# MOLECULAR DETECTION AND CHARACTERIZATION OF AVIAN BORNAVIRUS

A Dissertation

by

NEGIN MIRHOSSEINI

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

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Veterinary Microbiology

Molecular Detection and Characterization of Avian Bornavirus

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# ABSTRACT

Molecular Detection and Characterization of Avian Bornavirus. (May 2011)

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Chair of Advisory Committee: Dr. Susan Payne

Proventricular dilatation disease (PDD) was first recognized during an outbreak among captive macaws in the late 1970s. The disease, also known as proventricular dilatation syndrome or macaw wasting disease can occur in any psittacine but the most commonly affected birds are macaws, cockatoos and conures. The disease causes inflammation of the central, peripheral and autonomic nervous systems, as well as weight loss associated with regurgitation and the passage of undigested food in the feces. Although a viral etiology for PDD has been suspected for almost 40 years, the etiologic agent of the disease was unknown until lately. Recently we cultured a novel bornavirus from brain tissue from birds clinically diagnosed with PDD. This finding supports data from other groups who, in 2008, identified bornavirus sequences among birds suffering from PDD. It was reported that more 60% of PDD affected birds were infected with the new virus, designated avian bornavirus (ABV). ABV is a negative sense, single stranded RNA virus related to Borna disease virus (BDV). ABV isolates differ dramatically from BDV isolates in their level of genetic variation. Using polymerase chain reaction (PCR) assays, we were able to detect ABV in feces and tissue of PDD birds. We also detected ABV shedding from clinically healthy birds housed in aviaries

with no history of the disease. We also determined the complete genome sequences of eight North American ABV isolates. Genotyping indicates that the majority of North American ABV isolates are genotype 4. We found one ABV, genotype 1, which is the first complete sequencing of this genotype. Moreover, we found ABV genotype 2, isolated from an apparently healthy cockatiel with no PDD clinical signs. In order to investigate whether this genotype is avirulent, the virus was grown in duck embryo fibroblasts and inoculated into two adult cockatiels by the oral and intramuscular routes. One bird developed clinical signs on day 33 and was euthanized on day 36. The second challenged bird developed clinical signs on day 41 and was euthanized on day 45. On necropsy, the proventriculus of both birds was slightly enlarged and microscopic examination showed lesions consistent with PDD in the brain, spinal cord, heart, adrenal gland and intestine. A control, uninoculated cockatiel was apparently healthy when euthanized on day 50. ABV2 is now the second ABV genotype to be formally shown to cause PDD.

# DEDICATION

This dissertation is gratefully dedicated to my mom, Parvin Bazargan and my husband, Maysam Pournik, who have been always there for me and supported me through all my endeavors. I would not have been able to achieve my goals including this PhD degree without their love and support.

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# CHAPTER I

# INTRODUCTION: PROVENTRICULAR DILATATION DISEASE AND THE DISCOVERY OF AVIAN BORNAVIRUSES

## Proventricular Dilatation Disease (PDD)

### *History*

In 1979, proventricular dilatation disease (PDD) was initially described in macaws. At that time the disease was called "macaw wasting or fading syndrome", "wasting macaw syndrome", and "gastric distension of macaws" (28). As the disease was reported in other psittacines more general terms such as "proventricular dilatation", "proventricular dilatation syndrome", "proventricular hypertrophy", "proventricular dilatation of macaws or psittacines", and "proventricular dilatation disease" were used (28). Discoveries into possible causes of the disease generated names that reflected the pathologic signs such as "neuropathic gastric dilatation of psittaciformes", "proventricular and ventricular myositis", "myenteric ganglioneuritis", and "psittacine encephalomyelitis" (28).

To date PDD has been reported to affect more than 50 species of psittaciformes, including members of the *Cacatuidae* (i.e., cockatoos and cockatiels) and *Psittacidae* (i.e., lovebirds, macaws, parakeets, parrots, Amazon parrots, and conures) families (28).

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This dissertation follows the style of Journal of Virology.

While PDD was first described in the United States, it is now a well-known and established disease of captive psittacines in the United States, Canada, the United Kingdom, Europe, and Australia (18).

### *Clinical Signs*

PDD causes inflammation of the central, peripheral, and autonomic nervous systems. The clinical signs of PDD may vary to some extent between individuals and species. Clinical laboratory findings are usually inconclusive and may include signs of gastrointestinal dysfunction such as hypoproteinemia and hypoglycaemia or signs of opportunistic infections such as heterophilia. There are two general syndromes that birds may exhibit: neurological and gastrointestinal. Some PDD birds exhibit only neurological signs, others exhibit only gastrointestinal signs and some birds display both neurological and gastrointestinal involvement. Neurological signs are related to effects on the central nervous system (CNS) and include, but are not limited to, ataxia, blindness, abnormal head movements, and seizure. Affected birds tend to lose their balance and may become paralyzed. Development of gastrointestinal signs are caused by paralysis of the proventriculus and include weight loss (associated with regurgitation and the passage of undigested food in the feces), proventricular enlargement, diarrhea, weakness, and starvation (65).

***Diagnosis***

A presumptive diagnosis of PDD is based on history, clinical signs, radiographic evidence of dilatation of proventriculus as well as duodenum descendens and extended transit times of ingestions, fluoroscopic evidence of gastrointestinal dysfunction, and the histopathological examination of biopsy specimens. Definitive diagnosis is based on the demonstration of characteristic lymphoplasmacytic infiltrates within autonomic nerves and ganglia at various levels in the digestive tract (1, 65). The most reliable location of infiltrates has been shown to be the ventriculus (93%) and heart (79%). Unfortunately biopsy is invasive and potentially fatal in debilitated birds (29). Another common diagnostic procedure, which is less invasive and risky, is to perform crop biopsy to look for the lymphocytic infiltration of nerves of the crop. However crop biopsy is not a sensitive indicator of PDD as in a study of 44 birds with clinical diagnosis of PDD, only 30 showed infiltrates in crop biopsies with no clear pattern of the infiltrates' location (31). Historically there have been no cases with infiltrates present in crop without concomitant lesions in the proventriculus or ventriculus. As crop biopsies are not done on healthy birds, these so-called healthy birds cannot be guaranteed to be free of PDD.

***Prognosis***

The prognosis of PDD affected birds is poor and no specific treatment is available at this time (42). However symptomatically treating the affected birds by using anti-inflammatory drugs reduces inflammation of the central and peripheral nervous system as well as the digestive system. Use of anti-inflammatory drugs, in

conjunction with antimicrobials to control secondary infections, can help extend survival for months to years (20, 42).

### *The Etiology of PDD*

There has been much speculation as to the cause of PDD. Evidence of transmissibility was obtained when healthy birds, receiving the tissue homogenates from diseased birds, developed both clinical symptoms and histopathological lesions associated with PDD (30). With no evidence of bacterial infection a viral etiology was suspected and several viral candidates were implicated. The first candidate for a viral etiology of PDD was a paramyxovirus related to Newcastle disease (32). This speculation was based upon observing, by transmission electron microscopy, inclusion bodies and enveloped virus-like particles of 30 to 250 nm in size in the myenteric plexus and celiac ganglion of affected birds. However, serological studies did not support the observation, as paramyxovirus-specific antibodies were not found in diseased birds (39). Many other viruses such as coronaviruses (25), equine encephalitis virus, avian herpes virus, polyomaviruses, adeno-like viruses, enteroviruses, reoviruses, and avian encephalitis virus were, at one time or another, implicated as etiologic agents of PDD (65). In view of the fact that no potential viral agents could be clearly linked to PDD it was proposed that the disease might be autoimmune in nature and initiated by virus infection or gangliosides (65).

Even though the etiology of PDD was unclear until recently, isolation of affected birds was recommended. This suggestion was originally based on the observation of

PDD outbreaks in aviaries, the demonstration that PDD could be transmitted experimentally, and the identification of virus-like particles in tissues and feces of birds affected by PDD (65).

In 2008 and 2009, advanced technologies using fast and unbiased searches for viruses in clinical specimens identified novel Borna disease-like viruses associated with PDD (34, 39). Honkavuori et al. identified at least 2 different bornavirus strains in brains from 3 PDD birds (34). RNA extracted from brain tissue of parrots with PDD was reverse transcribed and directly subjected to high throughput sequencing. By searching for sequence similarities to known viruses, the group found genetic material with striking similarity to Borna disease virus (BDV) (34). In an independent study Kistler et al. also found evidence of bornaviruses in 62% of PDD cases. In this study, RNA extracted from parrots with PDD was hybridized to a pan-viral microarray that carried multiple cDNA probes from known viruses. The initial finding was confirmed using high throughput sequencing (39). Subsequent studies have provided additional support for avian bornavirus (ABV) as the etiological agent of PDD. Ouyang et. al (47) used an antiserum directed against the ABV nucleoprotein (N-protein) to perform immunohistochemistry and observed that 13 out of 13 cases of PDD contained ABV N-protein in their CNS while only one out of 11 reportedly negative cases contained this protein (47). In another study, the clinical and pathological signs of PDD were induced by inoculation of cockatiels via multiple routes (intramuscular, intraocular, intranasal, and oral) with brain homogenates derived from an ABV-infected bird (21). In a more defined study ABV was isolated from the brain of a PDD positive bird and passed in

duck embryo fibroblasts (DEF). The ABV infected cells were administered via oral and intramuscular routes to two healthy Patagonian conures and classical PDD was induced in these two birds by 66 days post-infection (26).

Epidemiological surveys also support the association of ABV with PDD. In one study, ABV was present in 71% of parrots with PDD and was absent in healthy animals (39). Other studies reported similar results, with detection of ABV in most, but not all, parrots with clinically suspected PDD (65). Given the known divergence among ABV strains identified to date (39), the inability of molecular or serological methods to detect ABV in some suspected cases of PDD might be due to strain differences although it cannot be ruled out that a second unrelated virus causes a similar disease.

Although there is a strong correlation between ABV infection and PDD, questions remain concerning routes of natural infection and transmission. It is not uncommon for birds exposed directly or indirectly to an affected bird to remain asymptomatic (28). For example, in a group of mixed species of wild-caught psittacine birds exposed to a bird confirmed by histopathology to have PDD, signs of the disease were not noted in any of the contact birds for up to a year following exposure (29). This might suggest innate resistance in some birds.

## Bornaviruses

### *Borna Disease Virus (BDV)*

BDV was the only known member of the family *Bornaviridae*, until the relatively recent discovery of avian bornavirus (ABV). BDV is a noncytolytic, enveloped RNA virus that most commonly affects horses and sheep in central Europe. The virus is highly neurotropic and infects the central, peripheral, and autonomic nervous systems (36). Thus a detailed examination of BDV may provide a better understanding of ABV and aid in predicting its structure and life cycle as well as aid in designing experiments in to enhance our knowledge of this virus.

Bornaviruses are non-segmented negative strand (NNS) RNA viruses (11) belonging to the family *Bornaviridae* (order *Mononegavirales*) whose prototype member is BDV (36). The BDV genome is approximately 9.9 kb which is significantly smaller than *Paramyxoviridae* (approximately 16 kb) or *Filoviridae* (approximately 19 kb) and quite similar to the *Rhabdoviridae* (approximately 11±1.5 kb)(36). BDV sequences obtained from various species have shown extraordinary sequence conservation uncommon amongst the RNA viruses (36). It has been suggested that the virus has a high degree of genetic adaptation to non-cytolytic replication in the CNS and restricted genetic flexibility (62). Comparison of nucleoprotein and phosphoprotein coding regions among widely distinct BDV isolates reveals variability of up to 4.1% at the nucleotide sequence level and 1.5% to 3% at the predicted amino acid level (36). Recently a new BDV genotype (designated No/98), which is 15% divergent in nucleotide sequence from other BDVs, was isolated from a naturally infected horse in

Austria (36) hinting that BDV sequences may be more genetically variable than previously recognized, an idea that is now supported by the genomic divergence seen among avian bornaviruses (39).

### *Virion Morphology and Physical Characteristics*

Using electron microscopy, BDV infectious particles appear to have a spherical morphology with a diameter ranging from 70 to 130 nm, covered with spikes approximately 7 nm long (14, 43). However, since BDV is very cell associated, it has been difficult to characterize particles any further. Virions or particles have not been seen in infected tissue. Virions are stable at 37ºC and at 4ºC; infectivity is stable for more than 3 months. Infectivity is lost at a pH below 5 or above 12 and by heat treatment at 56ºC. Organic solvents, detergents, formaldehyde, and exposure to ultra violet radiation also inactivate virus infectivity (14, 43).

### *Genome Organization and Encoded Proteins*

The BDV genome is organized into three transcription units (I, II, III) defined by three transcription initiation signals (S1–S3) and four transcription termination signals (T1-T4) which encode at least six open reading frames (ORFs) (Fig. 1)(36). BDV polypeptides, with the exception of N, are translated from polycistronic mRNAs (4). Hence, unlike other members of the order *Mononegavirales* that have open reading frames clearly separated by adjacent termination and transcription start signals, BDV transcription units and transcription signals overlap (13, 62). The first transcription unit

starts at position +44 (or 32) from the genomic 3'-end and encodes the Nucleoprotein p40 (N). The second transcription unit contains overlapping ORFs for p10 (X) and the p24 phosphoprotein (P). The third transcription unit contains overlapping ORFs for the matrix protein (M), the glycoprotein (G), and the L- polymerase (L) (36, 62). In the case of mutlicistronic mRNAs, expression of downstream proteins can be obtained by a leaky ribosome-scanning mechanism, recommencement of scanning after termination of an upstream ORF, or cap-independent internal initiation (4).

Nucleoprotein

The BDV nucleoprotein has two isoforms, p40 and p38. The *in vivo* significance of the two isoforms is unknown. While p40 is derived from the full length ORF, p38 translation starts at the second in-frame AUG codon, thus lacks 13 amino acids at the amino terminus. The two isoforms may also be encoded by two different mRNA species (40). There is a nuclear localization signal (NLS) in the first 13 amino acids of p40 resulting in its nuclear localization whereas p38 primarily localizes to the cytoplasm. Both N isoforms have an internal leucine-rich motif, $L_{128}$TELEISSIFSHCC$_{141}$, which is a nuclear export signal (NES). Both isoforms bind the viral P protein and to RNA to form ribonucleoprotein (RNP) complexes. Interestingly, the NES region of N overlaps the site of interaction with P and it has been shown that the nuclear export activity of p38N is blocked by co-expression of P. These results suggest that BDV N plays a key role in the nucleocytoplasmic transport of RNP complexes in combination with other viral proteins such as P (40, 43, 67). The nucleoprotein (p40) is also a major target for CD8+-T-cell-mediated immune response (52).

