice of administering NSAIDs to birds with clinical PDD. Since

NSAID treatment of birds may have a synergistic effect on gastrointestinal dysfunction resulting in toxicity and undesirable side effects such as gastrointestinal irritation and bleeding, this is an entirely inappropriate treatment modality for PDD cases. It may be argued that NSAIDs, while totally ineffective in treating cockatiels, may be of benefit in treating other parrot species. This is highly unlikely. Unless proven otherwise NSAID use in PDD cases should be discontinued immediately.

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APPENDIX A

		Weights																																		
		Days of inoculation																																		
Bird ID		-31	6	19	26	33	37	41	43	45	48	54	55	61	63	69	74	76	79	84	90	97	104	111	118	125	132	139	146	153	160	168	173±2			
Control	*237	102	104	108	101	100		101			98.5	95																								
	*302	100	89.5	95	89	89		87			86.5		91.5	74.8																						
	236	115	113	118	116	107		104			107.5		100.5		102	105.5		109			110	110	112.5	112	111	110	108.5	108.5	107.5	105.5	105	103	107	102		
	239	81	78	79	76.5	75		68.5			69.5		70.5		71	72		75			77.5	78	77.5	77	77	81	85	85	84.5	86	83	82.5	83	80.5		
	791	127	117	123	120	95		94.5			102.5		106		107	108.5		108.5			109	115	119	122	122	122	122.5	122.5	124.5	124	112.4	122	128	121.2		
	9491	91	94.5	98	94	94		93.5			96		94		93	92.5		95			99	99.5	99	98.5	99	100	98	97.5	97	96	98.5	95.5	97.5	99.2		
	92	96	88	90.5	87	85		85			83.5		82		86.5	84		84			85	84	85	85	85	86.5	84.5	85	86.5	83.5	84.5	82	83	86		
	2820	83	91	90	85	89		88			86		84.5		84	85		86.5			86.5	85.5	86.5	84	84	89	89	89.5	88.5	87	87	87	85	87		
	1460	96	94.5	100	98.5	95		94.5			90		82		92	96.5		100.5			102.5	103	104.5	102	102	104	105	105.5	101	106	105.5	103.5	103.5	93.9		
Meloxicam	*10	88	91.5	94	86	76																														
	*2725	114	112	119	104	93		96			92.5		94.5		107.5	112		121	117.6																	
	1662	82	83	88.5	77	74		72			78		78.5		79.5	79.5		82			84	85	87.5	87.5	88.5	88.5	89.5	88.5	88.5	89	89.5	87	90	93.6		
	254	98	93.5	95	88.5	84		87.5			86.5		87		93.5	97.5		97.5			98.5	101	99	104	104	102.5	102	94	86	84	87.5	93	100	98.5		
	691	100	108	112	106	98		100			95		101.5		105	104		102.5			103.5	102	103	101	103	105	103.5	103.5	106	105.5	105.5	109.5	112.5	106.5		
	210	109	93	105	94.5	94		97			95		90.5		90.5	89		91			95	95.5	97.5	96	98.5	100	93	90	96	91	93	92	92	85		
	229	112	107	107	103	102		92			94.5		93.5		94	91		96			97.5	96	98	94.5	97.5	98.5	98.5	98.5	101	100.5	102	103	104	101		
	221	98	91	98	90	84		92			90		89.5		91	93		92.5			94	94	94.5	91.5	91.5	92	93	92	93.5	94	94	95	97.5	94.6		
	261	125	107	112	100	80		85			87.5		90		87	88.5		90			91	90	91	91.5	95.5	97	99	99.5	100.5	102	105.5	110	112	107.5		
Celecoxib	*204	122	111	121	116	92	97																													
	*241	86	85	84	83.5	69		72	74.5																											
	*2441	105	97	101	97	79		89.5	83																											
	*196	79	76.5	78.5	75.5	75		72			69.5		69		71	70	63.6																			
	265	145	124	134	132	124		105			109		110		108	106		103.5			105	106	107.5	108.5	109	110	112.5	109.5	109.5	111	112.5	112	112.5	115		
	297	91	92	92	90	87		86.5			88.5		83.5		85	86		90			92	92	93.5	90	90	89	88	88.5	89	88.5	89	88.5	90	85.9		
	215	100	93.5	92	88.5	89		95			93		93		96	98		96			98	97.5	99	100	103	102	103.5	106.5	106.5	108.5	103.5	98.5	97	93.8		
	247	104	96	99	95	91		93			95		91.5		93.5	95		94			96.5	97.5	96.5	94	95	95	95	96	94	93.5	93	94.5	94.5	93.5		
279	91	92	95	92	85		90			90		91		96	95		93			96	96	95.5	94	96.5	95	96.5	98	97	98	98	96	96.5	95			

(g) grams (*) euthanized prior to the end of study

Body condition score

		Days of inoculation																														
Bird ID		-31	6	33	33	37	41	43	45	48	54	55	61	63	69	74	76	79	84	90	97	111	118	125	132	139	146	153	160	168	173±2	
Control	*237	3	3	4			3			4	3																					
	*302	3	3	3			3			3		3	2																			
	236	3	3	3			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	4	
	239	2	2	2			2			2		2		2	2		2		2	2	2	2	2	2	3	3	2	2	2	2	3	
	791	4	3	3			3			4		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	4	
	9491	2	3	3			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	2	
	92	3	3	3			3			3		3		3	3		3		3	3	3	2	3	2	3	3	3	3	3	3	4	
	2820	1	2	2			2			2		2		2	2		2		2	3	2	2	2	3	2	2	2		2	2	3	
	1460	2	3	3			4			3		2		2	3		3		3	3	3	3	3	3	3	3	3	3	3	3	3	
Meloxicam	*10	2	3	1	1																											
	*2725	3	3	2			3			3		3		3	2		2	2														
	1662	3	2	3			2			3				2	3				3	3	3	3	3	3	3	3	3	3	3	3	4	
	254	3	3	3			3			3		2		3	3		3		3	3	3	3	3	3	3	2	2	2	2	2	3	
	691	2	3	3			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	4	
	210	4	2	3			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	2	
	229	3	3	3			2			3		2		3	2		3		3	3	3	3	3	3	3	3	3	3	3	3	4	
	221	2	2	2			2			3		2		3	3		3		2	3	3	2	3	3	2	2	2	3	2	2	3	
	261	4	3	2			2			3		3		3	3		3		3	3	3	3	3		3	3	3	3	3	3	3	
Celecoxib	*204	4	3	2		2																										
	*241	3	2	2			2		2																							
	*2441	3	3	3			3	3																								
	*196	2	2	2			2			2		2		2	2	1																
	265	3	5	4			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	4	
	297	3	3	3			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	3	
	215	3	2	3			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	4	
	247	3	3	3			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	3	
	279	3	3	3			3			3		3		3	3		3		3	3	3	3	3	3	3	3	3	3	3	3	4	

(*) euthanized prior to the end of study

APPENDIX B

Schubot Necropsy Report

*TAMU 237- *Nymphicus hollandicus*

Necropsy done on 14 May 2014 by Paulina Escandon at 11:15AM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group

1. Bird found off balance; possible neurologic symptoms. Bird was euthanized by Dr.

Heatley at 11:00AM. Blood was collected.

- Necropsy Report: Weight- ~95
- Air sacs enlarged
- Body condition score- 3 of 5
- Crop- normal
- PV- normal
- V - normal
- Intestines- normal
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney- normal
- Heart- normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testes (male- 1 undeveloped testis)
- Brain- visible blood surrounding brain
- Spinal cord- normal
- Eye- normal

Schubot Necropsy Report
*RAATX 302- *Nymphicus hollandicus*

Necropsy done on 21 May 2014 by Paulina Escandon at 11:15AM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 1. Bird found experiencing regurgitation with a distended crop. Bird was euthanized by Paulina. Bird regurgitated during euthanasia. Blood was collected.

Necropsy Report:

- Air sacs clear
- Body condition score- 2 of 5
- Crop- Enlarged
- PV-slightly distended.
- V - abnormal
- Intestines- discoloration
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney- normal
- Heart- normal
- Lung- discoloration
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- discoloration
- Gonads- testes (male- 1 undeveloped testis)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 74.8g

Schubot Necropsy Report

TAMU 236- *Nymphicus hollandicus*

Necropsy done on 12 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 1 . End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- normal
- Excellent fat stores
- Body condition score- 3 of 5
- Crop-normal
- PV- normal
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-normal
- Spleen- enlarged
- Kidney-normal
- Heart-1.2g enlarged
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-102g

Schubot Necropsy Report

TAMU239- *Nymphicus hollandicus*

Necropsy done on 12 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group

1. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- normal
- Excellent fat stores
- Body condition score- 3 of 5
- Crop-normal
- PV- normal
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-normal
- Spleen- normal
- Kidney-normal
- Heart-0.6g
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testis (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-80.5 g

Schubot Necropsy Report

RAATX 791- *Nymphicus hollandicus*

Necropsy done on 12 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group

1. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- normal
- Body condition score- 4 of 5
- Crop-normal
- PV- slightly dilated
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-normal
- Spleen- slightly enlarged
- Kidney-normal
- Heart-1.2g enlarged
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-121.2 g

Schubot Necropsy Report

MRTFL 9491- *Nymphicus hollandicus*

Necropsy done on 12 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 1. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- normal
- Body condition score- 3 of 5
- Crop-normal full of seed
- PV- slightly dilated
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-normal
- Spleen- slightly enlarged
- Kidney-normal
- Heart-1.1g enlarged
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-99.2 g

Schubot Necropsy Report

RAATX 92- *Nymphicus hollandicus*

Necropsy done on 12 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group

1. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- normal
- Excellent fat stores
- Keel score- 3
- Crop-normal
- PV- normal
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-mottling, blood vessels presently visible
- Spleen- enlarged
- Kidney-normal
- Heart-0.8g
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testis (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-86g

Schubot Necropsy Report

MRTFL 2820- *Nymphicus hollandicus*

Necropsy done on 12 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group

1. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- normal
- Keel score- 2
- Crop-normal
- PV- normal
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-normal
- Spleen- normal
- Kidney-normal
- Heart-0.9g enlarged
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female) active follicle
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-87 g

Schubot Necropsy Report

MRTFL 1460- *Nymphicus hollandicus*

Necropsy done on 12 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group

1. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- normal
- Excellent fat stores
- Keel score- 3
- Crop-normal
- PV- normal
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-normal
- Spleen- enlarged
- Kidney-normal
- Heart-1.1g
- Lung- normal enlarged
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-93

Schubot Necropsy Report

RAATX 10- *Nymphicus hollandicus*

Necropsy done on 24 April 2014 by Dr. J. Jill Heatley at 2:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group

2. Bird was found at 9:00AM on 24 April 2014 with a drooping right wing. Bird was later found dead at 2:00PM.

Necropsy Report:

- Air sacs clear
- Free fluid in left coelom; fluid was swabbed
- At time of necropsy, distinct swelling was noted in the right wing
- Keel score- 1
- Crop- distended
- PV- discolored; Slightly dilated
- V - normal
- Intestines- blackened
- Cloaca- normal
- Adrenals- normal
- Liver- small white mass on liver
- Spleen- normal
- Kidney- pale with articulate pattern
- Heart- mottling
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- very white/pale in color
- Gonads- testes (young male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-76.0g

Schubot Necropsy Report

MRTFL 2725- *Nymphicus hollandicus*

Necropsy done on 08 June 2014 by Paulina Escandon at 9:30AM

History: Bird was previously inoculated with Parrot bornavirus strain M24 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. Bird found with visible regurgitation, enlarged crop, and puffed up with head low/tilted at 9:00AM on the bottom of the cage. Also wobbly body and lethargic. Bird was euthanized by Paulina at 9:15AM. Blood was collected.