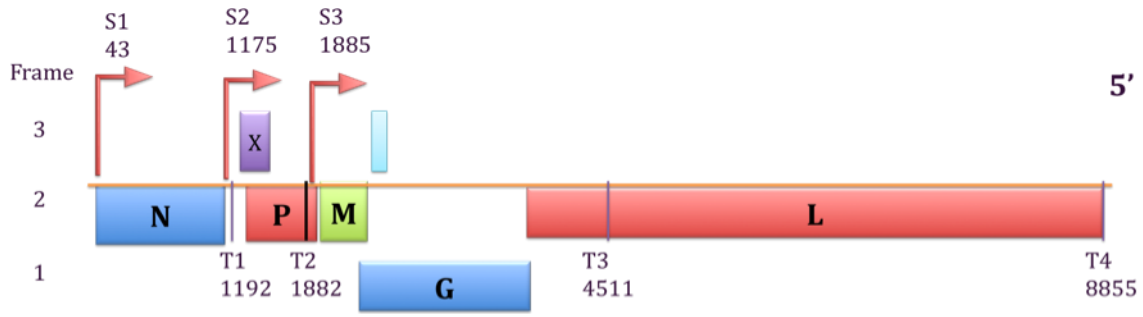


**Fig. 1** Structure of the BDV genome. The BDV genome is organized into three transcription units (I, II, III) defined by three transcription initiation signals (S1–S3) and four transcription termination signals (T1-T4) which encode at least six open reading frames (ORFs). N, nucleoprotein; X, protein X; P, phosphoprotein; M, putative matrix protein; G, glycoprotein; L, polymerase.

X Protein

The smallest transcript of BDV encodes the X and P proteins. The X ORF begins 49 nucleotides upstream from P ORF and overlaps the 71 N-terminal codons of P in a different reading frame (67). The 10-kDa X protein is present in both nuclear and cytoplasmic locations and binds directly or indirectly to P and N. The function of X is unclear, however through binding to P, X gains nuclear localization where it may play a role in negative regulation of BDV polymerase activity (54). Moreover, it has been recently shown that X represents the first mitochondrion-localized protein of an RNA virus and that it inhibits apoptosis in cultured cells and infected animals (53).

Phosphoprotein

The second transcription unit of BDV also encodes a 24-kDa phosphoprotein, P. P has two proline containing NLS, $P_{29}$RPRKIPR$_{36}$ and $P_{181}$PRIYPQLPSAPT$_{193}$ that are found in the amino and carboxyl termini of P (10). It has been shown that P interacts with X, N, and L, as well as itself. While the interaction of P with X, N, and itself occurs at non-overlapping regions, the region mediating homo-oligomerization was found to overlap with the region of interaction with L (43, 67). Oligomerization of P is necessary for polymerase activity based on assays of mutants that could interact with L but that had lost the ability to homo-oligomerize. P is phosphorylated at serine residues by cellular kinases, and phosphorylation influences its ability to form homomultimers, bind other viral proteins, and serve as a transcriptional activator (43, 67).

Matrix Protein

The M protein of BDV is a 16 kDa protein that oligomerizes and stably forms tetramers and octamers. M has been co-purified with N and colocalized with other nucleocapsid proteins, including P, suggesting the association of M with viral RNPs. Moreover, it has been shown that M binds to P but not N and does not inhibit viral transcription. Therefore, M binds to RNPs without inhibiting the viral polymerase (5).

Glycoprotein

The BDV G gene expresses a precursor polypeptide with a calculated molecular mass of 56 kDa. As a result of posttranslational modification by N-glycosylation with high-mannose oligosaccharides, the G precursor (gp94) migrates with a mass of 84 to 94

kDa. Cleavage by the cellular protease furin yields GP-1and GP-2 that correspond to the N- and C-terminal regions, respectively, of gp94. While gp94 accumulates in the endoplasmic reticulum, the GP-1 product is transported to the plasma membrane where it is anchored through its C-terminal transmembrane domain (8, 43). Antibodies to BDV gp94 exhibit neutralizing activity, suggesting that GP-1 or GP-2 or both participate in viral entry (24).

RNA-dependent RNA Polymerase

RNA-dependent RNA polymerase (RdRp) or L is a 190 kDa protein translated from an open reading frame generated by splicing a small upstream ORF to the large 3′ ORF. While the N-terminal half of the L is more closely related to rhabdoviruses, the C-terminal half is more closely related to paramyxoviruses. Similar to other L-polymerases, BVD- L includes RdRp motifs, is phosphorylated by cellular kinases, and interacts with P. Nuclear localization of L is either via its NLS motif or through a complex with P (43).

### *Replication and Life Cycle*

As isolation and purification of ABV is not possible due to the highly cell-associated nature of virus, thus traditional assays were not used to determine the replication and life cycle of BDV. Instead researchers employed various novel techniques and strategies to detect of the early steps of BDV replication and transcription (24). For example, pseudotyping experiments using recombinant vesicular stomatitis

virus were used to study viral entry (51) and a reverse-genetic system for BDV was developed (16, 50, 71).

BDV enters cells by receptor-mediated endocytosis (24). Surface glycoproteins GP1 and GP2 have important roles in receptor recognition and cell entry (8, 24). Using a pseudotyping method, Perez and his colleagues discovered that GP1 is sufficient for virus receptor recognition and cell entry (51). Using a cell surface biotinylation assay and applying lysosomotropic agents (ammonium chloride chloroquine, and amantadine), allowed Gonzalez-Dunia and his colleagues to show that GP2 mediates the pH-dependent fusion event (24). As the virus fuses to host membrane, its ribonucleoprotein RNP is released into the cytoplasm of infected cells (16). BDV replicates primarily in neurons but can infect many other tissues and organs by spreading through the axoplasm of peripheral nerves (16).

Replication and transcription of the BDV genome occurs in the nucleus of infected cells, which is unique among the *Mononegavirales*. The nucleocytoplasmic trafficking of BDV macromolecules is mediated by NLS or NES present in these molecules and has an important role in the virus life cycle (16). The untranscribed sequences at 5’ and 3’ end are of the BDV genome are promoters for expression of the viral positive-sense antigenome and viral transcripts, and expression of the negative-sense genome during replication, respectively. Although there is not any published data on regulation of BDV transcription and replication, the ratio or abundance of viral proteins during the replication cycle in the cell likely regulates the switch from

transcription to production of full length viral anti-genomes as in other *Mononegavirales* (36).

BDV mRNAs are polyadenylated and have homogeneous and genome encoded 5' ends which are thought to be 5' capped (16, 36). Unique among the *Mononegavirales*, BDV uses RNA splicing to regulate gene expression. RNA splicing allows BDV to efficiently use its genome, and may have implications for neurotropism and pathogenesis (16, 36). Differential splicing of the two transcription units regulates expression of the M, G, and L proteins. Splicing of transcription unit I places the amino acid residue in position 13 of the M ORF in frame with a stop codon, abolishing M expression and facilitating G expression. Splicing of transcription units I and II results in an mRNA containing the p8.3 and p190 ORFs, with the p190 ORF encoding the BDV L protein. Moreover, splicing of transcription unit III encodes two new viral proteins with predicted molecular masses of 8.4 and 165 (p165) kDa. The p165 is a truncated form of the BDV L polymerase (13, 16, 36).

### *Cell Tropism of BDV*

A variety of cell lines derived from organs and tissues of different species such as MDCK (Madin-Darby Canine Kidney cells), MDBK (Madin-Darby Bovine Kidney cells), Vero (African green monkey kidney epithelial cells), CrFK (Crandel Feline Kidney cells), C6 (rat glial cells), OL (mouse oligodendrocyte cells), PC12 (rat phaeochromocytoma cells), SK-N-SH (human neuroblastoma cell line) and 293 (human embryonic kidney cells) support the replication of BDV (67). BDV can establish

persistent infection without any cytopathic effects in the cultured cells, therefore infected cells cannot be distinguished from uninfected cells by simple light microscopic examination (67).

Although BDV infections are most commonly observed in horses and sheep, experimental infections of other mammals, such as rabbits, rats, and mice, have been successful (65). BDV infection causes inflammation of the central nervous system (CNS) (67). In naturally infected animals, the most severe inflammation is found in the olfactory bulb, gyrus, caudate nucleus, and the hippocampus, while only moderate changes occur in the spinal cord (67).

***BDV and the Immune Response***

BDV causes severe non-purulent meningoencephalitis with massive perivascular and parenchymal infiltration in naturally infected animals. Monocytes, T lymphocytes ($CD4^+$ and $CD8^+$) and to a lesser extent plasma cells, form the perivascular cuff (17). Despite severe encephalitis, degeneration of neurons is rare in naturally infected hosts, although astrocytosis is commonly observed in the areas of inflammation. In experimentally infected rats and mice, it has been demonstrated that $CD8^+$ T cells that recognize viral antigen play a critical role in both antiviral defense and virus-triggered disease (45). Virus-specific $CD8^+$ T cells induced by immunization can provide protection from infection. However, antiviral $CD8^+$ T cells can be harmful if the virus has infected an extensive number of cells in the CNS, as $CD8^+$ T cells attack infected cells causing severe meningoencephalitis (65, 67). It has been hypothesized that 5'-

terminal genome trimming of BDV might be a strategy used by BDV to down-regulate replication and evade the antiviral host response (63).

### *Transmission*

Intranasal infection by BDV is efficient and the olfactory bulbs of naturally infected horses show inflammation and edema early in the course of disease suggesting the olfactory route as a candidate for BDV transmission. Additionally, experimental infection of neonatal rats shows the presence of viral gene products and infectious virus in saliva, urine, and feces, which are known to be important in transmission of other pathogenic viruses such as lymphocytic choriomeningitis virus and Hantaviruses. Moreover, it has been demonstrated that normal adult rats can become infected if housed in cages separate from, but adjacent to those of neonatally infected rats, suggesting the possibility of aerosol transmission. There is also a potential for hematogenous transmission of BVD as its nucleic acid and proteins have been found in peripheral blood mononuclear cells (PBMC) (38, 60).

### *Avian Bornavirus (ABV)*

In 2008 ABV was reported in association with PDD positive birds (39) and genome sequences were obtained using pyrosequencing (34). Shortly thereafter two additional ABV genomes were sequenced by a second group using ultra high throughput sequencing of tissues from PDD birds (39). The genome organization reported by these two independent research groups was highly reminiscent of BDV but the new virus

showed a higher degree of nucleotide divergence than previously described for BDV isolates. To date, five distinct ABV genotypes have been isolated in psittacine birds (39) and also one additional distinct genotype of ABV was identified in a canary (*Serinus canaria)* suffering from PDD-like disease (70).

***Host Range***

While PDD was first reported in captive parrots of North America and Europe, it has now been observed in psittacines worldwide, with the spreading of disease linked to intensive trading (65). Even though more than 50 species of *Psittaciformes* have shown signs of PDD, most reporting of the disease has been in African gray parrots, blue and gold macaws, cockatoos, and Amazon parrots (65). This distribution may reflect a population bias rather than an actual species predisposition. PDD-like disease has also been observed in species other than psittacine birds (65). Examples include a report of clinical and pathological signs in a canary (*Serinus canaria*), greenfinch (*Carduelis chloris*), long-wattled umbrella bird (*Cephalopterus penduliger*), bearded barbet (*Lybius dubius*), and falcon (*Falco peregrinus*) as well as nonsuppurative encephalitis and ganglioneuritis in wild Canada geese (*Branta canadensis*) (65). Furthermore, lesions suggestive of PDD have been reported for toucans, honeycreepers, weaver finches, and a roseate spoonbill (65).

### *Detection of ABV in Captive Birds*

Molecular Detection

Reverse transcription polymerase chain reaction (RT-PCR) has proven to be a robust technique to detect ABV in tissue samples, collected at necropsy, from birds with a diagnosis of PDD (20, 35, 59). Primers designed for conserved areas of the L, M, and N genes have been used to detect at least 40 ABV isolates of 5 distinct genotypes (20). Quantitative real-time PCR, based on primers and probes within the P gene, has also been used to quantify ABV in different tissues (34). ABV RNA has most often been detected in brain, crop, proventriculus, ventricles, and adrenal glands (57). However, the distribution of the virus in tissues may vary from bird to bird. Some birds may have ABV RNA present in most major organs, as well as in the plasma, while others show a more restricted distribution of virus in their tissues (57). Thus, it is important that several types of tissues, or at least brain and gastrointestinal tract be tested. RT-PCR has also been used to detect ABV RNA *ante mortem* from specimens such as crop biopsy samples, blood, choanal and cloacal swabs, and feces (20, 35). However it appears that testing any one sample may not be sufficient, as preliminary data show that ABV-infected birds shed the virus only intermittently in their saliva or feces (35). Even crop biopsy tissue may test negative in some patients with characteristic signs of PDD (27) as small biopsy samples may not contain any, or enough, nerve ganglia to make a diagnosis. It may be even more significant to note that ABV sequences have been detected in some captive birds with no history of clinical signs (20, 35). In one of our studies, about 20% of purchased, apparently healthy, cockatiels were ABV positive (by

cloacal swabs) and one of those birds showed the presence of the virus in every tissue tested at necropsy (Mirhosseini, unpublished data). These findings suggest that false-negative and false-positive results must be carefully considered when RT-PCR is used to detect ABV infection.

Serological Detection

Since animals produce a detectable amount of antibody to BDV, serology has been applied to its diagnosis (52). The most common tests employed for detection of mammalian BDV have been immunofluorescence, enzyme-linked immunosorbent assay (ELISA) and western blot assays using persistently infected cells, lysates of such cells, or cloned recombinant proteins (68). In birds western blotting has also been employed successfully, as the serum of PDD birds contains antibodies against the ABV nucleoprotein, N (52, 68). From a limited number of observations it appears that a detectable antibody response may develop late, with respect to infection, with antibody predicting development of clinical PDD signs (35, 68). On the other hand we have also, on occasion, detected ABV antibody in apparently healthy birds (35, 68).

***Comparison of ABV and BDV***

Generally, ABV strains show about 65% nucleotide sequence identity with BDV (39). However the gene order as well as the structure of transcription initiation and termination signals and the location of splice donor and acceptor sites are well conserved (39). Thus the first predicted transcription unit encodes the nucleoprotein (N), the second predicted transcription unit encodes the regulatory protein (X) and polymerase co-factor

(P), and the third transcription unit is predicted to code for matrix protein (M), surface glycoprotein (G) and polymerase (L) (39). Similar to BDV, ABV X and P are encoded from overlapping reading frames and the primary transcript of the third transcription unit is likely formed by splicing (39). Additionally, ABV has been found to have a high degree of sequence conservation in the terminal non-coding and intergenic regions of the viral genomes, as seen in BDV (39). The region between the N and X gene in ABV is shorter than in BDV, lacking a 22-nucleotide fragment that serves a regulatory function for the expression of the viral proteins X and P. The absence of these elements in ABV suggests that fine-tuning of the X protein expression may be achieved by other means in ABV-infected cells (39, 59, 65).

While BDV is reported to exhibit a high preference for the CNS in naturally or experimentally infected animals, RT-PCR data and the results of immunohistochemistry show that ABV is present in many, if not all organs, of PDD positive birds (26, 48, 59). Immunohistochemical analyses revealed the presence of ABV antigens in intestinal villi, epithelial cells, lamina propria cells and myocytes supporting the conclusion that ABV can productively infect non-neuronal cells (59). In contrast with BDV, ABV does not appear to be well adapted for growth in cell lines of mammalian origin such as Vero cells or MDCK cells. Instead, ABV has a high preference for avian cells in tissue culture. The quail fibroblast cell line CEC32, the quail skeletal muscle cell line QM7 and DEF are suitable for the isolation of ABV (26).