Necropsy Report:

- Air sacs clear
- Dirty vent
- Body condition score- low 2
- Crop- enlarged; filled with seed
- PV- enlarged
- V- normal
- Intestines- black portions; visible seeds
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney- enlarged
- Heart- normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 117.6g

Schubot Necropsy Report

MRTFL 1662- *Nymphicus hollandicus*

Necropsy done on 09 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Body condition score- 4
- Crop-mild distended
- PV- normal
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-pale mottling
- Spleen- normal
- Kidney-normal
- Heart-0.8g 1x1.6cm enlarged
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testis (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-93.6g

Schubot Necropsy Report

TAMU254- *Nymphicus hollandicus*

Necropsy done on 09 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Body condition score- 3 of 5
- Crop-mild distended
- PV- dilated
- V- normal
- Intestines-mild distended
- Cloaca- normal
- Adrenals- normal
- Liver-Green mottled
- Spleen- normal
- Kidney-normal
- Heart- dilated 1.58g slightly enlarge
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- not recorded
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 98.5g

Schubot Necropsy Report

CWI 691- *Nymphicus hollandicus*

Necropsy done on 09 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Body condition score- 4 of 5
- Crop-normal
- PV- normal
- V- normal
- Intestines-pale GI tract
- Cloaca- normal
- Adrenals- normal
- Liver-slight mottling
- Spleen- normal
- Kidney-normal
- Heart-0.7g normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testis (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-106.5g

Schubot Necropsy Report

RAATX 210- *Nymphicus hollandicus*

Necropsy done on 09 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus strain-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Dirty vent
- Body condition score- 2
- Crop- undigested seed
- PV- dilated, undigested seed
- V- undigested seed
- Intestines- undigested seed
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney-normal
- Heart- dilated 1.2x1.7cm slightly enlarge
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testis (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 85g

Schubot Necropsy Report

TAMU229- *Nymphicus hollandicus*

Necropsy done on 09 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Body condition score- 4 of 5
- Crop-mild distended with seed
- PV- mild dilated
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-pale mottling
- Spleen- normal
- Kidney-normal
- Heart- normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (Female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-101g

Schubot Necropsy Report

TAMU221- *Nymphicus hollandicus*

Necropsy done on 09 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- not clear
- Body condition score- 3
- Crop-mild distention with undigested seed
- PV- mild dilation
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-normal
- Spleen- normal
- Kidney-normal
- Heart-0.7g normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testis (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-94.6g

Schubot Necropsy Report

TAMU261- *Nymphicus hollandicus*

Necropsy done on 09 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs- not clear
- Body condition score- 3 of 5
- Crop-normal
- PV- mild dilation with seed
- V- normal
- Intestines-normal
- Cloaca- normal
- Adrenals- normal
- Liver-pale yellow modeling suspect hepatic lipidosis
- Spleen- normal
- Kidney-normal
- Heart-0.5g normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight-107.5g

Schubot Necropsy Report

*TAMU 204- *Nymphicus hollandicus*

Necropsy done on 28 April 2014 by Dr. J. Jill Heatley at 9:00AM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group

3. Bird was found dead at 8:30AM on the bottom of the cage.

Necropsy Report:

- Air sacs clear
- BCS- 2 of 5
- Crop- distended and attached to pectoral muscle
- PV- enlarged; black surrounding area
- V - normal
- Intestines- blackened
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney- mottled
- Heart- normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (inactive female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 97.0g

Schubot Necropsy Report

*MRTFL 2441- *Nymphicus hollandicus*

Necropsy done on 03 May 2014 by Jeann Leal and Paulina Escandon at 11:15AM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. Bird found perching but extremely lethargic with head tilted, body wobbly, and eyes closed. Bird was euthanized by Dr. Heatley at 11:00AM. Blood was collected from right jugular.

Necropsy Report:

- Air sacs clear
- Body condition score- 3 of 5
- Crop- normal; digestion visible
- PV- slightly dilated (pale)
- V - normal
- Intestines- normal
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney- normal
- Heart- normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- not identified
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 83.0g

Schubot Necropsy Report

*TAMU 241- *Nymphicus hollandicus*

Necropsy done on 05 May 2014 by Paulina Escandon

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 3. Bird found perching but extremely lethargic with head tilted, body wobbly, and eyes closed. Bird was euthanized by Paulina. Blood was collected.

Necropsy Report:

- Air sacs clear
- Body condition score- 2 of 3
- Crop- normal
- PV- slightly enlarged
- V - normal
- Intestines- great amount of undigested seed
- Cloaca- normal
- Adrenals- normal
- Liver- multifocal yellow areas on surface
- Spleen- normal
- Kidney- normal
- Heart- normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- pale
- Gonads- testes (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 74.5g

Schubot Necropsy Report

*CWI 196- *Nymphicus hollandicus*

Necropsy done on 03 June 2014 by Paulina Escandon

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 2. Bird found lethargic, wobbly body, head tilted down, and eyes closed. Bird had visible enlarged crop and feces was noted to be dark in color. Bird was euthanized by Paulina.

Blood was collected.

Necropsy Report:

- Air sacs clear
- Body condition score- 1
- Crop- enlarged
- PV- discoloration
- V- discoloration; black on inside upon opening
- Intestines- black portions
- Cloaca- normal
- Adrenals- normal
- Liver- discoloration
- Spleen- very small in size
- Kidney- normal
- Heart- normal
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- discoloration
- Gonads- testes (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 63.6g

Schubot Necropsy Report

TAMU265- *Nymphicus hollandicus*

Necropsy done on 08 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 3. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Dirty vent
- Keel score- 4
- Crop- normal
- PV- normal
- V- normal
- Intestines- normal
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney-normal
- Heart- 0.9g slightly enlarged
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 115g

Schubot Necropsy Report

TAMU215- *Nymphicus hollandicus*

Necropsy done on 08 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 3. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Body condition score- 4 of 5
- Crop- normal
- PV- normal
- V- Contents was bright green
- Intestines- normal
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney-normal
- Heart- dilated 0.4g slightly enlarge
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 93.8g

Schubot Necropsy Report

TAMU247- *Nymphicus hollandicus*

Necropsy done on 08 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 3. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Body condition score- 3 of 5
- Crop- normal
- PV- normal
- V- normal
- Intestines- normal
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney-normal
- Heart- dilated 1.58g slightly enlarge *****
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- immature ovaries (female)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 93.5g

Schubot Necropsy Report

TAMU297- *Nymphicus hollandicus*

Necropsy done on 08 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 3. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Dirty vent
- Body condition score- 3 of 5
- Crop- normal
- PV- normal
- V- normal
- Intestines- normal
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney-normal
- Heart- 0.6g slightly enlarge
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testis (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 85.9g

Schubot Necropsy Report

TAMU279- *Nymphicus hollandicus*

Necropsy done on 08 September 2014 by Dr. Gentry at 1:30PM

History: Bird was previously inoculated with Parrot bornavirus-2 and was part of the Meloxicam/Celecoxib study; housed in isolation building 1044 in BSL2 room 6, Group 3. End of study necropsy performed. Blood and urine was collected prior to euthanasia.

Necropsy Report:

- Air sacs clear
- Dirty vent
- Body condition score- 4 of 5
- Crop- normal
- PV- Moderate amount of fluid
- V- moderate amount of fluids
- Intestines- normal
- Cloaca- normal
- Adrenals- normal
- Liver- normal
- Spleen- normal
- Kidney-normal
- Heart- 0.8g slightly enlarged
- Lung- normal
- Sciatic nerve- normal
- Brach. plex.- normal
- Pancreas- normal
- Gonads- testis (male)
- Brain- normal
- Spinal cord- normal
- Eye- normal
- Weight- 95g

APPENDIX C

Immunohistochemistry

Bird #	Brain	Prov/Ventr	Intestine	Crop/Eso	Others (+)	Others (-)
A-MRTFL 1662 (Mel)	++	++	++	+		Liver, Lung:
B-TAMU 254 (Mel)	++	++	+++	+	Adrenal, Kidney, Pancreas. ++	Liver, lung, Spl.
C-TAMU 229 (Mel)	+++	++	++	+	Adrenal ++, ovary (stroma) +	Pancr, Spl, Kid, oviduct
D-RAATX 210 (Mel)	+++	+	-	-	Eye: ON, Ret ++	Skin, Pancr, Adr, Testis
E-TAMU 261 (Mel)	-	-	-	-		Pancr, skin
F-MRTFL 9491 (Control)	+++	++	++	++	Eye: ON, Ret, Uvea +++, Heart +++, Kid, ovary, oviduct +	Tr, Pancr, Skel Mus
G-RAATX 92 (Control)	+++	++	++	++	Heart, Kid +++, Liv, Lung, Pancr +	Skel Mus
H-CWI 691 (Mel)	+++	++	++	+	Kid +++, Heart, Pancr, Lung, Liv ++	
I-TAMU 221 (Mel)	+++	++	++	+	Kid, Heart, Lung, Liver ++	
J-TAMU 236 (Control)	+++	+++	+++	++	Pancr, Adr, +++, Kid, Ht, ++, Lg, Parathyr, Aorta, Spl (LN), Ovary (stroma) +	Liver, Trach, Skel muscle, thyroid
K-MRTFL 1460 (Control)	+++	+++	+++	+	Pancr, +++, Ht, oviduct ++, Liv, Lg, Spl +	Kid, Skel muscle
L-TAMU 239 (Control)	+++	+++	++	++	PN, Testis, +, Kid, Skn, Cl, FF, Epid ++, Pancr +++	
M-MRTFL 2820 (Control)	+	+++	++	++	Lg, Ht, Trch (muc) +, Kid, Oviduct ++, Pancr +	Liver
N-RAATX 791 (Control)	++	++ (SM)	++	++	Lg, +, Ov (Str, Fol) ++, Adr +	Trach, Skel Musc
O-TAMU 265 (Cel)	+++	++	++	+	Adr, Lg, Ovary (Str), Ovid (Muc), Clo +, Panc ++, Kid +	Liv, Skel Musc.
P-TAMU 215 (Cel)	++	+++	++	++	Lg, +, Liv, Panca, Sk (FF) ++, Kid +	
Q-TAMU 247 (Cel)	+++	+++	++	++	Ht, Lg, Kid, Panc ++	
R-TAMU 297 (Cel)	+++	++	++	++	Uropyg, Clo + Lg, Adr ++	
S-TAMU 279 (Cel)	+++	+	++	+	Liv, GB, Pancr ++, Kid +++	

IHC staining score for ABV, T1402503: - negative, + mild, ++ moderate, +++ severe

Histopathology



California Animal Health & Food Safety Laboratory System

18830 Road 112
Tulare, CA 93274-9042
(559) 688-7543

Preliminary
Version 1
*This report supersedes all
previous reports for this case*

CAHFS Case #: T1402232
Referral #: 2014-11211-SPED
Date Collected: 04/24/2014
Date Received: 08/20/2014
Case Coordinator: H. L. Shivaprasad
BVSc, PhD
Preliminary Report Sent By:
Shivaprasad, H.L. on 9/3/2014 3:04:03PM

Email To:
TIZARD, IAN
itizard@cvm.tamu.edu

Collection Site:
SCHUBOT CENTER
VET 4467 TAMU
COLLEGE STATION TX
77843
County: BRAZOS

Specimens Received: 256 Issues - Fixed;

Comments: Carrier: FedEx

Case Contacts

Bill To	TEXAS A & M UNIVERSITY	979-862-1501	VET PATHOBIOLOGY - ATTN: ACCOUNTS PAYABLE4467 TAMU COLLEGE STATION, TX 77843
Submitter	TIZARD, IAN	979-845-5941	4467 TAMU COLLEGE STATION, TX 77843

Specimen Details

Animal/Source	ID Type	Taxonomy	Gender	Age
T1402232-01	CAHFS Internal ID	Cockatiel		

Laboratory Findings/Diagnosis

See histopathology description

Case Summary

09/03/14: I have completed the histopathology evaluation of all issues from the eight birds. IHC on the brain is pending. In the meantime if you have any questions please give me a call.

Clinical History

Cockatiels inoculated ABV strain and blind study treated with either control, celecoxib or meloxicam.

Gross Observations

Histology

2232-A (Euth - 4/24/14).

Brain, spinal cord, peripheral nerves: NSL;

Gizzard, intestine, crop/esophagus: NSL

Proventriculus has mild infiltration of lymphocytes in the mucosa.

Liver: has foci of eosinophilia characterized by hepatic cords that have hypertrophied hepatocytes.

Kidneys: have mild multifocal dilation of tubules some of which contain cellular debris and eosinophilic material.

Heart, lungs, spleen, pancreas, eye, skeletal muscles, bone, bone marrow, tongue/salivary glands, trachea, are free of significant lesions.

Skin, skeletal muscles and one bone: There is severe fibrosuppurative inflammation associated with bacteria in the subcutis that extends in to the skeletal muscles and bone marrow.

2232-B (Euth - 4/28/14).

Brain: Has multifocal mild to moderate perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum and

medulla oblongata.

Spinal cord: has mild multifocal infiltration of lymphocytes in the cord nerves/ganglia.
Peripheral nerves have similar infiltration.

Gizzard, proventriculus, intestine, crop/esophagus: have minimal or no infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia.
In addition proventriculus has moderate infiltration of lymphocytes in the mucosa and large numbers of megabacteria in the mucosa.

Heart has infiltration of a few lymphocytes in and around the epicardial ganglia.
Adrenal has a few scattered lymphocytes.

Liver: has multifocal hepatic cords that have hypertrophied hepatocytes

Kidneys: have moderate to severe multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens most prominent in the collecting tubules. Other changes such as dropout of tubules, interstitial inflammation and fibrosis, are also noted.

Eye: has diffuse infiltration of lymphocytes in the conjunctiva.

Skeletal muscles, lungs, eye, ovary, oviduct, bone, bone marrow, trachea, skin with feathers are free of significant lesions.

2232-C (Euth - 5/03/14).

Brain: Has multifocal moderate to severe perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum.

Gizzard, proventriculus, intestine, crop/esophagus: have mild to moderate to severe infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia most prominent in the gizzard and minimal in crop and proventriculus.

Crop has multifocal necrosis of epithelium, ulceration and inflammation.

Heart has infiltration of similar cells in the epicardial ganglia and occasionally mild infiltration in and around Purkinje cells.
There is extensive degeneration of myofibers and infiltration of mononuclear cells.

Liver: has mild periportal lymphocytic inflammation, hepatic eosinophilia and locally extensive lipidosis and interstitial fibrosis.

Kidneys: mild multifocal dilation of tubules

Skeletal muscles, pancreas, spleen, lungs, bone, bone marrow, trachea, skin, are free of significant lesions.

2232-D (Euth - 5/05/14)

Brain: Has multifocal mild to moderate perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum.

Spinal cord: has mild infiltration of lymphocytes in the spinal nerves/ganglia One section has malacia of the neuropil.

Gizzard, proventriculus, intestine, crop/esophagus: have mild infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia most prominent in the gizzard and minimal in crop and proventriculus.
Esophagus and proximal proventriculus have moderate lymphocytic and giant cell infiltration.

Heart has degeneration of myofibers with occasionally mild infiltration of mononuclear cells.

Kidneys: have moderate multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens.
Evidence of mild to moderate multifocal interstitial inflammation, and mineralization are also noted.

Skeletal muscles, peripheral nerves, liver, spleen, pancreas, lungs, bone, bone marrow, tongue/salivary glands, trachea, larynx, are free of significant lesions.

2232-E (Euth - 5/14/14)

Spinal cord: one section has a few glial nodules in the grey matter,

Kidneys: have moderate multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens.
Evidence of mild to moderate multifocal interstitial inflammation, fibrosis, loss or regenerating tubules and mineralization are also noted.

Brain, GI tract, heart, skeletal muscles, peripheral nerves, liver, spleen, pancreas, lungs, bone, bone marrow, tongue/salivary glands, trachea, larynx, are free of significant lesions.

2232-F (Euth - 5/21/14)

Kidneys: have moderate multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens. Evidence of mild to severe multifocal interstitial fibrosis especially around the collecting tubules are noted. Subjacent to one such area are irregular dilation of tubules some of which contain debris in their lumens. These tubules appear smaller and hyperchromatic.

Liver has foci of lymphocytes scattered throughout.

Crop, proventriculus, gizzard and intestine and a nerve: have occasional foci of lymphocytic infiltration in the serosal/subserosal ganglia and nerves

Brain, spinal cord, eye, heart, skeletal muscles, lungs, spleen, pancreas, bone, bone marrow, tongue/salivary glands, trachea and larynx, are free of significant lesions.

2232-G (Euth - 6/03/14)

Brain: Has multifocal mild to moderate perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum. Cerebrum also has moderate lipofuscinosis: granular brown pigment in the cytoplasm of neurons scattered here and there.

Spinal cord: has mild infiltration of lymphocytes in the spinal nerves/ganglia

Gizzard, proventriculus, intestine, crop/esophagus: have mild to moderate to severe infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia most prominent in the gizzard and minimal in crop and proventriculus.

Heart has infiltration of similar cells in the epicardial ganglia and occasionally mild infiltration in and around Purkinje cells.

Liver: has mild periportal lymphocytic inflammation.

Pancreas has focal acinar cell hyperplasia.

Kidneys: mild multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens. Evidence of mild to moderate multifocal necrosis (eosinophilia) of tubular epithelium noted. Other changes such as hemosiderosis, interstitial inflammation, mineralization and focal tubular hyperplasia/adenoma are also noted.

Thyroid has accumulation of amorphous basophilic material within the follicles.

Eye: has diffuse infiltration of lymphocytes in the conjunctiva.

Skeletal muscles, bone, bone marrow, tongue/salivary glands, trachea, are free of significant lesions.

2232-H (Euth - 6/08/14)

Brain: Have multifocal mild to severe perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum. Spinal cord: has mild infiltration of lymphocytes in the grey and white matter and occasionally in the spinal nerves/ganglia

Gizzard, proventriculus, intestine, crop/esophagus: have mild to moderate to severe infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia most prominent in the gizzard and minimal in crop and proventriculus. In addition proventriculus has moderate infiltration of lymphocytes in the mucosa associated with megabacteria.

Heart has infiltration of similar cells in the occasional epicardial ganglia and minimal infiltration in and around Purkinje cells.

Liver: has moderate periportal granulocytic and/or lymphocytic inflammation. There is one focus of eosinophilia and multifocal vacuolar changes in hepatocytes.

Kidneys: have mild multifocal dilation of tubules and interstitial inflammation.

Lungs have a few aggregates of mononuclear cells scattered.

Pancreas, skeletal muscles, bone, bone marrow, tongue/salivary glands, trachea, eye, are free of significant lesions.

DECALCIFICATION			
Animal/Source	Specimen	Specimen Type	Results
T1402232-01	A-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	B-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	C-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	D-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	E-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	F-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	G-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	H-decal tissue	Tissue - Fixed	COMPLETED

I m m u n o H i s t o C h e m i s t r y

Avian Bornavirus Immunohistochemistry			
Animal/Source	Specimen	Specimen Type	Results
T1402232-01	Block A-3	Brain Tissue	Pending
T1402232-01	Block B-2	Brain Tissue	Pending
T1402232-01	Block C-1	Brain Tissue	Pending
T1402232-01	Block D-1	Brain Tissue	Pending
T1402232-01	Block E-1	Brain Tissue	Pending
T1402232-01	Block F-1	Brain Tissue	Pending
T1402232-01	Block G-1	Brain Tissue	Pending
T1402232-01	Block H-1	Brain Tissue	Pending



California Animal Health & Food Safety

Laboratory System
18830 Road 112
Tulare, CA 93274-9042
(559) 688-7543

FINAL REPORT

This report supersedes all previous reports for this case

CAHFS Case #: T1402232
Referral #: SC14-TIEL1-8PEB
Date Collected: 04/24/2014
Date Received: 08/20/2014
Case Coordinator: H. L. Shivaprasad, BVSc, PhD
Electronically Signed and Authorized By: Shivaprasad, H.L. on 11/6/2014 4:13:45PM

Email To: TIZARD, IAN
tizard@cvm.tamu.edu

Collection Site: SCHUBOT CENTER
VET 4467 TAMU
COLLEGE STATION TX
77843
County: BRAZOS

Specimens Received: 256 Tissue - Fixed;

Comments: Carrier: FedEx

Case Contacts

Table with 4 columns: Field, Submitter, ID, and Address. Rows include TIZARD, IAN and TEXAS A & M UNIVERSITY.

Specimen Details

Table with 5 columns: Animal/Source, ID Type, Taxonomy, Gender, and Age. Row 1: T1402232-01, CAHFS Internal ID, Cockatiel.

Laboratory Findings/Diagnosis

- 1. See histopathology description.
2. IHC for ABV on the brain and GI tract, see the test results

Case Summary

09/03/14: I have completed the histopathology evaluation of all issues from the eight birds. IHC on the brain is pending. In the meantime if you have any questions please give me a call.

11/06/14: IHC on the brain and GI tract is complete now. Summary on the brain: Groups B (+), D (++) and F, G and H (+++) are positive for ABV. Summary on the GI tract: Only group G (++) is positive for ABV.

This completes all the tests on this case. If you have any questions please give me a call.

Clinical History

Cockatiels inoculated ABV strain and blind study treated with either control, celecoxib or meloxicam.

Gross Observations

H i s t o l o g y

2232-A (Euth - 4/24/14).

Brain, spinal cord, peripheral nerves: NSL:

Gizzard, intestine, crop/esophagus: NSL

Proventriculus has mild infiltration of lymphocytes in the mucosa.

Liver: has foci of eosinophilia characterized by hepatic cords that have hypertrophied hepatocytes.

Kidneys: have mild multifocal dilation of tubules some of which contain cellular debris and eosinophilic material.

Heart, lungs, spleen, pancreas, eye, skeletal muscles, bone, bone marrow, tongue/salivary glands, trachea, are free of significant lesions.

Skin, skeletal muscles and one bone: There is severe fibrous suppurative inflammation associated with bacteria in the subcutis that extends in to the skeletal muscles and bone marrow.

2232-B (Euth - 4/28/14).

Brain: Has multifocal mild to moderate perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum and medulla oblongata.

Spinal cord: has mild multifocal infiltration of lymphocytes in the cord nerves/ganglia.

Peripheral nerves have similar infiltration.

Gizzard, proventriculus, intestine, crop/esophagus: have minimal or no infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia.

In addition proventriculus has moderate infiltration of lymphocytes in the mucosa and large numbers of megabacteria in the mucosa.

Heart has infiltration of a few lymphocytes in and around the epicardial ganglia.

Adrenal has a few scattered lymphocytes.

Liver: has multifocal hepatic cords that have hypertrophied hepatocytes

Kidneys: have moderate to severe multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens most prominent in the collecting tubules. Other changes such as dropout of tubules, interstitial inflammation and fibrosis, are also noted.

Eye: has diffuse infiltration of lymphocytes in the conjunctiva.

Skeletal muscles, lungs, eye, ovary, oviduct, bone, bone marrow, trachea, skin with feathers are free of significant lesions.

2232-C (Euth - 5/03/14).

Brain: Has multifocal moderate to severe perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum.

Gizzard, proventriculus, intestine, crop/esophagus: have mild to moderate to severe infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia most prominent in the gizzard and minimal in crop and proventriculus.

Crop has multifocal necrosis of epithelium, ulceration and inflammation.

Heart has infiltration of similar cells in the epicardial ganglia and occasionally mild infiltration in and around Purkinje cells. There is extensive degeneration of myofibers and infiltration of mononuclear cells.

Liver: has mild periportal lymphocytic inflammation, hepatic eosinophilia and locally extensive lipidosis and interstitial fibrosis.

Kidneys: mild multifocal dilation of tubules

Skeletal muscles, pancreas, spleen, lungs, bone, bone marrow, trachea, skin, are free of significant lesions.

2232-D (Euth - 5/05/14)

Brain: Has multifocal mild to moderate perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum.