# CHAPTER II

# PCR-BASED SCREENING FOR AVIAN BORNAVIRUS DETECTION

## Introduction

To advance research into this devastating disease, a group of donated PDD positive birds were assembled in the aviary of the Schubot Exotic Bird Health Center at the College of Veterinary Medicine and Biosciences at Texas A&M University beginning in 2007. Moreover, local veterinarians were encouraged to send samples from birds that died of PDD. To identify the virus associated with PDD, two approaches, virus culture and serology, were initially used. In an attempt to grow the virus, chicken eggs and duck embryo fibroblasts (DEF) were inoculated with sera and tissues from PDD positive birds. Immunofluorescence of infected DEF and immunohistochemical staining of brain of PDD positive birds indicated the growth of an infectious agent (69). Subsequently proteins believed to be viral antigens, were identified using sera from PDD birds in western blot analyses. However, our efforts to purify or visualize virus particles had not been successful when, in 2008, both Honkavuori and colleagues (34) and Kistler and colleagues (39) identified Borna disease virus-like sequences in PDD positive birds using high throughput sequencing and hybridization methods. These breakthroughs enabled us to use polymerase chain reaction (PCR) assays to look for similar sequences and viruses in tissues from PDD birds as well as in cultures inoculated with brains of PDD positive birds.

PCR is an advanced molecular technique which can be used to diagnose infectious diseases with high sensitivity and ease (58). The power of PCR is due to its ability to synthesize millions of copies of a specific gene fragment *in vitro*, starting with a very few copies of target DNA, an amount which is undetectable by other methods (Figure 2). The power of PCR to amplify DNA templates is easily extended to RNA templates, such as ABV, by simply using reverse transcriptase in the initial step. We initially used PCR to detect avian bornavirus sequences in DEF inoculated with brain tissue from PDD birds, and in brain tissues collected at necropsy from PDD birds. This was followed by use of PCR to detect ABV in other tissues of both naturally and experimentally infected birds (35). Using PCR we also detected ABV in fecal samples from both PDD birds and healthy birds (35). Detecting ABV in the feces of healthy birds was unexpected, and led us to initiate some large-scale screening of local aviaries and archived fecal samples.

## Materials and Methods

### *Birds*

The Schubot Exotic Bird Health Center at Texas A&M University had available a collection of 23 psittacine birds of seven species, donated in the belief that they suffered from or were in close contact with cases of PDD. The donated birds included two *Ara chloropterus* (Red-and-green Macaw), two *Ara araraunae* (Blue-and-yellow Macaw), four *Psittacus erithaca* (African Grey Parrot), four *Ara macao* (Scarlet Macaw), two *Pionus chalcoptera* (Pionus), two *Ara auricollis* (Yellow-Collared

Macaw), three *Cyanoliseus patagonus* (Patagonian Conure), two *Nandayus nenday* (Nanday Parakeet) and two *Ara nobilis* (Red-shouldered Macaw). While they remained in good condition these birds were a source of blood and fecal samples; tissue samples were collected from birds eventually requiring humane euthanasia. In addition to the donated birds, fifteen apparently healthy cockatiels (*Nymphicus hollandicus*) were purchased from a single breeder.

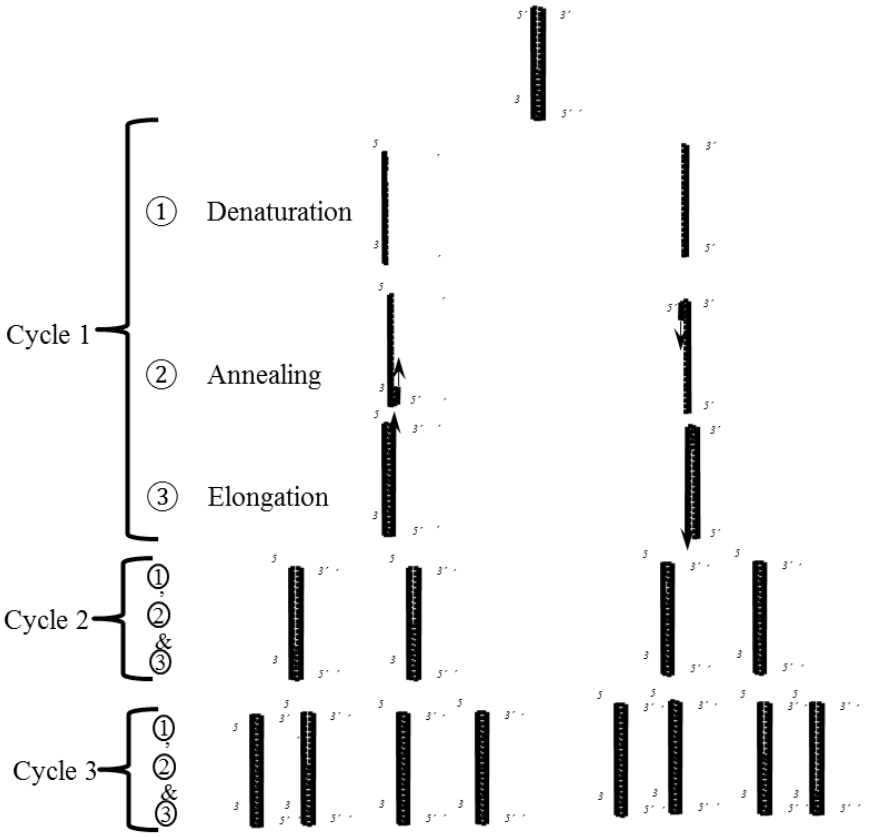


**Fig. 2** PCR cycles. (1) Denaturing at 94–96 °C. (2) Annealing at ~55 to 65 °C (3) Elongation at 72 °C. Three cycles are shown here. The first two lines represent the DNA template to which primers anneal that are extended by the DNA polymerase, to give DNA products, which are used as templates as PCR progresses.

### *Infected DEF*

DEF cell cultures were inoculated with brain tissues from PDD positive birds as described by Gray and colleagues (26). These cultures were maintained in Leibowitz L15–McCoy 5A (LM) Medium, containing 5% new borne calf (NBC) serum and 50 µg/ml penicillin-streptomycin, until processed for RNA purification.

### *Fecal Samples*

To obtain feces from birds housed in the aviary, clean paper sheets were set under the cage of each bird. The birds were kept under observation and as soon as a fecal drop fell onto the paper it was collected with a clean swab and suspended in about 500 to 750 µl of sterile phosphate buffered saline (PBS). Alternatively, the bird was caught, a swab of the cloaca was performed and the swab was re-suspended in PBS. The vials containing fecal material were transported on ice and either immediately processed for RNA isolation or frozen at -80ºC.

### *Tissue Collection*

Tissues from PDD positive or healthy birds were collected for ABV testing. Birds were humanely euthanized and upon necropsy, small pieces of different tissues (brain, spinal cord, heart, lung liver, proventriculus, ventriculus, spleen, crop, and adrenal gland among others) were immediately frozen in dry ice and stored at -80 °C until use.

***RNA Isolation***

Fecal sample processing: Fresh or previously frozen fecal samples, suspended in 500–750 µl of sterile saline, were resuspended by vortexing and the solids were pelleted by brief centrifugation at 2100× gravity (g) in a microcentrifuge. RNA was purified from the supernatants using either the Ambion MagMAX™ Viral RNA Isolation Kit (Ambion) or the QIAamp Viral RNA Mini kit (Qiagen), according to the manufacturer's recommendations. The MagMAX Viral RNA Isolation Kit utilizes a standard method of disrupting samples in a guanidinium thiocyanate-based solution that rapidly releases viral RNA and DNA (6, 7). Paramagnetic beads with a nucleic acid binding surface are then added to the sample to bind to nucleic acids. The beads/nucleic acids are captured on magnets, and proteins and other contaminants are washed away. The beads are then washed again to remove residual binding solution. Nucleic acids are eluted in a small volume of elution buffer. The QIAamp Viral RNA Mini kit combines the selective binding properties of a silica gel-based membrane with the speed of microspin technology. Using this kit, the samples are first lysed under highly denaturing conditions in proprietary buffers containing guanidine salts to inactivate RNases. The samples are then loaded onto the QIAamp Mini spin column where the RNA binds to the QIAamp silica-gel membrane in the presence of chaotropic salts. After a wash step, the RNA is eluted, under low- or no-salt conditions in an RNase-free buffer. Both kits demand use of carrier RNA in their lysis solution in order to improve the binding of viral RNA to beads or membranes. The kits recover total nucleic acids, which means cellular DNA and RNA will be recovered along with the viral RNA from any samples containing cells.

RNA isolation from tissues or infected cell cultures: Total RNA was extracted from tissues or infected DEF using the RNeasy Mini Kit (Qiagen). Tissue aliquots were 0.2 to 0.5 g pieces that were disrupted and homogenized in guanidine isothiocyanate-containing buffer using VWR® Disposable Tissue Grinders (Cat. 47732-446 or 47747-366). To process about $10^6$ DEF cells, the growth medium was aspirated and the cells were washed one time with PBS. The PBS was then aspirated, and PBS containing 0.22% trypsin was added to flasks to detach the cells from the dish. The cells were then pelleted by centrifuging at 300×g for 5 min. Cells were washed with PBS and pelleted again. Cells were then lysed in guanidine isothiocyanate-containing buffer and the lysate was passed through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe at least 5 times, or until a homogeneous lysate was achieved. Cell and tissue lysates were then applied to an RNeasy mini column (Qiagen), washed and eluted as described above.

The concentration of RNA was measured using a NanoDrop® ND-1000 UV-Vis Spectrophotometer by applying 1.5 µl of RNA. The RNA was either immediately used for cDNA synthesis or frozen at -80 °C for later use.

***cDNA Synthesis***

To synthesize single-stranded cDNA from total RNA, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. Briefly the procedure was as follows: A 2X Reverse Transcription Master Mix was prepared as recommended. To create a 1X mix, 10 µl of RNA sample, containing approximately 250 ng to 1 µg of RNA, was added to 10 µl of the 2X RT Master Mix. The final concentration of components for cDNA synthesis were as follows: 1X RT Buffer, 4mM dNTP Mix, 1X

RT Random Primers, 50 units MultiScribe™ Reverse Transcriptase, 20 units RNase Inhibitor, and 250 ng to 1 μg of RNA. The samples were then incubated at 25°C for 10 min, followed by 37°C for 120 min and 85°C for 5 min. The cDNA then was used for PCR.

***Primer Design and PCR***

The primers 1322For and 1322Rev (Table 1) were identical to those of Honkavuori et al. (34). Other primers (Table 1) were designed as additional ABV sequences were collected in our laboratory and/or were available in Genbank (Accession No.: FJ169441.1, FJ169440.1, FJ620690.1, FJ603683.1, FJ002318.1, FJ002326.1, FJ002327.1 and FJ002317.1). Tables 1 and 2 show the list of primer sequences and PCR conditions that were used to detect ABV from feces, tissues or infected cell cultures. The final PCR reactions contained: 1X of Platinum® Taq DNA Polymerase Buffer (minus Mg, Invitrogen), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 1 to 5μl of cDNA and 1.0 unit of Platinum® Taq DNA Polymerase. The PCR products were analyzed by fractionation on 0.8-1% agarose gels containing 0.002% ethidium bromide (10 mg/ml stock) or 1X SYBR® Green (Invitrogen) and were visualized under UV light.

**Table 1 Primers used for detecting ABV.**

| Primer Name* | Bases | Sequence | Tm °C** | Reference |
|---|---|---|---|---|
| Borna 1322For | 21 | CAG ACA GCA CGT CGA GTG AGA | 59 | (39) |
| Borna 1322Rev | 21 | AGT TAG GGC CTC CCT GGG TAT | 59 | (39) |
| Borna 1430Rev | 24 | GGC TCT TGG TCT GAG ATC ATG GAA | 58 | This study |
| Borna 5For | 21 | GCG GTA ACA ACC AAC CAG CAA | 58 | This study |
| Borna 827For | 20 | CAG AGC ACG GGG ACA TGT TT | 58 | This study |
| Borna 1472Rev | 20 | GCC CCT CTG CCT CGA TCA TA | 59 | This study |
| Borna 1212Rev | 25 | GTT CAT TAG TTT GCR AAT CCR GTT A | 54 | This study |
| Borna 1200For | 25 | GTA ACY GGA TTY GCA AAC TAA TGA A | 54 | This study |
| Borna 1200For | 25 | GTA ACY GGA TTY GCA AAC TAA TGA A | 54 | This study |

*Sequence names roughly based on the position in the ABV genome.
**$T_m$s were calculated using OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/)

**Table 2 Primer mixes and PCR conditions to detect ABV.**

| Primer Mix | Forward | Reverse | Target Region | Product Size | PCR Conditions* | |
|---|---|---|---|---|---|---|
| | | | | | 30-40 cycles | |
| | | | | | Annealing Step | Extension Step |
| 2 | Borna 1322For | Borna 1322Rev | X and P genes | 68 bp | 55°C; 30 sec | 72°C; 10 sec |
| 3 | Borna 1322For | Borna 1430Rev | X and P genes | 130 bp | 54°C; 30 sec | 72°C; 15 sec |
| N | Borna 5For | Borna 1212Rev | N gene | 1200 bp | 54°C; 30 sec | 72°C; 1 min |
| 5 | Borna 827For | Borna 1212Rev | N gene | 301 bp | 54°C; 30 sec | 72°C; 10 sec |
| 6 | Borna 1200For | Borna 1322Rev | N and X genes | 200 bp | 54°C; 30 sec | 72°C; 10 sec |
| 21 | Borna1322For | Borna 1472Rev | X and P genes | 223 bp | 55°C; 30 sec | 72°C; 20 sec |
| 22 | Borna 827For | Borna 1472Rev | N, X and P genes | 645 bp | 55°C; 30 sec | 72°C; 40 sec |

*For all reactions the PCR conditions were as follows: Initial denaturation, 94°C for 2 min, followed by 30 to 40 cycles of 94°C, 30 s combined with the annealing and extension conditions listed in the table, followed by a final extension of 5 min at 72°C.

### *DNA Sequence Analysis*

PCR products were cloned into a TOPO-TA vector (Invitrogen) and individual clones were sequenced after transformation into *E. coli*. Alternatively, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Cat. 28104) if only a single product was detected, or gel purified using QIAquick Gel Extraction Kit (Cat. 28704) when more than one band was obtained, according to the manufacturer's recommendations. The purified PCR products were used for direct sequencing using one of the primers used for the PCR. DNA sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and sequences were generated with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were assembled and aligned by using Geneious Pro 4.6.2 software (www.geneious.com) and Sequencher software (http://www.idmb. tamu.edu/gtl/sequencher.htm).

## Results

### *Detecting an Association between PDD and ABV*

Infection of DEF with brain tissue from PDD birds did not show any obvious cytopathic effects typical of viral infection, but immunofluorescence assays using sera from PDD birds indicated the growth and spread of an infectious agent throughout the cultures (69). In addition, proteins believed to be viral antigens were identified using sera from PDD birds in immunoblot assays of brain tissue homogenates (69). While

these assays were well controlled and provided strong evidence of a common viral agent in PDD birds, we were not able to visualize virus particles using electron microscopy (data not shown). However, within 48 h of the first report of BDV-like sequences in the brains of PDD birds (34), we were able to use PCR to screen numerous infected cell cultures and tissues from clinically confirmed and suspected PDD cases.