Spinal cord: has mild infiltration of lymphocytes in the spinal nerves/ganglia One section has magna of the neuropil.

Gizzard, proventriculus, intestine, crop/esophagus: have mild infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia most prominent in the gizzard and minimal in crop and proventriculus. Esophagus and proximal proventriculus have moderate lymphocytic and giant cell infiltration.

Heart has degeneration of myofibers with occasionally mild infiltration of mononuclear cells.

Kidneys: have moderate multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens. Evidence of mild to moderate multifocal interstitial inflammation, and mineralization are also noted.

Skeletal muscles, peripheral nerves, liver, spleen, pancreas, lungs, bone, bone marrow, tongue/salivary glands, trachea, larynx are free of significant lesions.

2232-E (Euth - 5/14/14)

Spinal cord: one section has a few glial nodules in the grey matter.

Kidneys: have moderate multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens. Evidence of mild to moderate multifocal interstitial inflammation, fibrosis, loss or regenerating tubules and mineralization are also noted.

Brain, GI tract, heart, skeletal muscles, peripheral nerves, liver, spleen, pancreas, lungs, bone, bone marrow, tongue/salivary glands, trachea, larynx, are free of significant lesions.

2232-F (Euth - 5/21/14)

Kidneys: have moderate multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens. Evidence of mild to severe multifocal interstitial fibrosis especially around the collecting tubules are noted. Subjacent to one such area are irregular dilation of tubules some of which contain debris in their lumens. These tubules appear smaller and hyperchromatic.

Liver has foci of lymphocytes scattered throughout.

Crop, proventriculus, gizzard and intestine and a nerve: have occasional foci of lymphocytic infiltration in the serosal/subserosal ganglia and nerves.

Brain, spinal cord, eye, heart, skeletal muscles, lungs, spleen, pancreas, bone, bone marrow, tongue/salivary glands, trachea, and larynx are free of significant lesions.

2232-G (Euth - 6/03/14)

Brain: Has multifocal mild to moderate perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum. Cerebrum also has moderate lipofuscinosis; granular brown pigment in the cytoplasm of neurons scattered here and there.

Spinal cord: has mild infiltration of lymphocytes in the spinal nerves/ganglia.

Gizzard, proventriculus, intestine, crop/esophagus: have mild to moderate to severe infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia most prominent in the gizzard and minimal in crop and proventriculus. Heart has infiltration of similar cells in the epicardial ganglia and occasionally mild infiltration in and around Purkinje cells.

Liver: has mild periportal lymphocytic inflammation.

Pancreas has focal acinar cell hyperplasia.

Kidneys: mild multifocal dilation of tubules with accumulation of eosinophilic and cellular debris in the lumens. Evidence of mild to moderate multifocal necrosis (eosinophilia) of tubular epithelium noted. Other changes such as hemosiderosis, interstitial inflammation, mineralization and focal tubular hyperplasia/adenoma are also noted.

Thyroid has accumulation of amorphous basophilic material within the follicles.

Eye: has diffuse infiltration of lymphocytes in the conjunctiva.

Skeletal muscles, bone, bone marrow, tongue/salivary glands, trachea, are free of significant lesions.

2232-H (Euth - 6/08/14)

Brain: Have multifocal mild to severe perivascular cuffing and gliosis in the cerebrum and mild in the cerebellum. Spinal cord: has mild infiltration of lymphocytes in the grey and white matter and occasionally in the spinal nerves/ganglia.

Gizzard, proventriculus, intestine, crop/esophagus: have mild to moderate to severe infiltration of lymphocytes and a few plasma cells in the serosal and subserosal nerves and ganglia most prominent in the gizzard and minimal in crop and proventriculus. In addition proventriculus has moderate infiltration of lymphocytes in the mucosa associated with megabacteria.

Heart has infiltration of similar cells in the occasional epicardial ganglia and minimal infiltration in and around Purkinje cells.

Liver: has moderate periports granulocytic and/or lymphocytic inflammation. There is one focus of eosinophilia and multifocal vacuolar changes in hepatocytes.

Kidneys: have mild multifocal dilation of tubules and interstitial inflammation.

Lungs have a few aggregates of mononuclear cells scattered.

Pancreas, skeletal muscles, bone, bone marrow, tongue/salivary glands, trachea, eye, are free of significant lesions.

DECALCIFICATION

Animal/Source	Specimen	Specimen Type	Results
T1402232-01	A-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	B-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	C-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	D-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	E-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	F-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	G-decal tissue	Tissue - Fixed	COMPLETED
T1402232-01	H-decal tissue	Tissue - Fixed	COMPLETED

Immunohistochemistry

Avian Bornavirus Immunohistochemistry (under validation)**

Animal/Source	Specimen	Specimen Type	Results
T1402232-01	Block A-3	Brain Tissue	Negative
T1402232-01	Block B-2	Brain Tissue	Positive
T1402232-01	Block C-1	Brain Tissue	Negative
T1402232-01	Block D-1	Brain Tissue	Positive
T1402232-01	Block E-1	Brain Tissue	Negative
T1402232-01	Block F-1	Brain Tissue	Positive
T1402232-01	Block G-1	Brain Tissue	Positive
T1402232-01	Block H-1	Brain Tissue	Positive
T1402232-01	Block A-4	Digestive Tract Tissue	Negative
T1402232-01	Block B-6	Digestive Tract Tissue	Negative
T1402232-01	Block C-4	Digestive Tract Tissue	Negative
T1402232-01	Block D-5	Digestive Tract Tissue	Negative
T1402232-01	Block E-4	Digestive Tract Tissue	Negative
T1402232-01	Block F-5	Digestive Tract Tissue	Negative
T1402232-01	Block G-3	Digestive Tract Tissue	Positive
T1402232-01	Block G-4	Digestive Tract Tissue	Positive
T1402232-01	Block H-3	Digestive Tract Tissue	Negative
T1402232-01	Block H-4	Digestive Tract Tissue	Negative



California Animal Health & Food Safety

Laboratory System

18830 Road 112
Tulare, CA 93274-9042
(559) 688-7543

Preliminary
Version 1
This report supersedes all
previous reports for this case

CAHFS Case #: T1402508
Referral #: SC14-11811-8 FEB
Date Collected: 06/23/2014
Date Received: 09/16/2014
Case Coordinator: H. L. Shivaprasad
BVSc, PhD
Preliminary Report Sent By:
Shivaprasad, H.L. on 2/19/2015
5:35:28PM

Email To:
TIZARD, IAN
tizard@cvm.tamu.edu

Collection Site:
SHUBOT CENTER
4467 TAMU
COLLEGE STATION TX
77843
County: BRAZOS

Specimens Received: 548 Issues - Fixed:

Comments: Carrier: FedEx

Case Contacts

Table with 4 columns: Submitter, Bill To, Phone, Address. Contains contact info for TIZARD, IAN at TAMU.

Specimen Details

Table with 5 columns: Animal/Source, ID Type, Taxonomy, Gender, Age. Contains details for animal T1402508-01.

Laboratory Findings/Diagnosis

- 1. Please see histopathology description, 19 birds.
2. IHC pending.

Clinical History

Cockatiels inoculated ABV strain and blind study treated with either control, celecoxib or meloxicam.

Gross Observations

Histology

A (MRTFL 1662): Brain, testes, lung, liver, kidney, heart, skeletal muscles, crop, proventriculus, gizzard, intestine, pancreas, nerves, bone, bone marrow, skeletal muscles, skin and eye are examined.
Crop, proventriculus, gizzard: and intestine to an extent have a few scattered lymphoid nodules in the serosa some subjacent to the ganglia. In addition proventriculus and intestine to an extent have occasional multifocal lymphoid foci deep in the mucosa of occasional section.
Kidney: scattered lymphoid nodules throughout, locally extensive basophilic of tubules (regeneration).
Bone marrow moderate and liver and pancreas have one or two scattered lymphoid nodules.
Lungs: scattered pneumoconiosis.
Testes: no spermatogenesis.

B (TAMU 254): Brain, spinal cord, testes, lung, liver, kidney, heart, skeletal muscles, crop, proventriculus, gizzard, intestine, pancreas, spleen, nerves, adrenal, bone, bone marrow, skeletal muscles, skin, and eye are examined.
Kidneys, liver, bone marrow, adrenal, pancreas, have numerous lymphoid nodules scattered throughout.
Heart has a few lymphoid nodules scattered in the epicardium/fat

Proventriculus and gizzard: have large numbers lymphoid nodules in the serosa some subjacent to the ganglia. In addition gizzard has lymphoid nodules in the muscular layers. **Intestine** have multifocal lymphoid foci deep in the mucosa.

C (TAMU 229): Brain, spinal cord, nerves lung, liver, kidney, heart, skeletal muscles, crop, esophagus, **proventriculus**, gizzard, intestine, pancreas, cloaca, spleen, adrenal, ovary, oviduct, bone, bone marrow, skeletal muscles, skin and eye are examined. Adrenal, ovary and liver have a few scattered lymphoid nodules. Spinal cord has occasional perivascular cuffing.

Proventriculus and gizzard: have a few lymphoid nodules on the serosa. In addition **proventriculus** has a few lymphoid nodules deep in the mucosa.

D (RAATX 210): Brain, spinal cord, nerves, lung, liver, kidney, heart, skeletal muscles, crop, esophagus, **proventriculus**, gizzard, intestine, pancreas, cloaca, spleen, adrenal, testes, bone, bone marrow, skeletal muscles, skin and eye are examined.

Brain and spinal cord have multifocal moderate to severe perivascular (PV) cuffing and mild gliosis randomly scattered throughout. Meninges in the brain are thickened with PV cuffing.

Nerves have occasional PV cuffing.

Adrenal has multifocal infiltration of lymphocytes in the medullary chords.

Lung: has sprinkling of a few lymphocytes in one ganglia and one large artery has perivascular infiltrate.

Kidneys: one section has mild multifocal interstitial infiltration of lymphocytes and a few adenovirus inclusions in the epithelial cells of collecting tubules.

Proventriculus and gizzard: have moderate numbers of lymphoid nodules in the serosa some in the ganglia. In addition gizzard has lymphoid foci in the muscular layers.

Esophagus is similarly but mildly involved.

Heart has a few foci of lymphocytes in the **epicardial** and myocardial ganglia.

Eye has a few PV cuffing in the optic nerve and lymphoid foci in the choroid.

E (TAMU 261): Brain, spinal cord, nerves, lung, liver, kidney, heart, skeletal muscles, crop, esophagus, **proventriculus**, gizzard, intestine, pancreas, cloaca, spleen, ovary, bone, bone marrow, skeletal muscles, skin and eye are examined.

Brain: one section has a few PV cuffs and gliosis.

Proventriculus: has large numbers of cryptosporidia in the glandular parts.

Gizzard: has infiltration of lymphocytes in the **subserosal** ganglia.

Liver: has **vacuolation** of hepatocytes in the cytoplasm interspersed by foci of hepatocytes.

Kidneys: occasional section has mild multifocal interstitial infiltration of lymphocytes.

F (MRTFL 9491): Brain, spinal cord, trachea, lung, liver, kidney, heart, skeletal muscles, crop, esophagus, **proventriculus**, gizzard, intestine, pancreas, cloaca, spleen, adrenal, ovary, oviduct, bone, bone marrow, skeletal muscles, skin and eye are examined.

Brain and Spinal cord: have mild increased glial cells and occasional PV cuffing.

Adrenal and kidneys have many lymphoid nodules. There are **large number** of lymphoid nodules subjacent to the kidney. One kidney has interstitial fibrosis, irregular and basophilic tubules, glomerular sclerosis.

There are lymphoid nodules scattered around the heart some in or subjacent to the ganglia.

Similar nodules seen in and around the gizzard, proventriculus, crop, esophagus, ovary, oviduct, spleen, bone marrow.

In addition **proventriculus** has large numbers of cryptosporidia and megabacteria in the mucosa associated with lymphoid nodules/lymphocytic inflammation.

G (RAATX 92): Brain, spinal cord, lung, liver, kidney, heart, skeletal muscles, crop, esophagus, **proventriculus**, gizzard, intestine, pancreas, bone, bone marrow, skeletal muscles, skin and eye are examined.

Brain, spinal cord optic nerve: have mild increased glial cells and brain has mild PV cuffing. Spinal ganglia have mild lymphocytic foci.

Kidneys have many lymphoid nodules scattered throughout. Lungs, feather follicle, bone marrow have similar lymphoid nodules.

Similar nodules are seen in and around the gizzard, **proventriculus**, crop and esophagus.

There are a few lymphoid nodules scattered around the heart some in or subjacent to the ganglia.

Liver: has multifocal **lipidosis**.

H (CWI 691). Brain, spinal cord, nerves, lung, liver, kidney, heart, skeletal muscles, crop, esophagus, **proventriculus**, gizzard, intestine, pancreas, cloaca, spleen, adrenal, testis, bone, bone marrow, skeletal muscles, skin and eye are examined.

Liver, spleen, kidneys, and bone marrow (numerous) and adrenal, dermis of skin (**a few**) have lymphoid nodules scattered throughout.

Optic nerve has increased glial cells.

I (TAMU 221): Brain, spinal cord, lung, liver, kidney, heart, skeletal muscles, crop, esophagus, proventriculus, gizzard, intestine, pancreas, bone, bone marrow, skeletal muscles, adrenal, testis, skin and eye are examined.

Brain, spinal cord optic nerve: have mild to severe (brain) PV cuffing, glial nodules and increased glial cells scattered throughout. Kidneys, adrenal, bone marrow have many lymphoid nodules scattered throughout. Similar nodules are seen in and around the serosa and ganglia of esophagus, gizzard, proventriculus, intestine and crop.

There are a occasional lymphoid nodules scattered around the heart some in or subjacent to the ganglia.

Lungs, and air sacs: lungs have severe proliferative bronchopneumonia and air sacs are thickened due to inflammation.

J (TAMU236): Brain, spinal cord, nerves, trachea, lung, liver, kidney, spleen, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, thyroids/parathyroid, bone, bone marrow, skeletal muscles, ovary, oviduct, skin and eye are examined.

Lymphoid nodules in a few to many in and around ganglia of crop, esophagus, proventriculus, gizzard, intestine are noted.

Heart has a few similar nodules around the epicardial and occasional myocardial ganglia.

Kidneys, spleen, pancreas, bone marrow have a few to many lymphoid nodules. In addition kidneys have prominent adenovirus inclusions in the collecting tubular epithelial cells.

Lung: there is a solitary ganglia surrounded by layers of lymphocytes.

K (MRTFL1460): Brain, spinal cord, trachea, lung, liver, kidney, spleen, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, bone, bone marrow, skeletal muscles, oviduct, skin and eye are examined.

Brain has mild to moderate perivascular cuffing and increased glial cells scattered throughout.

Proventriculus and gizzard to a moderate extent and intestine, crop and esophagus to a milder extent have lymphoid nodules in and around the isolated ganglia.

Heart has a few lymphoid nodules in and around the epicardial ganglia. Moderate atherosclerosis of the aorta noted.

Liver and spleen to a moderate extent and kidney, pancreas and bone marrow to a milder extent have lymphoid nodules scattered here and there.

L (TAMU239): Brain, spinal cord, trachea, lung, liver, kidney, spleen, heart, pharynx, crop, esophagus, proventriculus, gizzard, intestine, pancreas, cloaca, bone, bone marrow, skeletal muscles, testis, skin and eye are examined.

Brain has mild occasional perivascular cuffing and increased glial cells.

Esophagus, proventriculus, gizzard and intestine have scattered lymphoid nodules in and around the serosa, isolated ganglia and muscular layers of gizzard and one section of the intestine.

Kidneys (moderate), epididymis, spleen, base of the heart, bone marrow, dermis have lymphoid nodules scattered here and there.

M (MRTFL 2820): Brain, spinal cord, nerves, lung, liver, kidney, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, cloaca, bone, bone marrow, skeletal muscles, ovary, oviduct, skin and eye are examined.

Proventriculus, gizzard and intestine have scattered lymphoid nodules in and around the serosa, isolated ganglia.

Heart has focal infiltration of lymphocytes in an isolated endocardial ganglia (Purkinje cells). Moderate atherosclerosis of the aorta noted.

Ovary, kidneys and bone marrow have a few lymphoid nodules scattered here and there.

N (RAATX 791): Brain, spinal cord, nerves, trachea, lung, liver, spleen, kidney, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, cloaca, bone, bone marrow, skeletal muscles, ovary, oviduct, skin and eye are examined.

Proventriculus, gizzard and intestine have many scattered lymphoid nodules in and around the serosa, isolated ganglia and occasionally in the muscular layers.

Heart has focal infiltration of lymphocytes in the myocardium and around isolated epicardial ganglia.

Kidney and ovary have a few lymphoid nodules.

O (TAMU 265): Brain, spinal cord, nerves, lung, liver, spleen, kidney, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, cloaca, bone, bone marrow, skeletal muscles, ovary, oviduct, adrenal, skin and eye are examined.

Brain has mild occasional perivascular cuffing and increased glial cells.

Proventriculus, gizzard, esophagus, and intestine have many scattered lymphoid nodules in and around the serosa, mucosa and isolated ganglia.

Heart has focal infiltration of lymphocytes around isolated epicardial ganglia and the epicardium.

Kidneys, ovary, oviduct, bone marrow, spleen, periphery of adrenal, have a few to many lymphoid nodules scattered

P (TAMU 215): Brain, spinal cord, nerves, trachea, lung, liver, spleen, kidney, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, cloaca, bone, bone marrow, skeletal muscles, ovary, oviduct, adrenal, skin and eye are examined.

Proventriculus, gizzard, esophagus, crop and intestine have many scattered lymphoid nodules in and around the serosa, isolated ganglia and occasionally in the muscular layers and in the mucosa.

Lungs have scattered foci of lymphocytes in the interstitium and lymphoid nodules scattered throughout.

Heart has multifocal infiltration of lymphocytes around isolated epicardial ganglia and epicardium

Kidney, ovary, oviduct, bone marrow, spleen, periphery of adrenal, have a few to many lymphoid nodules scattered have a few lymphoid nodules.

Liver has fatty change and a few lymphoid nodules

Q (TAMU 247): Brain, spinal cord, nerves, lung, liver, spleen, kidney, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, cloaca, bone, bone marrow, skeletal muscles, ovary, oviduct, skin and eye are examined.

Brain: rare mild PV cuffing and rare gliosis.

Proventriculus, gizzard, esophagus, crop and intestine have a few scattered lymphoid nodules in and around the serosa, isolated ganglia and occasionally in the muscular layers of the gizzard.

Heart has multifocal infiltration of lymphocytes/nodules around isolated epicardial ganglia and epicardium

Kidney, ovary, spleen, have a few lymphoid nodules scattered. In addition kidney has locally extensive dilation of tubules, interstitial inflammation and eosinophilia of tubules.

R (TAMU 297): Brain, spinal cord, nerves, lung, liver, spleen, kidney, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, cloaca, bone, bone marrow, skeletal muscles, testis, adrenal, skin and uropygial gland are examined.

None have any significant lesions except for a few lymphoid nodules in the kidneys, bone marrow, and occasionally around the epicardium and myocardium, near Purkinje cells.

S (TAMU 279): Brain, spinal cord, nerves, lung, liver, spleen, kidney, heart, crop, esophagus, proventriculus, gizzard, intestine, pancreas, cloaca, bone, bone marrow, skeletal muscles, testis, adrenal, skin are examined.

Except for a few lymphoid nodules in the liver, kidneys, spleen and bone marrow there are no significant lesions.

DECALCIFICATION

Animal/Source	Specimen	Specimen Type	Results
T1402508-01	A-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	B-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	C-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	D-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	E-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	F-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	G-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	H-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	I-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	J-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	K-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	L-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	M-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	N-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	O-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	P-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	Q-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	R-Decal tissue	Tissue - Fixed	COMPLETED
T1402508-01	S-Decal tissue	Tissue - Fixed	COMPLETED

APPENDIX D

PROTOCOLS

Cell Culture

Cells require an appropriate temperature of 37°C and 5% CO₂ mixture for growth and maintenance, which can be accomplished with a cell incubator. In addition to temperature and gas conditions, different cell cultures require different media that contains factors for cell growth and promotion. The precise medium will vary between cell lines but for the most part media requires having a specific pH, glucose concentration, and growth factor supplements usually derived from animal blood serum. To prevent for any bacterial or fungal contamination there are additional supplements such as antimycotic, and, actinomycin D, penicillin streptomycine, that can also be added.

(A) Isolating primary cell lines.

The primary cell cultures that were established in these protocol were isolated from the eggs of duck, quaker parrots, and zebra finches.

Harvesting duck embryo fibroblast cells from duck eggs.

1. 20 fertile Mallard duck eggs were purchased from Metzger Farm in California.
2. Prior to arrival of the eggs, the egg incubator was cleaned, turned on, and filled with distilled water in order to allow temperature to reach 37.5°C and approximately 60% humidity.

3. Immediately after delivery, the fertile Mallard eggs were numbered 1-20 and inserted in the incubator's rotating platform in automatic speed setting for approximately 12 days.
4. On the 12th day, the eggs are candled using a flashlight to observe embryo development.
5. If the eggs are ready to be harvested they will be sprayed with 70% ethanol and chilled at 4°C refrigerator for one hour. (This process humanely euthanizes the embryo)
6. After being chilled for one hour, the eggs will be sprayed again with 70% ethanol.
7. The egg tops (the area with the big end of the shell) are removed with sterile scissors under a biological safety cabinet. Using the sterile scissors, tap the shell to create a hole and then use the pointy end to cut around the egg to remove the tops.
8. The embryos were then extracted using sterile forceps and placed into sterile petri dishes. The eggs were then decapitated using sterile blades and the heads were placed in 1.5mL sterile vials numbered 1-20 respective to each egg. Heads were then tested using reverse transcriptase polymerase chain reaction (PCR), as a control to confirm the absence of parrot bornavirus (PaBV) within the embryo.
9. The embryos then had their limbs and internal organs removed with a sterile blade.

10. Two embryos were then eviscerated and placed into a trypsinizing glass flask containing a magnetic stir bar.
11. To remove red blood cells, add 25mL of PBS, wash by swirling for a minute, then remove PBS without discarding the embryo body parts. (This process is repeated two more times.)
12. Add 25mL of trypsin-EDTA 0.05% at room temperature to a trypsinizing flask and stir it at room temperature for 6 minutes. **NOTE:** You can use 15mL of trypsin-EDTA 0.05% if the amount and size embryo is greatly decreased.
13. Decant the trypsin from the trypsinizing flask through 8 layers of sterile cheesecloth into a beaker containing complete medium (DMEM with 5%FBS and 1% Pen Strep). The beaker is placed on ice and the cheesecloth is presaturated with complete medium in the central region only. (The medium stops the enzymatic digestion performed by trypsin.)
14. Repeat steps 12, 13 until the remaining embryo tissue is no longer visible.
15. The contents of the beaker were then separated into 50mL centrifuge tubes and spun at a 2,000 rpm for 10 minutes at 10°C.
16. The supernatant is then removed and the cell pellet is resuspended in 50% FBS and 50% complete medium containing 20% dimethyl sulfoxide (DMSO).
Amount of solution will depend on the cell to cryogenic vial contents wanted.
17. Solution will then be divided into 1mL cryogenic vials and slow freeze using Nalgene Freezing container at -89°C. (Cryogenic vials are labeled with Metzer Farm, Clean DEF cell, mm/dd/yyyy, # of egg.)

Harvest Quaker parrot embryo fibroblast cells from Quaker parrots eggs

The quaker parrot eggs were collected from our Schubot aviary. Quaker eggs were candled every day until two days prior to hatching. The rest of the process was exactly the same as the duck egg protocol above. Cryogenic vials are labeled with Schubot, Clean QEF cells, mm/dd/yyyy, # of egg.