PCR was first used to determine if DEF cells inoculated with brain tissue from birds with a clinical diagnosis of PDD were, in fact, infected with ABV. We performed reverse transcription (RT) PCR on RNA isolated from infected and control DEF cultures. As shown in Fig. 3**,** using primer mix N (Borna 5 forward and Borna 1212 reverse), a DNA fragment of the expected size (1200 bp) was readily amplified from six DEF cultures originally inoculated with brain tissue from six different PDD birds, but not from two DEF cultures inoculated with material from control birds. Sequence analysis of the positive PCR products revealed that they were 80 to 99% identical, at the nucleotide sequence level, with each other and the initial ABV sequences in deposited in Genbank (Table 3).

**Table 3 Percent nucleotide identity between partial N genes from ABV isolates.**

| | ABV Type 1[a] | M25 | M20 | AG5 | M15 | M10 | M14 | M24 | 06 | ABV Type 4[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| ABV Type 1[a] | 100 | | | | | | | | | |
| M25 | 89.4 | 100 | | | | | | | | |
| M20 | 80.9 | 82 | 100 | | | | | | | |
| AG5 | 80.9 | 82 | 99.7 | 100 | | | | | | |
| M15 | 80.9 | 82 | 99.9 | 99.7 | 100 | | | | | |
| M10 | 80.9 | 82 | 99.6 | 99.5 | 99.7 | 100 | | | | |
| M14 | 81.1 | 81.7 | 99.6 | 99.6 | 99.7 | 99.6 | 100 | | | |
| M24 | 81.1 | 81.7 | 95 | 94.8 | 94.7 | 94.5 | 94.5 | 100 | | |
| O6 | 81.4 | 81.8 | 94.5 | 94.5 | 94.4 | 94.3 | 94.2 | 99.3 | 100 | |
| ABV Type 4[b] | 80.6 | 81.4 | 94 | 94.7 | 94.7 | 94.7 | 95 | 92.7 | 94.5 | 100 |

Isolates were assigned to previously defined ABV groups by generating Neighbor-Joining trees using a 115 nucleotide region of the N gene.

[a] ABV serotype 1 accession No. FJ002329

[b] ABV serotype 4 accession no. FJ603687

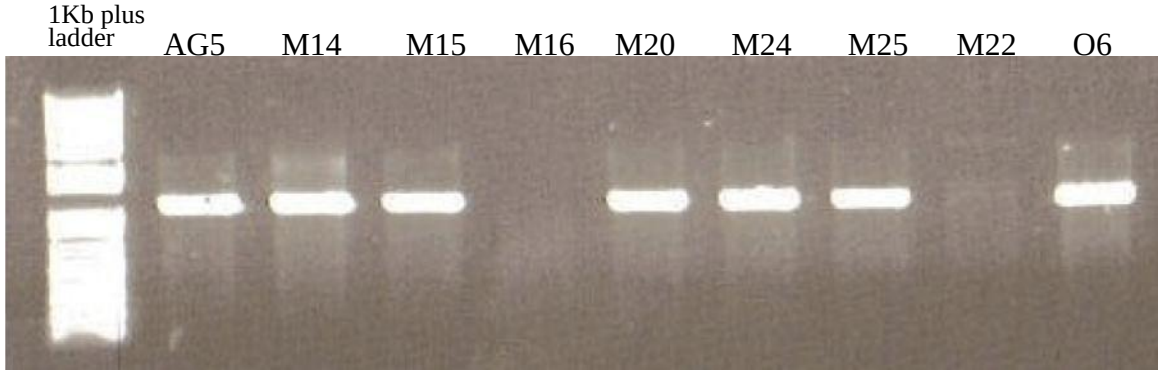


**Fig. 3** Cultured material from brains of PDD positive birds is ABV positive. Performing PCR using primer mix N (Borna 5For and Borna 1212Rev), a band of the expected size (1200 bp) was readily amplified from six DEF cultures originally inoculated with brain tissue from PDD birds (AG5, M14, M15, M20, M24, M25, and O6) but not from two DEF cultures inoculated with material from control birds (M16 and M22).

PCR was next used to detect ABV sequences in tissues of birds with a clinical diagnosis of PDD. Tissue samples from bird O1, a Jenday conure (*Aratinga jandaya*), were processed as described in the Materials and Methods and RNA was used for RT-PCR to detect ABV. Figure 4 shows ABV in central nervous system samples as well as the proventriculus. The presence of ABV in these tissues correlates well with the common clinical manifestations of PDD. Sequence analysis was done to confirm that PCR product is ABV. In other studies, tissues from 30 birds were tested for the presence of ABV. Using PCR, ABV sequences were detected from the brains of 21 birds. Ten of the ABV positive brains were from birds with a clinical diagnosis of PDD. These birds were identified as AG5, M10, M14, M15, M19, M20, M24, M25, O6, and O1. One positive brain sample (CT15) was obtained from a cockatiel euthanized for health reasons that were not apparently PDD-related. The rest of ABV positive brain samples were from birds (ducks and cockatiels) that had been experimentally infected with ABV. More information about all birds tested for ABV is provided in Table 4.

**Table 4 List of the birds checked for the presence of ABV in the CNS.**

| Bird | English name | Scientific name | ABV[a] | WB[b] | History |
|---|---|---|---|---|---|
| AG5 | African gray parrot | *Psittacus erithacus* | + | + | Clincal signs and crop biopsy positive |
| M4 | Blue & Yellow Macaw | *Ara ararauna* | - | - | No clinical signs |
| M10 | Green-wing Macaw | *Ara chloropterus* | + | (-)/+* | Clinical signs but crop biopsy negative |
| M14 | Scarlet Macaw | *Ara macao* | + | + | Clincal signs and crop biopsy positive |
| M15 | Blue & Yellow Macaw | *Ara ararauna* | + | + | Clincal signs and crop biopsy positive |
| M16 | Scarlet Macaw | *Ara macao* | - | - | Presented with a liver mass |
| M19 | Scarlet Macaw | *Ara macao* | - | - | Crop biopsy negative but some abnormal behavior |
| M20 | Blue & Yellow Macaw | *Ara ararauna* | + | N/A | Clincal signs and crop biopsy positive |
| M21 | Blue & Yellow Macaw | *Ara ararauna* | - | - | Clinical signs but crop biopsy negative |
| M22 | Blue & Yellow Macaw | *Ara ararauna* | - | N/A | Clincal signs and crop biopsy positive |
| M24 | Yellow-collared Macaw | *Ara auricollis* | + | + | Clincal signs and crop biopsy positive |
| M25 | Yellow-collared Macaw | *Ara auricollis* | + | + | Clincal signs and crop biopsy positive |
| O1 | Jenday conure | *Aratinga jandaya* | + | N/A | No clinical signs |
| O6 | Goffin's Cockatoo | *Cacatua goffini* | + | + | Crop biopsy positive |

**Table 4 continued.**

| Bird | English name | Scientific name | ABV[a] | WB[b] | History |
|---|---|---|---|---|---|
| CT15 | Cockatiel | *Nymphicus hollandicus* | + | + | Presented with a wing mass, crop biopsy negative |
| CTI2 | Cockatiel | *Nymphicus hollandicus* | - | N/A | Bird was inoculated with untreated DEF; PDD negative |
| CTI6 | Cockatiel | *Nymphicus hollandicus* | - | - | Bird was inoculated with untreated DEF; PDD negative |
| CTI8 | Cockatiel | *Nymphicus hollandicus* | + | N/A | Infected with ABV; PDD-like lesions at necropsy |
| CTI9 | Cockatiel | *Nymphicus hollandicus* | + | N/A | Infected with ABV; PDD-like lesions at necropsy |
| CTI14 | Cockatiel | *Nymphicus hollandicus* | + | N/A | Infected with ABV; PDD-like lesions at necropsy |
| CTI10 | Cockatiel | *Nymphicus hollandicus* | - | N/A | Uninfected bird; PDD negative |
| CTI1 | Cockatiel | *Nymphicus hollandicus* | + | N/A | Infected with ABV; PDD-like lesions at necropsy |
| CTI13 | Cockatiel | *Nymphicus hollandicus* | + | N/A | Infected with ABV; PDD-like lesions at necropsy |
| PG5 | Patagonian Conure | *Cyanoliseus patagonus* | - | N/A | Bird was inoculated with untreated DEF; PDD negative |
| PG7 | Patagonian Conure | *Cyanoliseus patagonus* | + | N/A | Infected with ABV; PDD-like lesions at necropsy |
| PG8 | Patagonian Conure | *Cyanoliseus patagonus* | + | N/A | Infected with ABV; PDD-like lesions at necropsy |
| CG8 | Mallards | *Anas platyrhincos* | - | N/A | Bird was inoculated with untreated DEF; PDD negative |
| IM-duck | Mallards | *Anas platyrhincos* | + | N/A | Infected with ABV; PDD negative |
| IO-duck | Mallards | *Anas platyrhincos* | + | N/A | Infected with ABV; PDD negative |
| IP-duck | Mallards | *Anas platyrhincos* | + | N/A | Infected with ABV; PDD negative |

[a] ABV in detection in brain

[b] Western blot using bird serum to detect antibody reactive with ABV antigens (nucleoprotein)

* Bird was seronegative initially; after 6 months, with the development of the clinical signs of PDD, it became seropositive.

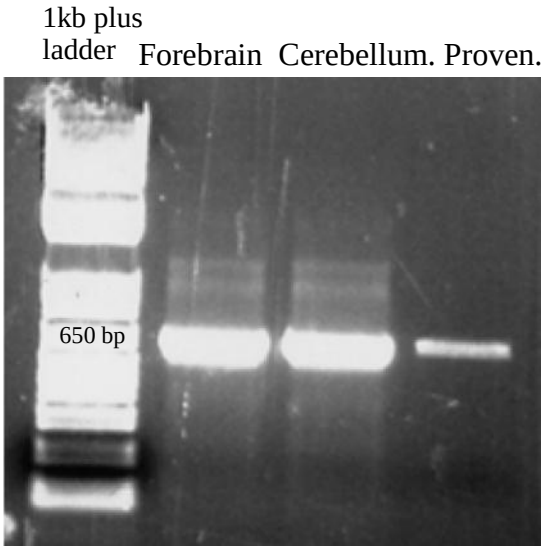


**Fig. 4** Detection of ABV in the CNS and proventriculus. Samples of forebrain, cerebellum and proventriculus (Proven.) of O1 bird were PCR amplified using primer mix 22. One major band near 650 bp appeared in all samples.

While ABV was always detected in brain or CNS tissue from PDD birds, the presence of ABV in other organs was more variable. Figure 5 shows two different extremes. ABV was detected in multiple tissues (heart, lung, liver, spleen, adrenal gland, ventriculus, kidney, and crop) from CT15, a cockatiel seropositive for ABV N antigen but not displaying symptoms characteristic of PDD (Fig. 5a). The CT15 results indicate that ABV may be present in many tissues. In contrast ABV was detected only in brain tissue from M19, a scarlet macaw, a bird with no history of PDD, but housed in the same aviary with other PDD positive birds (Fig. 5b). However the PCR assay alone cannot determine the specific types of cells infected in various tissues.

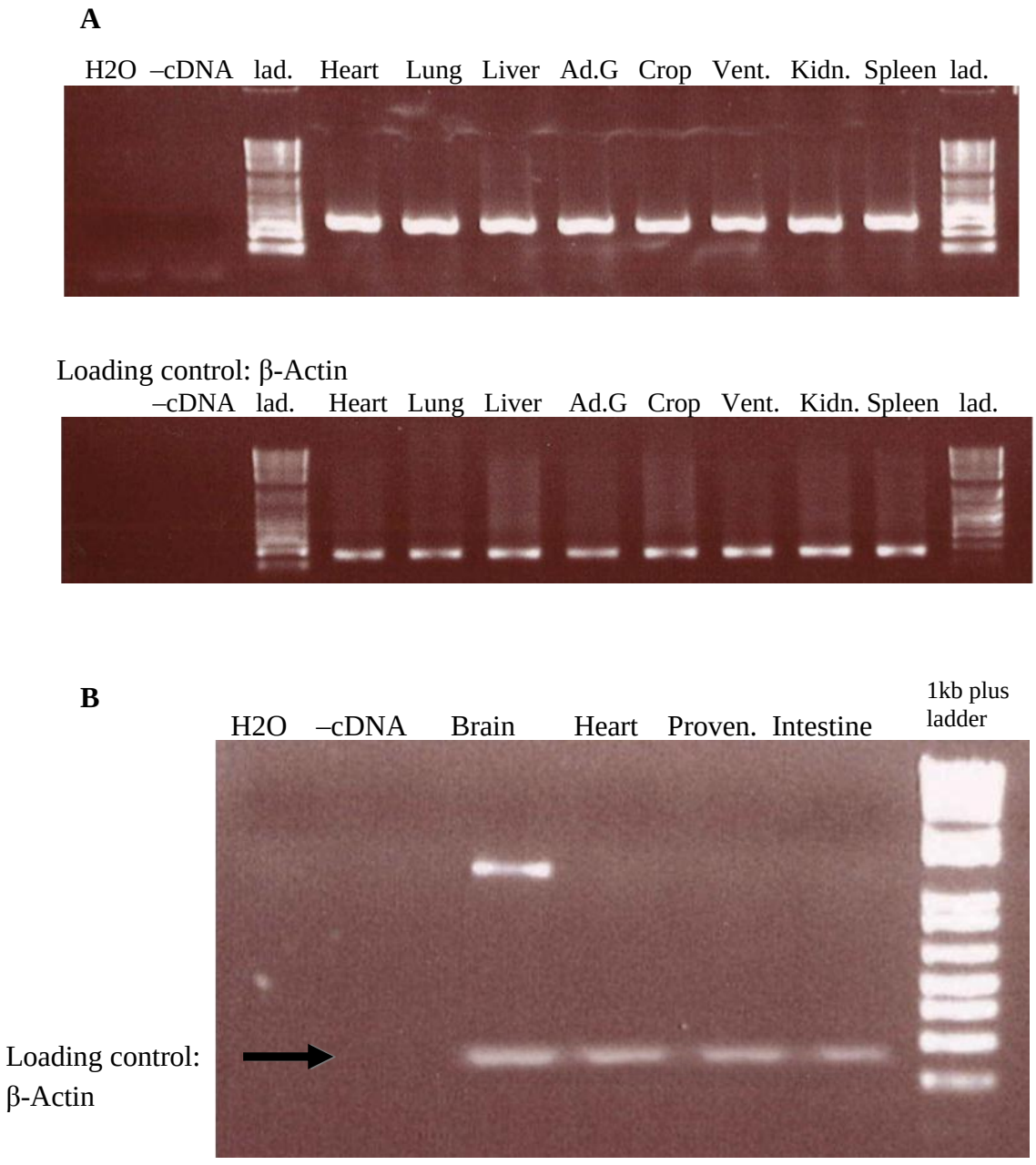


**Fig. 5** Detection of ABV in tissue samples. **a** PCR was performed using primer mix 5 on tissues from CT15. Brain and proventriculus from CT15 were also PCR positive, but are not shown in this figure. **b** PCR using primer mix N on a tissue from a scarlet macaw (M19) showed that ABV can be restricted to the CNS. Amplification of β-actin was used as a loading control in these assays. The DNA ladder (1kb plus) was purchased from Invitrogen.