Harvest Zebra finch embryo fibroblast cells from Zebra finch eggs

The zebra finch eggs were donated from one of our veterinary pathobiology professors. Zebra finch eggs are much smaller than quaker eggs so the process had to be modified to obtain the maximum amount of zebra finch embryo fibroblast cells.

1. Zebra finch eggs were candled on a daily basis until two days prior to hatching (when the light from the flashlight isn't visible thru the egg).
2. If the eggs are ready to be harvested they will be sprayed with 70% ethanol and chilled at 4°C refrigerator for one hour. (This process humanely euthanizes the embryo)
3. After being chilled for one hour, the eggs will be sprayed again with 70% ethanol.
4. The egg tops (the area with the big end of the shell) are removed with sterile scissors under a biological safety cabinet. Using the sterile scissors, tap the shell to create a hole and then use the pointy end to cut around the egg to remove the tops.
5. The embryos were then extracted using sterile forceps and placed into sterile petri dishes. The eggs were then decapitated using sterile blades and the heads

- were placed in 1.5mL sterile vials numbered 1-3 respective to each egg. Heads were then tested using reverse transcriptase polymerase chain reaction (PCR), as a control to confirm the absence of parrot bornavirus (PaBV) within the embryo.
6. Each embryo was then eviscerated and its limbs and organs were removed if they were visible.
 7. The embryo was then rinsed with PBS three times using a syringe to remove all red blood cells.
 8. The embryo was exposed to 2mL of trypsin in a 15mL conical vial and swirled by hand for 3 minutes.
 9. To stop the enzymatic digestion of trypsin, 3mL of complete medium were added to the 15mL conical vial.
 18. Centrifuge the 15mL conical vial at 1,000 rpm at 10°C for 10 minutes.
 10. Remove the supernatant and resuspended the cell pellet with complete medium and inserted in a 25mL volumetric flask. The 25mL volumetric flask is then placed in a cell incubator at 37°C, 5% CO₂, and with 95% humidity.
 11. Incubation of zebra finch embryo fibroblast cells continued until the flask reached 90% cell confluence, then the cells in the flask were divided into two 25mL flasks.
 12. Step 11 was repeated 4 more times for propagation of zebra finch embryo fibroblast cells. (This process is described on section (B) of cell culture protocols)

13. Zebra finch embryo fibroblast cells from each of the ten 25mL flasks were detached from the 25mL flask floor by using 0.25% trypsin-EDTA.
 - a. Use 1.5mL of 0.25% trypsin-EDTA into a 25mL flask and wash (by swirling trypsin on flask floor) for 2 seconds.
 - b. Immediately after wash remove the 1.5mL of 0.25% trypsin-EDTA and discard in beaker with 10% bleach solution. **NOTE:** This step removes any excess complete media and begins the enzymatic digestion of trypsin.
14. 0.5mL of trypsin then is added and swirled until the complete flask floor is covered.
15. The 25mL flask is then placed in the clean cell culture incubator for 2 minutes or until cells are seen detached by using a cell culture microscope.
16. To stop trypsin's enzymatic digestion, add 2mL of complete media solution and gently agitate flask with your hand to loosen any adhered cells. **NOTE:** steps 13-16 are repeated with the rest of the nine 25mL flask containing zebra finch embryo fibroblast cells.
17. Combine the 2mL of complete media and zebra finch embryo fibroblast cells from each of the 10 flask into one 25mL flask and swirl.
18. Pour the 25mL of complete media and zebra finch embryo fibroblast cells into a 50mL conical tube.
19. Centrifuged solution at 1,000 rpm and 10°C for 10 minutes in an Allegra X-15 centrifuge. **NOTE:** Make sure to balance centrifuge with another 50mL conical tube containing 25mL of tap water.

20. Remove the supernatant from the conical tube and discard it in the beaker with 10% bleach.
21. To resuspend the pelleted cells, add 20mL of freezing media solution and mix slowly with the electronic pipettor.
22. In 20 cryogenic vials add 1mL of zebra finch embryo fibroblast cells and freezing media solution and slow frozen using Nalgene Freezing container at -89°C. (Cryogenic vials are labeled with Dr. Payne, Clean Zebra Finch EF cells, mm/dd/yyyy, #of egg.) **NOTE:** To slow freeze leave cryogenic vials for at least 5 days in the Nalgene Freezing container at -89°C.

(B) Propagation of cell culture line.

Propagation of a cell culture line can be from a previously harvested cell culture line from an egg that has been frozen multiple times with freezing media (50% FBS and 50% complete medium containing 20% dimethyl sulfoxide (DMSO) in a Nalgene Freezing container at -89C. Growing cell culture lines by the process of passaging cells results in the increase of cell culture line density which allows for further research studies or creation of a cell line stock. Passaging cells involves growing a cell culture line and transferring a small portion of the cells to another flask after a 80-90% confluence, to prolong high cell density. The propagation process is similar for different species' cell lines, however, it differs in the amount of time it takes for each cell line to reach optimal confluence which is 80-90%.

Propagation of duck embryo fibroblast (DEF) cells from DEF cells vial stock

To propagate a DEF cell line, we will use one cryogenic vial of previously frozen DEF cells. This cryogenic vial could have come from a primary line or it could have previously been propagated. This process will be divided into four parts. **NOTE:** These duck embryo fibroblast cells are not experimentally immortalized, therefore, this may result in a decline in the quality and quantity of the cell line after a number of propagations.

1. Prepare cell culture cabinet (BSL II cabinet) with an electronic pipettor, complete media solution (DMEM media that contains 10% fetal bovine serum (FBS) and 1% penicillin streptomycin), (manufacturer: Life technologies) and a discard beaker containing 10% bleach solution. **NOTE:** DMEM media needs to be at room temperature prior to use to prevent rupturing of cells.
2. Insert 1 vial containing 1mL of clean DEF cells into a clean latex glove and tie a knot to minimize the amount of water exposure.
3. Quick thaw the vial in a 37°C water bath by swirling the vial in water just until thawed to ensure a higher proportion of cells survive. This will take about 2-3 minutes.
4. Remove vial from clean latex glove and spray vial with 10% bleach before placing it in cell culture cabinet.
5. Add 10mL of complete media solution into a 15mL conical tube.
6. Pour the 1mL of clean DEF cells into the 15mL conical tube with 10mL complete media solution.

7. Solution will then be centrifuged at 1,000 rpm and 10°C for 10 minutes in an Allegra X-15 centrifuge. **NOTE:** Make sure to balance centrifuge with another 15mL conical tube containing 11mL of tap water.
8. Remove the supernatant from the conical tube and discard it in the beaker with 10% bleach.
9. To resuspend the pelleted cells, add 5mL of complete media solution and mix slowly with the electronic pipettor.
10. Finally, add the 5mL of resuspended cells into a 25mL flask and incubate in a clean cell culture incubator at 37°C and 5% CO₂.
11. Check cells daily for about 3-4 days or until cell confluence is 80-90%. When 25mL flask has an 80-90% cell confluence, the cells will be passaged into a 75mL flask. **NOTE:** Cell confluence progression changes depending on cell type or amount of cells per unit square.

Part II

1. To begin separating cells from the 25mL flask into a 75mL flask, the 5 mL of complete media are removed and discard in a beaker with 10% bleach solution.
2. Cells are then detached from the 25mL flask floor by using 0.25% trypsin-EDTA.
 - a. Use 1.5mL of 0.25% trypsin-EDTA to wash (by swirling trypsin on flask floor) for 2 seconds.

- b. Immediately after wash remove the 1.5mL of 0.25% trypsin-EDTA and discard in beaker with 10% bleach solution. **NOTE:** This step removes any excess complete media and begins the enzymatic digestion of trypsin.
3. 0.5mL of trypsin then is added and swirled until the complete flask floor is covered.
4. The 25mL flask is then placed in the clean cell culture incubator for 2 minutes or until cells are seen detached by using a cell culture microscope.
5. To stop trypsin's enzymatic digestion, add 15mL of complete media solution and gently agitate flask with your hand to loosen any adhered cells.
6. Transfer the mixture of 15mL complete media with cells to a 75mL flask and incubate in a clean cell culture incubator until 80-90% cell confluence (approximately 3-4 days).

Part III

When the 75mL flask reaches 80-90% confluence, DEF cells are passaged into two 75mL flask. The following steps are performed to passage cells into two 75mL flasks.

1. Removed the 15mL of complete media and discarded into a beaker with 10% bleach solution. **NOTE:** If cells begin to over grow on bottom of flask you may lose some cells when removing the 15mL of complete media. That is why it is best to separate before 100% confluence.
2. Cells are then detached from the 75mL flask floor by using 0.25% trypsin-EDTA.

- a. Use 3.0mL of 0.25% trypsin-EDTA to wash (by swirling trypsin on flask floor) for 2 seconds.
 - b. Immediately after wash remove the 3.0mL of 0.25% trypsin-EDTA and discard in beaker with 10% bleach solution. **NOTE:** This step removes any excess complete media and begins the enzymatic digestion of trypsin.
3. 1.5mL of trypsin is then added and swirled until the complete flask floor is covered.
 4. The 75 mL flask is then placed in the clean cell culture incubator for 2 minutes or until cells are seen detached by using a cell culture microscope.
 5. To stop trypsin's enzymatic digestion, add 10mL of complete media solution and gently agitate flask with your hand to loosen any adhered cells.
 6. Add 5mL of complete media with cells to each 75mL flask and then add 10mL of fresh complete media to each 75mL flask. (Making a total volume of 15mL per flask) **NOTE:** Original 75mL flask can be reused once.
 7. Incubate both 75mL flasks in clean cell culture incubator until 80-90% cell confluence (approximately 3-4 days).

Part III is repeated two more times for each of the 75mL flask, concluding with eight 75mL flask of 80-90% cell confluence.

Part IV

1. The 15mL of complete media are removed from each of the eight 75mL flasks and discarded in a beaker with 10% bleach solution.

2. Cells are then detached from the 75mL flask floor by using 0.25% trypsin-EDTA.
 - a. Use 3.0mL of 0.25% trypsin-EDTA to wash (by swirling trypsin on flask floor) for 2 seconds.
 - b. Immediately after wash remove the 3.0mL of 0.25% trypsin-EDTA and discard in beaker with 10% bleach solution. **NOTE:** This step removes any excess complete media and begins the enzymatic digestion of trypsin.
3. 1.5mL of trypsin is then added and swirled until the complete flask floor is covered.
4. The flasks are then placed in the clean cell culture incubator for 2 minutes or until cells are seen detached by using a cell culture microscope.
5. To stop trypsin's enzymatic digestion, add 5mL of complete media solution and gently agitate flask with your hand to loosen any adhered cells.
6. The 5mL of each of the 75mL flask were then combined in a 50mL conical vial.
7. The 50mL conical vial was then centrifuged for 10 minutes at 1,000 rpm and 10°C in an Allegra X-15 centrifuge.
8. The supernatant is then removed and the cell pellet is resuspended in 50% FBS and 50% complete medium containing 20% dimethyl sulfoxide (DMSO).
Amount of solution will depend on the amount of cells to vial ratio wanted.
NOTE: One 75mL flask approximately makes two-three 1mL cryogenic vials

9. The solution will then be divided into 1mL cryogenic vials and slowly frozen in a Nalgene Freezing container at -89°C. (Cryogenic vials are labeled with Clean DEF cell, mm/dd/yyyy, vial stock)

Harvest muscle Quail cells from muscle Quail cell vial stock

The process was exactly the same as the propagation of duck embryo fibroblast (DEF) cells from the DEF cells vial stock depicted above. Muscle quail cell lines have been shown to grow at a higher rate than duck embryonic fibroblast cells, meaning this will change the times for each of the incubation periods. **NOTE:** Checking on our flask's % confluence on a daily basis will help anticipate the timing in which to divide the flask for propagation. Cryogenic vials are labeled with Schubot, Clean mQuail cells, mm/dd/yyyy.