***ABV Shedding***

To investigate the possibility that ABV is shed by PDD positive birds, fecal material was obtained from three African gray parrots (*Psittacus erithacus*), Juniper (AG1), Quincy (AG2), and George (AG3), housed in the Schubot aviary. These birds had been identified as affected with PDD, and were crop biopsy positive and sero-positive for ABV N antigen, but were apparently healthy at the time of fecal sample collection. Figure 6a shows the presence of ABV in all three samples, but not in the fecal material from a healthy African gray parrot, Zelda (AG8). AG8 was also seronegative and had a negative crop biopsy.

To check the pattern of ABV shedding from PDD positive birds, the three African gray parrots were monitored over a period of 15 weeks. The results showed that these birds shed ABV at levels detected by our PCR assay almost continuously (Fig. 6b and Table 5). ABV was detected at all sampling dates from AG1 and in 12 of 13 samples from AG2 and AG3. This study also provided information about processing of fecal samples for ABV. We determined that ABV sequences could be recovered from cloacal swabs as well as fecal swabs (Fig. 6b) and showed that ABV can be recovered after samples are frozen and thawed. In this study ABV was detected from frozen fecal material after 2 weeks.

**Table 5 ABV shedding by three PDD positive African gray parrots.**

| Collected date | Sample type | RNA Processing Date | Number PCR positive |
|---|---|---|---|
| 8/5/09 | Feces | The same day | 3/3 |
| 2/24/09 | Feces | The same day | 3/3 |
| 3/2/09 | Feces and cloacal | The same day | 3/3 |
| 3/9/09 | Feces | A week later | 2/3 |
| 3/16/09 | Feces | The same day | 2/3 |
| 3/24/09 | Feces | 10 days later | 3/3 |
| 3/31/09 | Feces | 3 days later | 3/3 |
| 4/6/09 | Feces | 10 days later | 3/3 |
| 4/13/09 | Feces | 3 days later | 3/3 |
| 4/20/09 | Feces | 2 weeks later | 3/3 |
| 4/27/09 | Feces | A week later | 3/3 |
| 5/4/09 | Feces | The same day | 3/3 |
| 6/10/09 | Feces | 5 days later | 3/3 |

In order to determine possible shedding of the virus through other sites, additional samples were collected from the three ABV fecal-positive African gray parrots. Using RT-PCR, ABV was detected in swabs of the nares, the choana, and the feathers in the axilla (Fig. 6c). All choanal swabs were positive at this sampling date, suggesting that this may be an important site of shedding and useful for ABV diagnosis in captive birds that can be easily handled. Shedding of virus from the feather follicles was somewhat unexpected and has implications for virus spread through shed feathers.

***ABV in Healthy Birds***

We accidentally discovered that a flock of healthy cockatiels, with no history of PDD, contained ABV-infected birds. These cockatiels were purchased from 'aviary A' for an ABV challenge study and were checked for viral shedding in preparation for that study. The first time fecal samples were tested, we were surprised to see that 7 out of the 15 birds were positive for ABV (Fig. 7; lanes1–10 and 16–23). The birds were checked three additional times, and each time a different set of birds was ABV positive. When we returned to the aviary and randomly checked 24 additional cockatiels by cloacal swab, sixteen percent were positive for ABV (Table 6).

To determine the extent of ABV infection among different flocks of cockatiels two more aviaries were checked for ABV (Table 6). One of the aviaries (aviary B) was, like aviary A, an "open" aviary into which new birds were frequently introduced. The third aviary (aviary C) was a "closed" breeding colony. In aviary B, 24 birds were randomly checked, and 20% of fecal samples were positive for ABV. However, in aviary C, we did not detect any ABV positive fecal samples.

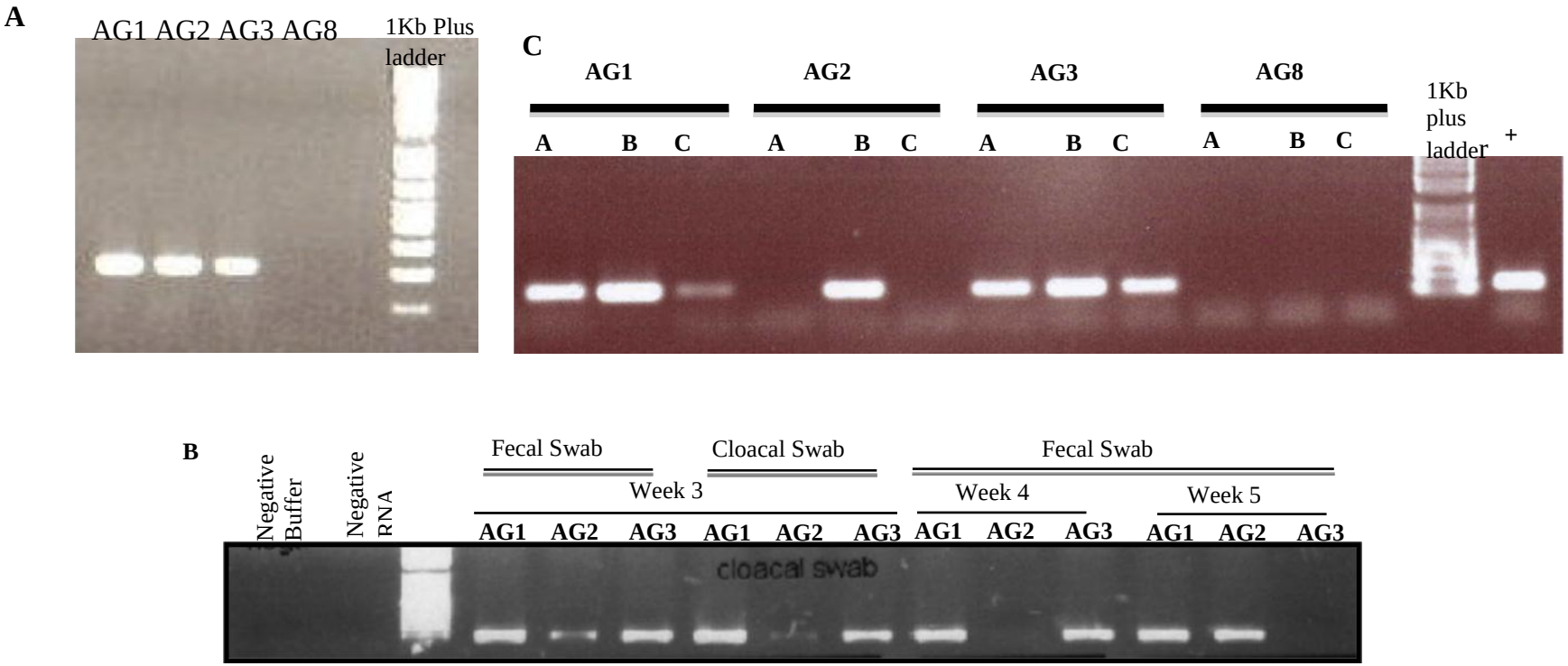


**Fig. 6** Use of PCR for detection of ABV in 3 African gray parrots. AG1, AG2, and AG3 were all crop biopsy positive and seropositive for ABV. AG8 was consistently negative on PCR, serology, and crop biopsy. **a** Performing PCR using primer mix 21, a band of the expected size was readily amplified from bird feces. **b** Fecal samples from 3 seropositive African gray parrots. PCR results show shedding to be continuous (AG1) or intermittent (AG2, or AG3). **c** Shedding of ABV is not restricted to fecal route. PCR with primer mix 3 shows that the AG1, AG2, and AG3 may shed the virus from different body sites such as nares (A), choana (B), and feathers (C).

**Table 6 Survey of three cockatiel aviaries for ABV.**

| Aviary | Samples | Collection date | # of tested samples | % positive birds |
|---|---|---|---|---|
| A* | cloacal swab | 3/26/09 | 15 birds | 46% |
| A* | cloacal swab | 4/10/09 | 14 birds | 42% |
| A* | cloacal swab | 4/20/09 | 14 birds | 42% |
| | | | Average of positive birds: | 43% |
| A** | cloacal swab | 4/15/09 | 24 fecal swab | 16% |
| B | feces | 5-14-20009 | 10 fecal swab | 20% |
| C | feces | 5/22/09 | 11 fecal swab | 0% |

*Purchased cockatiels
** Samples collected from aviary

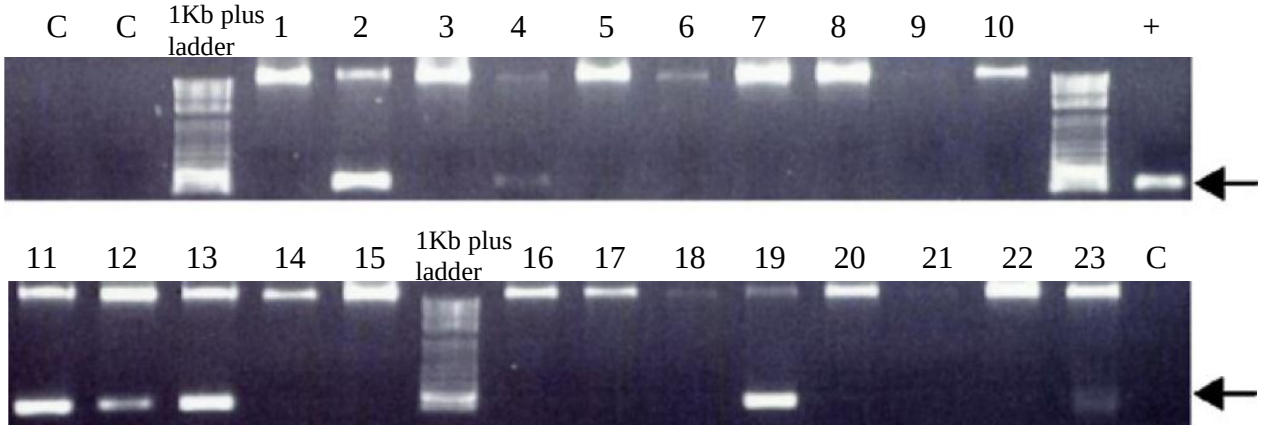


**Fig. 7** PCR for the detection of ABV sequences in bird feces using primer mix 3. Lanes1–10 and 16–23: PCR products from fecal samples from healthy cockatiels. Lanes 11–15: PCR products from fecal samples from PDD African gray parrots. Lanes 2, 4, 11, 12, 13, 19 and 23 show the presence of a band corresponding to the expected 131 bp fragment using primer mix 3. Lanes labeled M contain 100 bp ladder. Lanes labeled C are negative reagent controls. The positive control (+) lane is PCR product from the brain of a PDD case. The high molecular weight band seen in most fecal samples is a nonspecific product.

### *Comparison of PCR with Serology for the Detection of ABV*

We compared results of PCR assays with the serology from 28 birds in our aviary in a single study. PCR results on the fecal samples of these birds using primer mix 3 showed that 18 out of 28 (64%) of the birds were shedding ABV. However only 6 (33%) of the PCR positive birds were seropositive by Western blot. The other 12 (63%) of the fecal positive birds were seronegative. Surprisingly, 3 out of 10 (33%) PCR negative birds were seropositive. Only 7 out of 28 birds were both PCR negative and seronegative. The results of this study have been published (68) and are summarized in Table 7.

**Table 7 Comparison of western blot and PCR for detection of ABV infection.**

| | Fecal PCR Positive | Fecal PCR Negative | Total |
|---|---|---|---|
| Seropositive | 6 (66%) | 3 (33%) | 9 |
| Seronegative | 12 (63%) | 7 (37%) | 19 |

## Discussion

### *Use of PCR for ABV Surveillance*

PDD is frequently a fatal disease in psitassines. Just recently, it has been discovered that ABV is associated with PDD and likely causes the disease (12, 21, 26).

Using PCR, we were able to identify ABV in PDD cases. While use of the PCR technique for disease diagnosis may appear easy and straightforward, in reality it can be tricky and unreliable as there are many variables that impact results.

One obvious variable is sample collection. In terms of ease, PCR can be performed on fecal samples, which can be obtained without harm or stress to the birds. When birds are caged individually, or in pairs, fresh fecal samples, attributed to specific birds, can be collected. However, as shown in Fig. 7A, some birds do not shed the virus in fecal material at detectable levels all the time. Virus shedding is not limited to fecal samples and virus can be detected from other body sites such as nares, the choana, and the feathers. While such samples might be more reliable for detection of ABV shedding, they cannot be readily collected unless the birds are caged, and can also be handled safely (particularly important for the large macaws).

Other variables that impact PCR results include the frequency and level of virus shedding and sample handling. For example, in the healthy cockatiel study (Fig. 8 and Table 6) detection of ABV in fecal samples was demonstrated to be intermittent. In addition, PCR failed to detect ABV in 33% of ABV seropositive birds housed in the Schubot aviary. Given that ABV, like BDV, is a chronic infection, we assume that most seropositive birds are virus carriers. Thus the 'false negative' rate of the PCR assay was as high as 33% among our aviary birds. Finally, while our results indicate that ABV can be detected from fecal samples frozen and stored at -80C, it is likely that sample shipping and storage conditions can contribute to degradation of viral RNA, impacting PCR results. Another confounding factor to be considered is ABV genome sequence

variability (see Chapter 3). There is not yet a universal primer set that can detect all ABV isolates. Hopefully by sequencing more ABV genotypes, that problem can be resolved. Taken together, the variables discussed above strongly suggest that use of PCR alone is inadequate for ABV diagnosis or surveillance in individual birds. However, as the technique is sensitive, non-invasive, inexpensive, rapid and generally easy to perform, PCR screening is a good method for ABV detection amongst flocks, where a positive result is a strong indicator of the presence of the virus among members of the flock.

Serology was also used to detect ABV infection in aviary birds with suspected or confirmed PDD. When the results of PCR and serology in this small study were compared, it was clear that neither assay alone is ideal because the results were discordant. Among 18 PCR positive birds, 12 (66%) were seronegative. Serology did detect ABV infection (past or present) in 3 of 10 (30%) PCR negative birds.

***ABV Infection of Clinically Healthy Birds***

ABV infection, as indicated by a positive PCR result may not correspond with PDD, as we detected ABV in 'healthy' cockatiels in two aviaries. During single surveys of three aviaries, evidence of ABV infection was found in aviaries A (16%) and B (20%) while there was no obvious evidence of clinically ill birds in the flocks on the sampling dates. Among a group of purchased cockatiels, ABV was detected by PCR in 63% of birds that were seronegative and clinically healthy (68). However, in the case of one cockatiel, CT15, a bird euthanized because of a tumor in its wing and a general diagnosis of 'poor performance', ABV was found in all tissues tested by PCR although gross

necropsy findings did not indicate PDD. Among birds donated to the Schubot aviary, several are PCR positive but show no signs of ill health. Finally, it has been demonstrated by others that healthy macaws can coexist with the virus for a long time without showing any noticeable clinical signs of disease (12).