Harvest Quaker embryo fibroblast cells from Quaker vial stock

The process was exactly the same as the propagation of duck embryo fibroblast (DEF) cells from the DEF cells vial stock depicted above. Cryogenic vials are labeled with Schubot, Clean QEF cells, mm/dd/yyyy.

Harvest Zebra finch embryo fibroblast cells from Zebra vial stock

The process was exactly the same as the propagation of duck embryo fibroblast (DEF) cells from the DEF cells vial stock depicted above. Cryogenic vials are labeled with Schubot, Clean ZFEF cells, mm/dd/yyyy.

C) Infect cell culture line from viral stock

Infecting cell culture lines is a useful application of cell cultures. Studying infected cell cultures is essential to better understand a virus' lifecycle, possible viral treatments/vaccinations, and how a virus is transmitted.

Infecting DEF cell culture line from parrot bornavirus genotype 4 (PaBV-4) viral stock.

To infect DEF cell culture line there are two parts. 1) A cell line has to be cultured and allowed to be grown into 50-60% confluence. 2) Infected DEF cell line is used to infect growing DEF cells. Infected DEF cells can be propagated to create a viral stock for future experiments while a portion of the infected cells are tested for parrot bornavirus detection, in order to confirm viral presence. Parrot bornavirus have been shown to replicate within the cell's nucleus. Parrot bornavirus method of transmission is by cell to cell contact. **NOTE:** Utilize two distinct cell culture incubators to prevent contamination of uninfected cells with virus: The "clean" cell culture incubator will house uninfected cells while the "dirty" cell culture incubator will house infected cells. When working in the cell culture cabinet, first work with uninfected cell culture prior to working with infected cell culture to prevent viral cross-contamination.

Part I

1. Prepare cell culture cabinet (BSL II cabinet) with an electronic pipettor, complete media solution (DMEM media that contains 10% fetal bovine serum and 1% penicillin streptomycin) (manufacture: Life technologies) and a discard

beaker containing 10% bleach solution. **NOTE:** DMEM media needs to be at room temperature prior to use to prevent rupturing of cells.

2. Insert 1 vial of clean DEF cells into a clean latex glove and tie a knot to minimize the amount of water exposure.
3. Quick thaw the vial in a 37°C water bath by swirling the vial in water just until thawed to ensure a higher proportion of cells survive. This will take about 2-3 minutes.
4. Remove vial from clean latex glove and spray vial with 10% bleach before placing it in cell culture cabinet.
5. Add 10mL of complete media solution into a 15mL conical tube.
6. Pour cryogenic vial containing 1mL of uninfected DEF cells into the 15mL conical tube with 10mL complete media solution.
7. Solution will then be centrifuged at 1000 rpm and 10°C for 10 minutes in an Allegra X-15 centrifuge. **NOTE:** Make sure to balance centrifuge with another 15mL conical tube containing 11mL of tap water.
8. Remove the supernatant from the conical tube and discard in beaker with 10% bleach.
9. To resuspend the pelleted cells, add 5mL of complete media solution and mix slowly with the electronic pipettor.
10. Finally, add the 5mL of resuspended cells into one 25mL flasks and incubate in a clean cell culture incubator at 37°C and 5% CO₂.

11. Check cells daily for about 3-4 days or until cell confluence is 50-60%. When 25mL flask has a 50-60% cell confluence, the cells will be infected with PaBV-4 DEF cells.

Part II

1. Insert 1 cryogenic vial containing 1mL of infected DEF cells into a clean latex glove and tie a knot to minimize the amount of water exposure.
2. Quick thaw the vial in a 37°C water bath by swirling the vial in water just until thawed to ensure a higher proportion of cells survive. This will take about 2-3 minutes
3. Remove cryogenic vial from clean latex glove and spray vial with 10% bleach before placing it in cell culture cabinet.
4. Add 10mL of complete media solution into a 15mL conical tube.
5. Pour the 1mL of infected DEF cells into the 15mL conical tube with 10mL complete media solution.
6. Solution will then be centrifuged at 1000 rpm and 10°C for 10 minutes in an Allegra X-15 centrifuge. **NOTE:** Make sure to balance centrifuge with another 15mL conical tube containing 11mL tap water.
7. Remove the supernatant from the conical tube and discard in beaker with 10% bleach.
8. To resuspend the pelleted cells, add 5mL of complete media solution and mix slowly with the electronic pipettor.

12. Finally, add the 5mL of resuspended cells into the 25mL flasks with uninfected DEF cells and incubate in a “dirty” cell culture incubator at 37°C and 5%CO₂.
13. Check cells daily for about 3-4 days or until cell confluence is 80-90%. When 25mL flask has an 80-90% cell confluence, the cells will be passaged into one 75mL flask for further propagation of infected cell culture line to create a viral cell culture stock.

D) Diagnostic test for PaBV

Indirect immunocytochemistry assay (ICC)

Indirect immunocytochemistry involves an unlabeled primary antibody which binds with an antigen and a labeled secondary antibody which binds with the primary antibody. Indirect immunocytochemistry is commonly used because of its higher sensitivity. This higher sensitivity is due to signal amplification caused by several secondary antibodies binding with different antigenic sites on the primary antibody. We used a secondary antibody labeled with an alkaline phosphatase enzyme.

Prep- Materials:

A) 0.02M Phosphate buffer solution (PBS)

1. Weigh out 35.49g of 0.5M Na₂HPO₄ and dissolve in 500mL of de-ionized water.
2. Weigh out 59.99g of 1M NaH₂PO₄ and dissolve in 500mL of deionized water.
3. Weigh out 116.88g of 4M NaCl and dissolve in 500mL of deionized water.
4. Mix 11.54 mL of 0.5M Na₂HPO₄ solution, 4.23mL of 1M NaH₂PO₄ solution, 37.5mL of 4M NaCl and add ultra pure water to make a total of 1,000mL

NOTE: Autoclave solution for lasting purposes.

B) 2% paraformaldehyde solution in PBS

1. Weigh out 1g paraformaldehyde powder (Sigma, St. Louis, MO) to 32 mL of 0.02M PBS.
2. Add 12.5uL of 2M NaOH. (2M NaOH: 20mL NaOH into 80mL of deionized water)
3. Heat to 60°C (**NOTE:** make sure solution is in a sealed container), mix frequently until the paraformaldehyde goes into solution. (**NOTE:** this will happen quickly as soon as the suspension reaches 70 °C) Allow the solution to cool to room temperature.
4. Adjust pH to 7.4 using 0.1 M NaOH or 0.1M HCl, if needed.
5. Adjust volume to 50 mL with PBS
6. Filter and store away from light at 4°C.

Indirect immunocytochemistry assay protocol:

1. Wash infected DEF cells 2 times for 5 minutes in 0.02M phosphate buffer solution (PBS). **NOTE:** wash by setting plate in bench rocker
2. Fix for 10 minutes in 2% paraformaldehyde in 0.02M PBS.
3. Wash 2 times for 5 minutes in 0.02M PBS.
4. Permeabilize cells in 1% Triton X-100/0.02 M PBS for 10 minutes.
5. Wash 3 times for 5 minutes each time in 0.03% Tween / 0.02M PBS. **NOTE:** Make sure to make a new batch of 0.03% Tween/PBS each time you do an immunocytochemistry assay.
6. Blocking performed for 2 hours in 5% dried milk/ (0.03% Tween /PBS)

NOTE: Immunocytochemistry assay take approximately 6 hours. You can leave the blocking overnight in a humidified chamber if you can't finish all at once.

7. Incubate in a humidified chamber for 30 minutes at 37°C with the primary antibody at a 1:500 dilution in 1% dried milk/ (0.03% Tween/PBS)

8. Wash 3 times for 5 minutes in 0.03% Tween/PBS.

9. Incubate in humidified chamber for 30 minutes at 37°C with the secondary antibody (AP- conjugated goat anti-macaw IgG) at a 1:500 dilution in 1% dried milk / (0.03% Tween/PBS).

10. Wash 3 times for 5 minutes in 0.03% Tween/PBS.

11. In 50 mL tube add two Sigma FAST BCIP/NBT tablet into 20 mL ultrapure water, then add 2 mL into each well.

****Look for color change (blue-purple) on the bottom of the plate where cells are fixed****

One-step TaqMan qRT-PCR

One-step TaqMan RT-PCR reagents are designed to detect a specific RNA target by performing reverse transcription (RT) and polymerase chain reaction (PCR) amplification simultaneously. Reverse transcription is when an enzyme catalyzes the mRNA or RNA to generate its complementary DNA (cDNA). Reverse transcriptase in PCR is used to qualitatively detect gene expression of RNA viruses through the creation of the virus mRNA's complementary DNA. Prior to using the One-step TaqMan RT-PCR master mix reagent kit we had to extract the mRNA from our samples. To extract the mRNA we used the QIAamp Viral RNA Mini kit. This kit purifies the viral mRNA from tissue, cell, and cloacal swab samples.

(**NOTE:** for detailed information use QIAamp Viral RNA Mini Handbook manufacture: QIAGEN)

RNA extraction procedures:

1. Mix volume of Buffer AVL and carrier RNA-Buffer AVE depending on the amount of samples being extracted. For optimal results you will want 5.6µg of carrier RNA per sample. **NOTE:** QIAamp Viral RNA Mini Handbook has a table with the volume of Buffer AVL and carrier RNA-Buffer AVE depending on the amount of samples. Mix solution by rotating 5-6 times, **do NOT use vortex.**
2. In a 1.5mL microcentrifuge tube add 560µL of Buffer AVL and carrier RNA-Buffer AVE mixture.
3. Add 140µL of your sample in microcentrifuge and mix for 20seconds using vortex, making a homogeneous solution.
4. Incubate at 21 °C (70 °F) for 10 minutes.
5. Centrifuge the tubes for 30 seconds at 8,000-10,000 rpm to remove any drops from the inside of the lid.
6. Add 560µL of ethanol (100% pure) to the sample and mix for 15 seconds using the vortex.
7. Centrifuge the tubes for 30 seconds at 8,000-10,000 rpm to remove any drops from the inside of the lid.
8. Insert QIAamp mini column filter and add 630µmL of the buffer AVL-carrier RNA, sample, and ethanol mixture into a 2mL microcentrifuge collection tube.

9. Centrifuge for 1 minute at 8,000 rpm, then place the QIAamp mini column filter into a new microcentrifuge and discard the liquid that has passed through the filter. **NOTE:** Repeat this step if there is a larger sample volume.
10. Add 500 μ L of Buffer AW1 into QIAamp mini column filter and centrifuge for 1 minute at 10,000 rpm, then place the QIAamp mini column filter into a new microcentrifuge and discard the liquid that has passed through the filter.
11. Add 500 μ L of Buffer AW2 into QIAamp mini column filter and centrifuge for 3 minutes at 14,000 rpm, then place the QIAamp mini column filter into a new microcentrifuge and discard the liquid that has passed through the filter.
12. Centrifuge for 1 minute at 14,000 rpm, and then place the QIAamp mini column filter into a new microcentrifuge and discard the liquid that has passed through the filter.
13. Add 60 μ L of Buffer AVE equilibrated to room temperature and let it rest for 1 minute, then centrifuge at 10,000 rpm for 1 minute. **NOTE:** Buffer AVE elutes viral mRNA from the QIAamp mini column filter.