***Assessing PCR False Positives or PCR Contamination***

Sensitivity of the PCR reaction makes it a powerful tool for pathogen detection. We checked the sensitivity and specificity of our PCR reactions at the level of cDNA for a single primer set and showed that this primer pair can detect as few as copies ten of ABV (data not shown). However, due to the sensitivity of the PCR technique some false positive results were observed at the initial stage of the study (data not shown). These were likely due to cross-contamination of samples during processing. In order to overcome this issue, the RNA isolation, cDNA synthesis and assembly of PCR reactions were performed in an isolated room that was kept free of PCR products or ABV-sequence containing plasmids. This strategy, along with the use of multiple negative controls to check reagents and sample processing helped to minimize false positive results.

When performing standard PCR reactions, false positive reactions can also result from amplification of non-ABV products of similar size to the expected products. Thus in order to make sure that the amplified fragments were part of ABV genome, the PCR products were routinely sequenced. Sequencing data showed that the majority of our primers were very specific for detecting ABV when the samples were obtained from captive psitticines. However in a survey of hunter-harvested waterfowl samples

consisting largely of blue-wing teal (*Anas discors*), green-winged teal (*A. crecca*), northern shovelers (*A. clypeata*) and gadwall (*A. strepera*) (19) only false positive PCR reactions were obtained (data not shown). In some cases the DNA products were not of the expected size and it was not surprising when sequence analysis did not yield ABV sequences. However, in some instances the PCR products were of the appropriate size but were not viral in origin.

Sequence analysis of PCR products was also used as a means to detect possible PCR contamination. In the waterfowl survey mentioned above, where approximately 600 RNA samples were screened for ABV sequences, 4 samples did yield PCR products that were ABV by sequence analysis. However closer examination showed that the sequences were identical to a laboratory isolate and one sample yielding ABV products was a negative control RNA sample. In these cases of possible (probable) laboratory contamination less than 1% of the overall test samples were positive. So in order to detect this level of contamination, at least 100 negative samples should be run.

***Tissue Tropism of ABV***

ABV was recovered from the CNS of all infected (either naturally or experimentally) birds, which, due to the neurotropic nature of bornavirus, was expected. However, our data also showed that the ABV may not be restricted to CNS but may also be present in other tissues. Currently, we do not have any explanation for the distribution of the virus in different tissues and in any case, the PCR assay on whole tissue samples does not provide any information about the cell types infected. In the case of BDV, although it has been reported that virus is restricted to the central nervous system in

naturally infected animals (horses and sheep), BDV-RNA was detected also in the spinal cord, eye, nasal mucosa, parotid gland, lung, heart, liver, kidney, bladder and ovaries of in naturally infected animals with clinical disease (41). In addition, BDV-specific RNA was detected in conjunctival fluid, nasal secretions and saliva of two naturally infected animals (41). Moreover, in naturally infected white-toothed shrews, *Crocidura leucodon*, BDV was detected in most of the tissues examined, without evidence of pathological lesions (56).

# CHAPTER III

# MOLECULAR CHARACTERIZATION OF AVIAN BORNAVIRUS GENOMES

## Introduction

Avian bornavirus was first detected and described in 2008 by Kistler et al. (39) and Honkavuori et al. (34) through efforts to identify the cause of proventricular dilatation disease (PDD) of captive psitticines. Over the past 3 years the links between ABV and PDD have been confirmed in numerous studies, and Koch's postulates have been fulfilled by demonstrating that laboratory cultured ABV causes PDD (26). PDD is a neurological disease frequently accompanied by the development of a severely dilated proventriculus. It is thought that virus replication in the enteric ganglia (where virus antigen in readily detected) causes a failure of gut motility (59). Stasis of the proventriculus leads to accumulation of food, impaction, a dilated proventriculus and starvation. The virus also replicates in the central nervous system where it can cause a severe encephalomyelitis with accompanying neurologic abnormalities (26, 59) .

Borna disease virus (BDV) is a close relative of ABV and is the prototype species in the family *Bornaviridae*. It is highly neurotropic and was first recognized as the etiologic agent of Borna disease among livestock, notably horses, in central Germany (23, 36). Characterizing BDV was challenging due to its highly cell-associated nature and the complete lack of cytopathic effects in cultured cells (36). Borna disease was recognized for at least 2 centuries before the infectious agent was cultured and

characterized in the latter half of the 20th century (23). Most isolates of BDV are highly related, showing less than 5% nucleotide sequence heterogeneity. Few reliable reports of the disease, or of virus isolation, originate from outside Saxony Germany, although serologic and molecular diagnostic surveys suggest that the virus may be very widespread in nature, infecting a variety of mammals, including sheep, llamas, ostriches, cats, and cattle (33).

The recent discovery of ABV as a disease agent of captive parrots was achieved by cutting-edge high-throughput sequence analysis of brain tissue from parrots with PDD (34, 39). Initial findings were quickly followed by recovery and molecular characterization of many distinct ABV strains (39). Based on partial sequence data of ABV isolates recovered from a variety of captive birds it appears that ABV is much more diverse than BDV. We have sequenced 8 ABV isolates from birds with a clinical diagnosis of PDD. These isolates were recovered from seven different species (*Psittacus erithacus*, *Ara macao*, *Ara ararauna*, *Nymphicus hollandicus*, *Aratinga jandaya*, and *Aratinga jandaya*) of psitticines. In addition, partial ABV sequences were recovered from numerous other isolates as part of a diagnostic service and surveillance program at the Schubot Exotic Bird Health Center at the College of Veterinary Medicine at Texas A&M University. We compare the nucleotide and translated protein sequences of our ABV isolates to each other, and to the type strain of BDV. Our data confirms the organization of two ABV isolates previously reported by Kistler (39) and Rinder (59). One of our sequenced ABV belongs to genotype 1 and we believe that this is the first report of a complete genome sequence for a member of this genotype.

## Materials and Methods

### *ABV Positive Birds*

Eight PDD positive birds belonging to seven species (one *Psittacus erithacus* (AG5), one *Ara macao* (M14), two *Ara ararauna* (M15 and M20), one *Nymphicus hollandicus* (CT15), one *Aratinga jandaya* (O1) and one *Aratinga jandaya* (O6) were used for isolation of ABV and the resulting virus isolates were completely sequenced. All birds but CT15 showed clinical and/or pathological signs of PDD related to the digestive tract (reduced body weight, diarrhea, undigested food in feces, or dilated proventriculus) or to the nervous system (tremor, ataxia, or convulsions) and were humanely euthanized. Upon necropsy, some brain samples were immediately processed for RNA isolation or for virus isolation in DEF. Alternatively, samples were snap frozen in dry ice and stored at -80 °C until they were processed.

### *Virus Isolation and Cell Culture*

Sections of the cerebrum and cerebellum were homogenized, minced, and then passed through an 18-gauge needle in Leibowitz L15–McCoy 5A (LM) complete medium. In instances where immediate inoculation of DEF cultures was not possible, frozen brain tissue was thawed in a 37 °C water bath immediately before inoculation. One milliliter of the brain suspension was used to inoculate DEF monolayers, obtained from 13-day old decapitated and eviscerated duck embryos by trypsinization (using 0.25% trypsin). DEF were incubated in complete LM medium containing 5% new born calf (NBC) serum and 50 µg/ml penicillin-streptomycin for 24 h. The inoculated DEF

cultures were then washed once with phosphate-buffered saline (PBS), replaced with fresh LM medium supplemented with 2% fetal calf serum, and incubated for 5–7 days. Infected DEFs were trypsinized using 0.25% trypsin and cocultivated with freshly plated DEFs. This procedure was repeated for a minimum of 3 passages.

***RNA Isolation***

Total RNA was extracted from brain tissue or infected DEF using the RNeasy Mini Kit (Qiagen). Tissue aliquots were 0.2 to 0.5 g pieces that were disrupted and homogenized in guanidine isothiocyanate-containing buffer using VWR® Disposable Tissue Grinders (Cat. 47732-446 or 47747-366). To process about $10^6$ DEF, the growth medium was aspirated and the cells were washed one time with PBS. The PBS was then aspirated, and PBS containing 0.25% trypsin was added to flasks to detach the cells from the dish. The cells were pelleted by centrifuging at 300 X g for 5 min and were washed with PBS and pelleted again. Cells were then lysed in guanidine isothiocyanate-containing buffer and the lysate was passed through a 20-gauge (0.9 mm) needle attached to a sterile plastic syringe at least 5 times–or until a homogeneous lysate was achieved. The samples were loaded onto the RNeasy mini column (Qiagen), washed and eluted as suggested by the manufacturor (Qiagen).

The concentration of RNA was measured using a NanoDrop® ND-1000 UV-Vis Spectrophotometer by applying 1.5 µl of sample. The RNA was either immediately used for cDNA synthesis or frozen at -80 °C for later use.

***cDNA Synthesis***

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize single-stranded cDNA from total RNA using random hexamer primers. Briefly the procedure was as follows: A 2X Reverse Transcription Master Mix was prepared as recommended by manufacturer. To create a 1X mix, 10 µl of RNA sample, containing approximately 250 ng to 1 µg of RNA, was added to 10 µl of the 2X RT Master Mix. The samples were then incubated at 25 °C for 10 min, followed by 37 °C for 120 min and 85 °C for 5 min. The cDNA is then used immediately for PCR or stored frozen at -80 °C.

***Primer Design and PCR***

Forty-eight primers were designed to amplify and sequence different regions of ABV. The forward and reverse primers initially used were designed based on available Borna sequences in GenBank® (Accession NO.: FJ169441.1, FJ169440.1, FJ620690.1 and NC_001607) (3, 39). Table 8 lists the primers that were used to amplify and sequence the ABV genomes. Table 9 indicates the amplified regions and the primers used for sequencing. The PCR conditions used to generate ABV fragments were as follows: Initial denaturation, 94 °C for 2 minutes, followed by 30 or 40 cycles of 94 °C, 30 s combined with the annealing at 50 to 55 °C (based on primer melting temperatures (Tm)) for 30 seconds and extension at 72 °C for 1 to 3 minutes (depending on fragment length), followed by a final extension of 10 minutes at 72 °C. The final PCR reactions contained: 1X of Platinum® Taq DNA Polymerase Buffer (minus Mg, Invitrogen), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 1 to 5µl of cDNA and 1.0

unit of Platinum® Taq DNA Polymerase. The PCR products were analyzed by fractionation on 0.8 to 1% agarose gels containing 0.002% ethidium bromide and were visualized under UV light.

***Rapid Amplification of cDNA Ends***

In order to obtain the 5'end of ABV genome, we performed rapid amplification of cDNA ends (RACE) using the Invitrogen™ 5´ RACE System, Version 2.0. In brief, a gene-specific primer, GSP-Borna 8150For (CTT CAT GGT GAT CCT T), was used to synthesize the first strand of cDNA according to manufacturer's protocol. This cDNA was purified using the QIAquick PCR purification kit (Qiagen, Cat. 28104) and terminal transferase (TdT, Invitrogen) tailing with dCTP was performed as recommended by the manufacturer. Tailed cDNA was then amplified by PCR with Platinum® Taq DNA Polymerase (Invitrogen) using 5-µl volumes of the tailing reaction and the Abridged Anchor primer (5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′, Invitrogen) and the gene-specific primer, GSP3-Borna 8260For (GTG GTG TTC ATA TTG GAG GAC CAT CKG ACT CAT). The conditions for amplifying the 5' end included an initial denaturation at 94 °C for 3 minutes, thirty-seven cycles at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 minute, followed by a final extension at 72 °C for 7 minutes.

In order to generate the 3' end of the ABV genome, cDNA synthesized with random hexamers was linearly amplified using the gene specific primer, GSP1-Borna 700Rev (GTC AGT AGT TAA TAA AGC AA PCR). Conditions for linear amplification of the 3'end were as follows: an initial denaturation at 94 °C for 2 minutes,

forty cycles at 94 °C for 30 sec, 40 °C for 30 sec, and 72 °C for 1 min and 40 sec, followed by a final extension at 72 °C for 10 min. Linear amplified products were then gel purified and tailed as described above. Tailed products were then amplified by PCR with Platinum® Taq DNA Polymerase (Invitrogen) using 5 µl volumes of the tailing reaction and the Abridged Anchor primer (5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′, Invitrogen) and gene-specific primer, GSP2-Borna 515Rev (GCG GAT GAG CCT ATG ACC ACT CCA AT). The conditions for amplifying the 3' end included an initial denaturation at 94 °C for 2 minutes, thirty-seven cycles at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min.

PCR products of the 5'- and 3'-RACE reactions were visualized in 0.9% agarose gels containing 0.002% ethidium bromide. Products were cloned into the pCR® 2.1 vector (Invitrogen) using the Invitrogen TA Cloning® Kit (Cat. KNM2040-01) and then TOP10 cells were transformed according to the manufacturer's protocol (One Shot® Chemical Transformation Procedure; Invitrogen). The transformed cells were spread on LB plates containing 50 µg/ml kanamycin and incubated overnight at 37 °C. The resulting colonies were PCR-screened to identify 5'- and 3'- RACE PCR products. The positive cells were then grown overnight at 37 °C in 3 ml of LB medium contacting 50 µg/ml kanamycin. The 5 Prime FastPlasmid Mini Kit (Invitrogen, Cat. 2300010) was used for plasmid purification.

***Sequence Analysis***

Some PCR products, including all of those generated by 5' and 3' RACE, were cloned into a TOPO-TA vector (Invitrogen) and individual plasmids were sequenced. Alternatively PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Cat. 28104) (if only a single product was detected) or gel purified using QIAquick Gel Extraction Kit (Qiagen, Cat. 28704) (when multiple bands were obtained), according to the manufacturer's recommendations. The purified PCR products were used for direct sequencing using one of the primers used for the PCR. All DNA sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and sequences were generated with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were assembled and aligned using Sequencher 4.8 (http://www.idmb.tamu.edu/gtl/sequencher.htm) and Geneious Pro 4.6.2 software (www.geneious.com). Available ABV genome sequences (FJ620690.1 and EU781967.1) were used to assist in contig assembly, however in all cases, overlapping PCR fragments were obtained and PCR primer sequences were removed from the data.

***Phylogeny***

The full-length nucleotide and deduced amino acid sequences of respective genes were used for phylogenetic analyses. Multiple sequence alignments were done by ClustalW and phylogenetic trees were constructed using the neighbor-joining method.

**Table 8 Primers used for sequencing the ABV genome.**

| Sequence Name | Bases | Sequence[a] | Tm °C |
|---|---|---|---|
| Borna 5For | 21 | GCG GTA ACA ACC AAC CAG CAA | 59 |
| Borna 1200For | 25 | GTA ACY GGA TTY GCA AAC TAA TGA A | 54 |
| Borna 1700For | 31 | GAA TGA RAC TAT GAA RAT GAT GAT GGA GAA A | 55 |
| Borna 3800Rev | 25 | CCC CAR GTY AKT GTA ACM ACT CKA A | 57 |
| Borna 5400Rev | 20 | GGY CGR AAW GCR TTA CAC CA | 57 |
| Borna 3310Rev | 21 | CGC TGA CCT GAR TTA ACA GTT | 54 |
| Borna 2430For | 19 | GGG GGT TCT TTG GWA GTT A | 52 |
| Borna 5190For | 21 | GAC TAT GAG YTC RAC BTC ACT | 53 |
| Borna 7580Rev | 21 | CCT TAG GTC CGC AAG AGA AAA | 55 |
| Borna 8640Rev | 27 | GCC TTC GAG TAA AAA TAA TAT GTA ATT | 50 |
| Borna 6880Rev | 25 | GGC AGT ATT TGA CCC TAA GTA CAA A | 55 |
| Borna 6100For | 23 | CCA AGT GTR TTT TTC CGY GGA AT | 56 |
| Borna 8690Rev | 24 | GGT ATC CTT CCA TTA ATC GTG ATT | 53 |
| Borna 8490Rev | 24 | CCA ATA ACT GTC CAC AAG TGC CTT | 58 |
| Borna 6540For | 20 | GGA AGA TGT GGT GCT TGA GA | 55 |
| Borna 7380For | 19 | GCC AGT ATC TGT GCC WGA A | 55 |
| GSP1-Borna 700Rev | 20 | GTC AGT AGT TAA TAA AGC AA | 45 |
| M25 | 22 | CCA ACT CTA CCC ACA GCT CCA A | 59 |
| M25-Borna 4740Rev | 21 | CGA TCT GTT GCA TCC TGG TTT | 56 |
| GSP1-CT15 Borna 705Rev | 18 | CCA CTA ATT TGA TCT GAT | 43 |
| GSP1-M25Borna 695Rev | 17 | GGT AGT TAG TAA CGC AA | 46 |
| Borna 1313Rev | 19 | GCT GTC TGC GGG TCG TCT T | 60 |

**Table 8, Continued**

| Sequence Name | Bases | Sequence[a] | Tm °C |
|---|---|---|---|
| GSP3-Borna 8260For | 33 | GTG GTG TTC ATA TTG GAG GAC CAT CKG ACT CAT | 63 |
| GSP2-Borna 515Rev | 26 | GCG GAT GAG CCT ATG ACC ACT CCA AT | 62 |
| GSP-Borna 8150For | 16 | CTT CAT GGT GAT CCT T | 45 |
| Abridged Anchor Primer (AAP) | 30 | GGC CAC GCG TCG ACT AGT ACG GGG GGG GGG | 74 |
| Borna 3000For | 19 | GGG ARG ACT GYG AAA TTT T | 50 |
| Borna 3700 (2)For | 20 | CGG MMR GTG GTC GGA ATA AA | 56 |
| Borna 3300Rev | 22 | GCC ARA YAA CMC CDA YYC CAT T | 57 |
| Borna 2710Rev | 20 | CTG TGC TGC ART CTG CAA AA | 55 |
| M25-Borna 7790Rev | 22 | GGG ATA TCA AAT CAC GCC TAT T | 53 |
| M25-Borna 6570For | 21 | GCA AGC CGT TAT AGA CCA TAT | 52 |
| M24-Borna 315For | 20 | GCC AGT CAA GCA AGG AGA AC | 56 |
| M24-Borna 444Rev | 20 | CGG ATG AGC CTA TGA CCA CT | 56 |
| M25-Borna 2710For | 24 | GCA CAG TCA ATG AGA AGG ATC TAT | 54 |
| M25-Borna 3950Rev | 23 | CAG CTC GGA TTA GTA CAC CAA TT | 55 |
| M25-Borna 6050For | 21 | GGT CCG TCA TTA GGT GGT CTT | 57 |
| M25-Borna 8460Rev | 19 | GGC TGT GAA TGG ACC GAT A | 54 |
| Borna 8550For | 19 | SCA TGT GGA GGA RTT TGA T | 51 |
| M25-Borna 3040For | 19 | GGT TCG TTT GGG AAT GGT A | 52 |
| M25-Borna 3540Rev | 20 | CCC GTA TCA GTT GAG GTT AA | 52 |

[a]All primers were purchased from Integrated DNA Technologies and were purified by standard desalting. In this table, standard IUB symbols are used to designate mixed bases (9)

**Table 9 Regions of ABV amplified by PCR and the primers used for sequencing**

| Region | Primer for PCR and sequencing | Internal primers for sequencing | |
|---|---|---|---|
| ABV- N region | Borna: 5For & 1313Rev | | |
| ABV-X, P, and M regions | Borna 1200For & Borna 1930Rev | | |
| ABV-G region | Borna: 1700For & 3800Rev | Borna 2430For | Borna 3310Rev |
| ABV-L1 region | Borna 3700Fo. & Borna 6300Rev | Borna 5190For | |
| ABV-L2 regions | Borna 6100For. & Borna 7580Rev | Borna 6880Rev | |
| ABV-L3 region | Borna 7380 For. & Borna 8490Rev | | |
| ABV-O1-G1 region | Borna 1700For. & Borna 3310Rev | | |
| M25-G1 region | M25-Borna 1800For. & Borna 3800Rev | | |
| M25-G2 region | M25-Borna 2710For & Borna 3950Rev | | |
| M25-L1 region | Borna 3700(2)For &M25- Borna 4740Rev | | |
| M25-L2 region | M25-Borna 6050For & Borna 8460Rev | | |

## Results

### *Organization of ABV Genomes*

The genome organization of the bornaviruses is unique among the *Mononegavirales*. Some genes, for example M and G, are overlapping and discrete transcription start and stop sites do not occur between each open reading frame. The ABV isolates from this study showed the same overall genome organization as previously characterized ABVs (39, 59) with six ORFs encoding homologs of the BDV N, X, P, M, G, and L genes (Fig 8). The relative position of each gene and the degree of gene overlap was also well conserved. The most notable difference in the organization of the ABV and BDV genomes was the deletion of an approximately 20 nt sequence between the N and X genes that was previously noted for ABV (39, 59).

### *Texas Isolates Belong to Three Different ABV Genotypes*

Phylogenetic trees were generated to determine the genotypes represented by the Texas ABV isolates. Initially, partial N sequences (397 nt) were used to generate a tree containing 12 ABV strains with previously designated genotypes (39) and the Texas isolates (Fig. 9). Seven of the Texas ABV genomes (NM-M10, NM-M14, NM-M15, NM-M20, NM-AG5, NM-01 and NM-06) formed two clusters in a branch of the tree containing other genotype 4 ABVs (ABV4). The Texas isolates were more closely related to one another than to the other ABV4s. One Texas ABV, NM-M25, was genotype 1 and one, NM-CT15, was genotype 2 (Fig. 9).

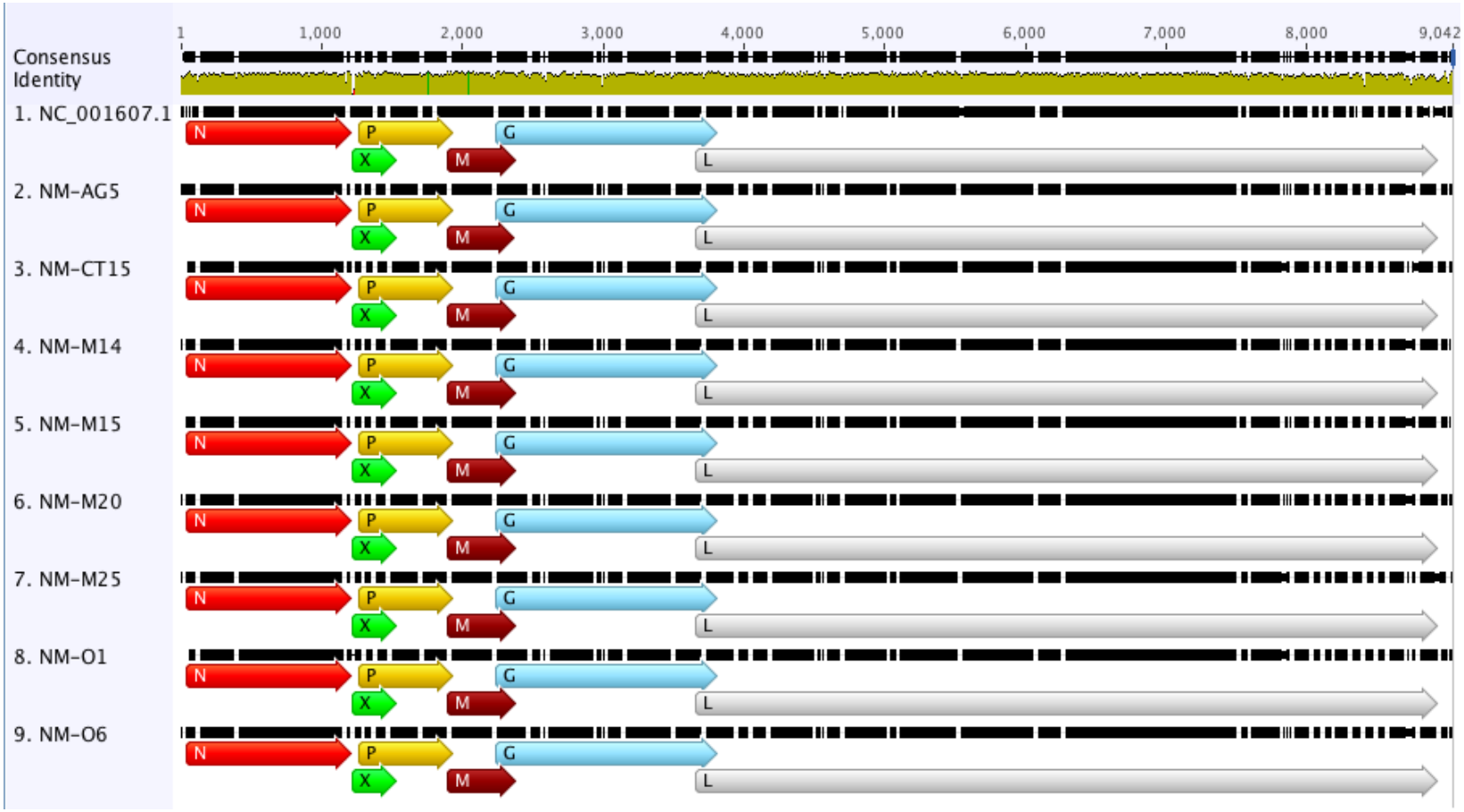


**Fig. 8** ABV genome organization. Alignments of entire genomes showing predicted open reading frames for the Texas ABV isolates. The overall gene organization of the Texas ABV isolates was identical to BDV and previously characterized ABVs (65).

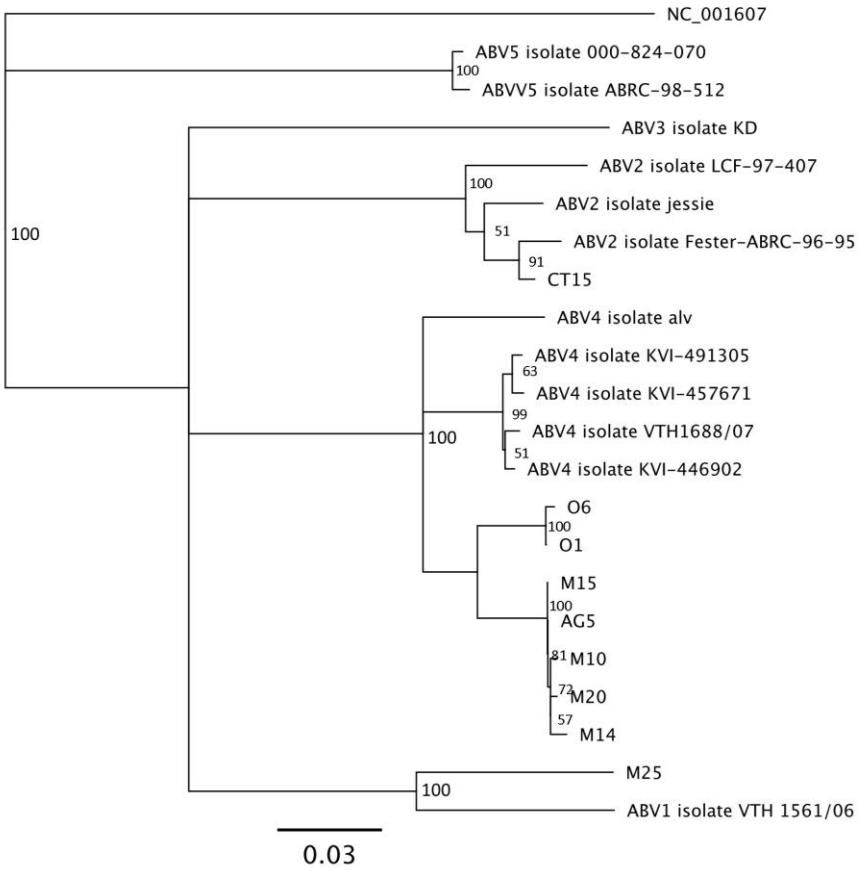


**Fig. 9** Neighbor joining tree for a 379 nt region N. NC-001607 (BDV) was used as the out-group. Isolates designated ABV1, ABV2, ABV3, ABV4, and ABV5 were previously assigned to these groups (39, 65). Texas isolates are: M25, M24, O6, M15, AG5, M10, M20 and M14. Node labels show consensus support.

Additional phylogenies were generated using complete X, P, M, G and L gene sequences (Fig. 10). Sequences from two complete ABV2 genomes (Accession Nos. FJ620690 and EU781967.1), were included in the analyses. At the time of the analysis complete genome sequences of other genotypes were not available. In each tree, NM-CT15 grouped closely with EU781967.1, an ABV2 isolated from crop tissue from a Sun Parakeet (*Aratinga solstitialis*) in the United States (39). NM-M25, designated an ABV1 based on N sequences, always appeared on its own branch, The Texas ABV4 isolates always split into two clusters, one containing NM-01 and NM-06 and the other containing the isolates NM-M14, NM-M15, NM-M20 and NM-AG5.

***Identification of Putative Regulatory Sequences***

Analysis of BDV transcripts has identified 3 transcription initiation sites (TIS) and 4 major transcription termination (TER) sites (61). At similar locations in the ABV genome we identified putative TIS (Fig. 11a-c). We predict that ABV TIS1 and TIS3 overlap the confirmed BDV TIS but that the transcript initiation sites are 2 to 3 nt closer to the start codon of the downstream ORFs. The region of the ABV and BDV genomes that is most divergent falls between the end of the N gene and the beginning of the X gene. The ABV genome lacks a 22 nt stretch present in BDV (Fig. 11a). We predict that the ABV TIS2 site is downstream of the mapped BDV site, placing the transcription initiation site 22 nt closer to the start codon of the X gene. Four transcription termination sites (T1-T4) have been mapped for BDV (61). The TER are U-rich sites in the BDV genome that serve as polyadenylation sites of viral transcripts. The ABV genomes are identical to BDV at all four termination sites (Fig. 11b, d, e).