One-step TaqMan RT-PCR procedures

Materials needed:

- PaBV matrix (M1) gene set, which contains: primer #1 and #2 (mix 18 μ M of each primer together to yield a final concentration of 36 μ M) and Taqman probe # 3 at 10 μ M.
 - M1 primers- #1: (5'- GGTAATTGTTTCCTGGATGG-3'), #2: (5'- ACACCAATGTTCCGAAGACG-3'), and Taqman probe #3 (5'- FAM-TCGATAACTG [Y]TCCCTTCCGGTC-BHQ-3')

- PaBV Phosphoprotein (P2) set, which contains: primer #9 and #10 (mix 18 μ M of each primer together to yield a final concentration of 36 μ M) and Taqman probe #11 at 10 μ M.
 - P2 primers- #9: (5' AAGAAGAACCTCCATGATCTC 3'), #10: (5'-AATGCCGAATAGTCATC- 3'), and Taqman probe #11: (5'-FAM-TCGATAACTG [Y]TCCCTTCCGGTC-BHQ-3') for the detection of PaBV Phosphoprotein mRNA.
- Taqman Fast virus master tough mix with ROX fluorescent dye. (Life Technologies)
- Ultrapure H₂O
- Samples mRNA (**NOTE:** these samples are the samples that were collected from the RNA extractions procedures described above)
- 24 well plate

NOTE: Mix Taqman fast virus 1-step master mix, ultrapure H₂O, mRNA of viral samples, PCR primers, and taqman probe for the desired target using the following template.

(This is per sample/well)

- a. Taqman fast virus master mix----- 6 μ L
- b. Primer mix (for either M1 (#1/2) or P2 (#9/10) set) ----- 0.3 μ L
- c. Probe (for either M1 (#3) or P2 (#11) set) ----- 0.3 μ L
- d. Ultrapure H₂O----- 0.4 μ L

e. mRNA per sample----- 5 μ L

Total= 12 μ L per well

1. To test for 24 samples, mix enough solution for 27 samples, with the M1 primer and probes in a 1.5mL microtube. (Label microtube: M1 MASTER MIX) **NOTE:** Always prepare master mix for at least 3 extra samples.

a. Taqman fast virus master mix----- 162 μ L

b. Primer mix (M1 (#1/2) set) ----- 8.1 μ L

c. Probe (M1 (#3) set) ----- 8.1 μ L

d. Ultrapure H₂O----- 10.8 μ L

2. To test for 24 samples, mix enough solutions for 27 samples, with the P2 primer and probes in a 1.5mL microtube. (Label microtube: P2 MASTER MIX) **NOTE:** Always prepare master mix for at least 3 samples extra.

a. Taqman fast virus master mix----- 162 μ L

b. Primer mix (P2 (#9/10) set) ----- 8.1 μ L

c. Probe (P2 (#11) set) ----- 8.1 μ L

d. Ultrapure H₂O----- 10.8 μ L

3. Distribute 7 μ L of the M1 and P2 master mix to each of the wells (Figure D.1).

4. Add 5 μ L of your mRNA sample.

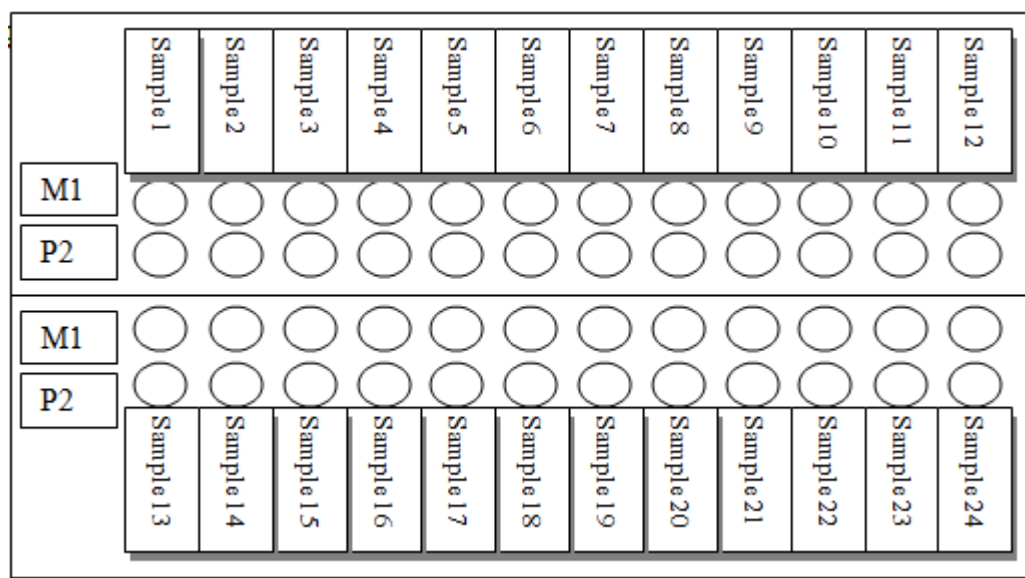
5. Seal plate, cover with foil, vortex, and centrifuge at 2000 rpm for 2 minutes.

6. Run the plates by using a real-time PCR machine (ABI 7900HT).

a. Use the Sequence Detection System (SDS) program template with the following settings: initial denaturing for 5 minutes at 48°C, followed by 20 seconds at 95°C, and then 45 amplification cycles using the following parameters; reverse transcriptase inactivation for 3 seconds at 95°C and polymerase activation for 30 seconds at 56°C.

7. Analyze results using the Sequence Detection System, Version 2.4.1 (SDS 2.4) software.

Figure D.1. Distribute 7µL of the M1 and P2 master mix to each of the wells.



E) Infect cell culture line from urine of PaBV positive shedding birds

This process consisted of three parts: 1) Collection of urine from two PaBV positive shedding African Grey parrots and one PaBV negative shedding African Grey parrot; 2) Detection of PaBV in urine using one-step taqman RT-PCR for viral P-protein

mRNA; 3) Infecting cell culture lines with the urine of the African Grey parrots. **NOTE:** We detecting the virus in cell culture using indirect immunocytochemistry by looking for viral N-protein and one-step taqman RT-PCR by looking for viral P-protein mRNA.

Part I

1. The three African grey parrots were sedated and given 10mL of fluids for the increase of urine production. (10 minute waiting period)
2. Each bird was carried in flight position for a better visualization of the cloacal vent.
3. A microtube containing a window was placed inside the cloacal vent; window was positioned towards the urodeum using visual confirmation.
4. Urine flowed from the urogenital papilla into the microtube and then suctioned with capillary tubes.
5. The urine was filtered using an Acrodisc syringe filter with Nylon membrane, diameter 13mm, pore size 0.45 micron.
6. Prior to infecting DEF cells, a portion of urine was tested with one-step taqman RT-PCR for the detection of PaBV P RNA to confirm viral presence.

Part II

1. Prepare cell culture cabinet (BSL II cabinet) with an electronic pipettor, complete media solution (DMEM media that contains 10% fetal bovine serum and 1% penicillin streptomycin) (Life technologies Co.) and a discard beaker containing 10% bleach solution. **NOTE:** DMEM media needs to be at room temperature prior to use to prevent rupturing of cells.

2. Insert 1 cryogenic vial containing 1mL of clean DEF cells into a clean latex glove and tie a knot to minimize the amount of water exposure.
3. Quick thaw the vial in a 37°C water bath by swirling the vial in water just until thawed to ensure a higher proportion of cells survive. This will take about 2-3 minutes.
4. Remove vial from clean latex glove and spray vial with 10% bleach before placing it in cell culture cabinet.
5. Add 10mL of complete media solution into a 15mL conical tube.
6. Pour cryogenic vial of clean DEF cells into the 15mL conical tube with 10mL complete media solution.
7. Solution will then be centrifuged at 1000 rpm and 10°C for 10 minutes in an Allegra X-15 centrifuge. **NOTE:** Make sure to balance centrifuge with another 15mL conical tube containing 11mL tap water.
8. Remove the supernatant from the conical tube and discard in beaker with 10% bleach.
9. To resuspend the pelleted cells, add 5mL of complete media solution and mix slowly with the electronic pipettor.
14. Finally, add the 5mL of resuspended cells into one 25mL flasks and incubate in a clean cell culture incubator at 37°C and 5% CO₂.
15. Check cells daily for about 3-4 days or until cell confluence is 80-90%. When 25mL flask has 80-90% cell confluence, the cells will be transferred into a 24 well plate.

Part III

1. To begin separating cells from the 25mL flask into a 24 well plate, the 5 mL of complete media are removed and discarded in a beaker with 10% bleach solution.
2. Cells are then detached from the 25mL flask floor by using 0.25% trypsin-EDTA.
 - a. Use 1.5mL of 0.25% trypsin-EDTA to wash (by swirling trypsin on flask floor) for 2 seconds.
 - b. Immediately after wash remove the 1.5mL of 0.25% trypsin-EDTA and discard in beaker with 10% bleach solution. **NOTE:** This step removes any excess complete media and begins the enzymatic digestion of trypsin.
3. 0.5mL of trypsin is then added and swirled until the complete flask floor is covered.
4. The 25mL flask is then placed in the clean cell culture incubator for 2 minutes or until cells are seen detached by using a cell culture microscope.
5. To stop trypsin's enzymatic digestion, add 12mL of complete media solution and gently agitate flask with your hand to loosen any adhered cells.
6. Add the 0.5mL of complete media with cells to each of the 24 wells in a plate and incubate in clean cell culture incubator until it reaches 50-60% cell confluence (approximately 2-3 days).

Part IV

To infect DEF cells with the urine of PaBV positive shedding African grey parrots.

Urine was divided into 2 categories: 1) Diluted urine 2) Concentrated urine (containing urates) and both were tested with one-step taqman RT-PCR for the detection of PaBV P mRNA in order to confirm viral presence. By the time the clean cell culture 24 well plate reached 50-60% cell confluence the diluted urine was filtered and treated with anti-fungal and anti-bacterial to prevent contamination.

1. The 24 well plate containing duck embryonic fibroblast cells was infected with 0.1 mL of filtered urine and allowed to incubate for 18 days (Figure D.2).

Infecting Cells in 24 Well plate

- In columns 1, add 1.0 mL of 2% FBS media to each well
 - In column 2, add 1.0 mL of post-filtered urine from PaBV negative shedding bird.
 - In column 3, add 1.0 mL of post-filtered urine from PaBV positive shedding bird-1.
 - In column 4, add 1.0 mL of post-filtered urine from PaBV positive shedding bird-2.
 - In columns 5, add 1.0 mL of 10^{-1} PaBV-4 to each well
 - In columns 6, add 1.0 mL of 10^{-2} PaBV-4 to each well
2. For our positive controls we used dilutions of duck embryonic fibroblast cells infected with PaBV-4.

PaBV Infected Cell Dilutions

Use 2% FBS media for this part

1. Quick thaw 1 vial of PaBV4 infected DEF cells in 37°C while swirling, then spray exterior with 70% alcohol.
2. Dilutions
 - i. 10^{-1} = Add 0.4 mL of thawed virus into 3.6 mL 2% FBS media
 - ii. 10^{-2} = Add 1.5 mL of 10^{-1} into 13.5 mL 2% FBS media
3. Each well in the 24 well plate had a sample removed every 2-3 days. With these samples we performed one-step taqman qRT-PCR in order to detect the presence of PaBV P mRNA. (Process repeated 6-9 times)
 1. The 1 mL of complete media in each of the 24 wells are removed and discarded in a beaker with 10% bleach solution.
 2. Cells are detached from the 24 well plate floor by using 0.25% trypsin-EDTA.
 - a. Use 0.5mL of 0.25% trypsin-EDTA to wash (by swirling trypsin on flask floor) for 2 seconds.
 - b. Immediately after wash remove the 1.5mL of 0.25% trypsin-EDTA and discard in beaker with 10% bleach solution. **NOTE:** This step removes any excess complete media and begins the enzymatic digestion of trypsin.

3. 0.2mL of trypsin then is added and swirled until the complete well floor is covered.
4. The plate is then placed in the clean cell culture incubator for 2 minutes or until cells are seen detached by using a cell culture microscope.
5. To stop trypsin's enzymatic digestion, add 1mL of complete media solution and gently agitate plate with your hand to loosen any adhered cells.
6. Remove 0.5mL of complete media with cells and insert into a cryo-vial and store in -80°C for the detection of PaBV P mRNA by one-step taqman qRT-PCR.
7. Finally, add 0.5ml of clean media to each 24 well and incubate in "dirty" cell culture incubator until 90-100% cell confluence (approximately 2-3 days).

Figure D.2. Twenty four well plate containing duck embryonic fibroblast cells was infected with 0.1mL of filtered urine.