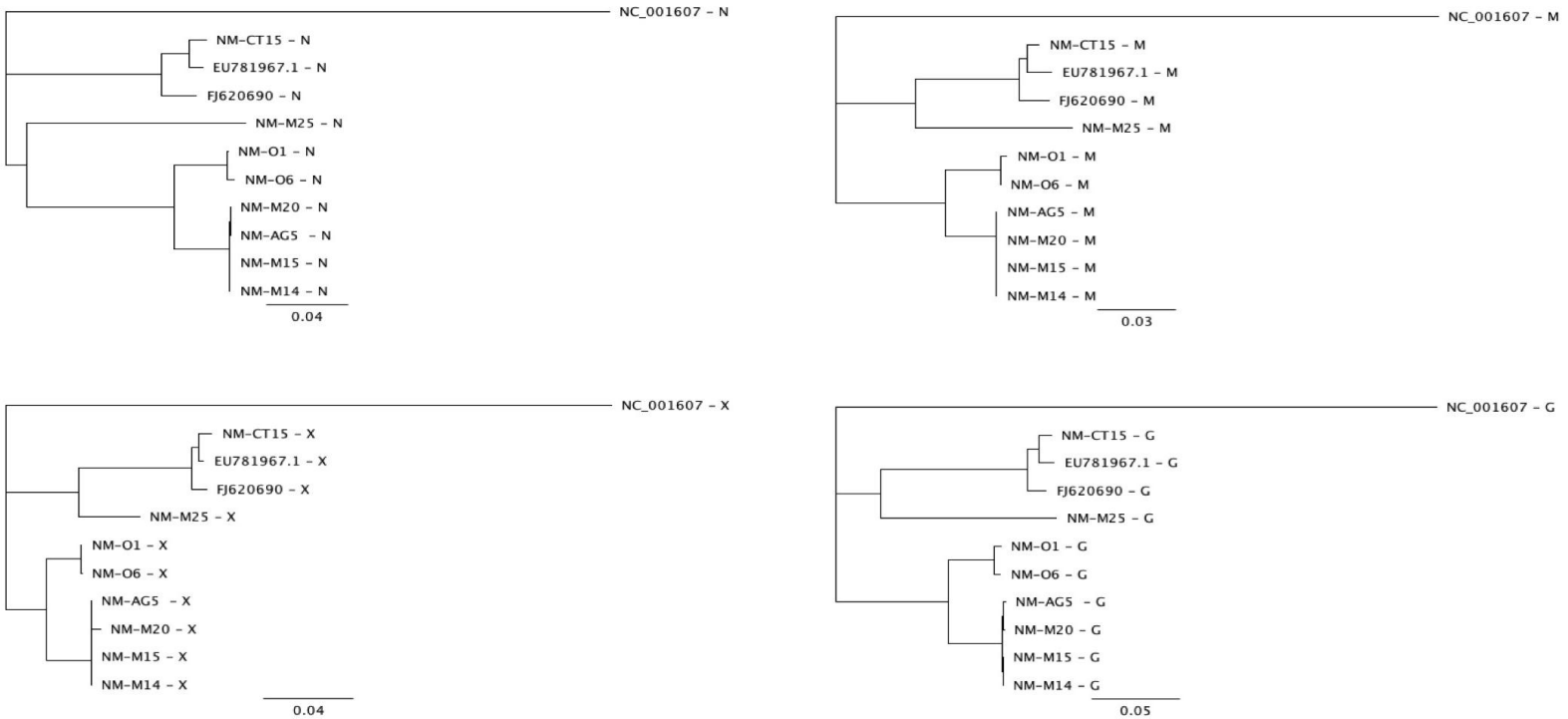


**Fig. 10** Neighbor joining trees generated using the complete nt sequence of N, X, P, M and G and L genes. NC_001607 (BDV) was used as the out-group. FJ620690 and EU81967.1 are genotype 2 ABVs whose complete sequences are available in Genbank. The Texas isolates all cluster as shown for the partial N gene sequences. Only ABV genotypes 1, 2, and 4 are included in this figure as complete genome sequences are not available for genotypes 3 and 5.

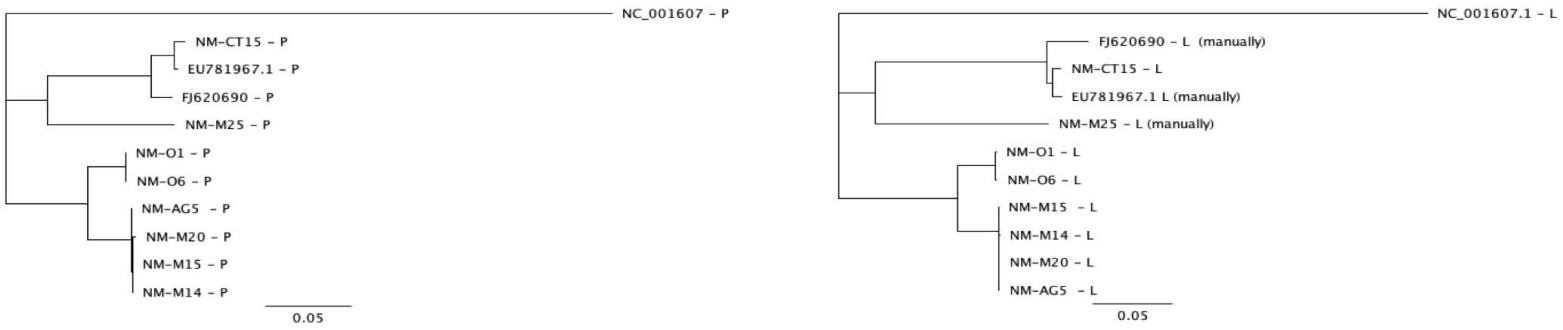


**Fig. 10,** Continued


**Fig. 11** Potential transcription initiation (TIS) and transcription termination sites (TER) for ABV. **a, b, c** The postion putative TIS in ABV. The region of the ABV and BDV genomes that is most divergent falls between the end of the N gene and the beginning of the X gene. The ABV genome lacks a 22 nt stretch present in BDV (a). **b, d, e** The ABV genomes are identical to BDV at all four termination sites.

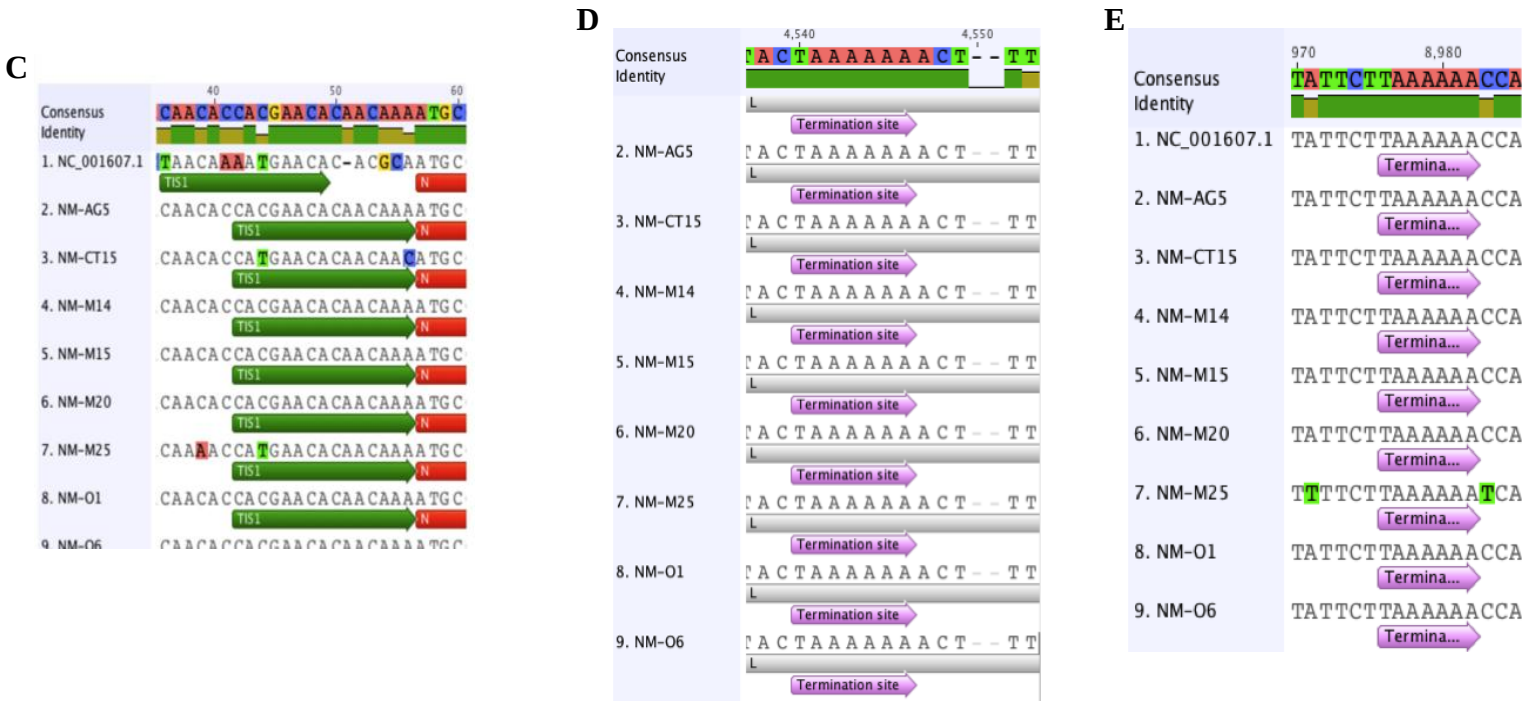


**Fig. 11**, Continued

***Pairwise Comparisons of ABV and BDV Sequences***

Pairwise comparisons of ABV and BDV sequences revealed that although the overall phylogenies agreed for each protein, the degree of nucleotide and amino acid sequence variation varied among the genes and their predicted proteins, as shown in Table 10. Overall, for comparisons of BDV to ABV genotypes, the percent amino acid identities were, from highest to lowest: M (~84%), N (75-77%), L (66-68%), G (65-67%), P (59-63%) and X (41-50%). At the nucleotide sequence level, identity of BDV to ABV genotypes was: M (71-75%), X (63-71%), N (63-68%), G (64-66%), P (63-65%) and L (61-63%). Pairwise sequence comparisons among the complete ABV genomes showed that all of the ABV isolates were more closely related to each other than to BDV, as expected. The percent amino acid identities were, from highest to lowest: M (95-97%), N (93-100), P (93-100%), L (91-100%), G (88-100%) and X (84-100%). Percent nucleotide identities among the ABVs were: X (88-100%), M (85-100%), P (83-100%), N (81-99%), G (80-100%) and L (79-100%). An unusual result in both sets of comparisons was the X protein, whose observed nucleotide sequence identity was greater than the predicted amino acid sequence identity. This occurred because X protein is short (87-88 aa) and rich in leucine and arginine.

**Table 10 Summary of nucleotide and amino acid identities from comparisons of ABV and BDV genes**

| | Percent Identity | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N (375-374 aa)[a] | | X (88-87 aa) | | P (202 aa) | | M (142aa) | | G (500-503 aa) | | L[b] (1702-1706 aa) | |
| Isolates compared: | aa* | nt** | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt |
| NM-ABV4[c] | *98-100* | 94-99 | *93-100* | 96-100 | *98-100* | 95-100 | *†97-100* | 95-100 | *97-100* | 94-100 | *98-100* | 95-99 |
| ABV2[d] | *99-100* | 95-98 | *95-98* | 98-99 | *98-99* | 96-99 | *98* | 97-98 | *97-98* | 97-98 | *98-99* | 96-98 |
| NM-ABV4[c] v. ABV2[d] | *93-95* | 81-82 | *74-79* | 87-88 | *93-95* | 83-85 | *96-97* | 86-87 | *88-90* | 80-82 | *89-90* | 79 |
| NM-ABV4[c] v. ABV1[e] | *94-95* | 81-82 | *84* | 90-91 | *94-95* | 84 | *95-96* | 85-86 | *88- 90* | 81 | *90-91* | 79-80 |
| ABV2[d] v. ABV1[e] | *97-98* | 81-82 | *84-87* | 91-92 | *94-95* | 85-86 | *97-99* | 89 | *91-92* | 82-83 | *91* | 80 |
| BDV[f] | *98* | 84 | *80* | 88 | *96* | 86 | *97* | 88 | *93* | 82 | *92* | 78 |
| BDV v. NM-ABV4[c] | *75-77* | 64-68 | *45-50* | 67-71 | *60-63* | 65-66 | *84-85* | 75 | *65-66* | 65-66 | *67-68* | 61-63 |
| BDV v. NM-CT15 | *75-76* | 63-67 | *41-47* | 63-68 | *59-62* | 63-66 | *83* | 72-74 | *66-67* | 65-66 | *67-68* | 61-63 |
| BDV v. NM-M25 | *76-77* | 64-68 | *47* | 70 | *60-62* | 64-65 | *83* | 71-74 | *65* | 64-65 | *66-67* | 61-62 |

**Table 10, Continued**

[a]For each protein, the range of amino acid lengths are shown.

[b]Data was calculated for only for the L gene second exon.

[c]Comparisons of Texas isolates NM -M10, -M14, -M15, -M20, -AG5, -01 and -06.

[d]Comparisons of Texas isolates NM-CT15, FJ620690 and EU781967.1.

[e]ABV1 is Texas isolate NM-M25.

[f]BDV sequences compared are AJ311524 and NC001706.1.

* Percent amino acid sequence identities are shown. These were calculated using CLUSTAL W (1.81) multiple sequence alignments with the BLOSUM matrix. In comparisons of groups of isolates, the values are presented as a range from the most divergent pair (lowest percent identity) in the group to the most conserved pair.

** Percent nucleotide sequence identities were calculated using CLUSTAL W multiple sequence alignments with the IUB matrix. In comparisons of groups of isolates, the values are presented as a range from the most divergent pair (lowest percent identity) in the group to the most conserved pair.

[†]Values for NM-AG5 are not included due to an unresolved frame-shift in the M protein.

***Characterization of Predicted ABV Proteins***

The ABV gene order is identical to BDV. Nucleotide and amino acid sequence similarities strongly suggest that predicted ABV proteins are homologous to those in BDV. A brief description of each protein follows.

N Protein

The BDV N protein is 371 amino acids (aa) and is expressed from an S1 mRNA transcript. The predicted ABV N proteins for the Texas isolates were 374 amino acids and shared 270 (~72%) identical sites with BDV. Among the ABVs the N proteins shared 344 identical sites. Two ABV N's from Genbank (FJ620690 and EU781967.1) have identical N protein sequences; among the Texas isolates NM-CT15 was the most closely related to those isolates, sharing 99% identical sites. BDV N has a nuclear localization signal (NLS) near its N-terminus and the 371 aa N product (p40) has a nuclear localization. A second BDV N protein, p38, initiates at a second methionine codon 14 aa downstream and thus lacks the NLS. The ABV N proteins of the Texas isolates shared 9 identical sites at their amino termini, predicting a motif that could function as proline-containing NLS. The ABVs also shared a methionine, at position 16, that could serve as a second translation start site to produce a shorter N protein.

X Protein

The BDV X protein is 88 amino acids. The BDV X gene starts upstream of the P gene, but overlaps it. BDV X and P are likely translated from transcripts that initiate at the S2 transcription initiation site. A comparison of the Texas ABVs and BDV showed

that overall the X proteins shared only 34 identical sites (39%). Among the Texas ABVs, where X is predicted to be 87 aa, the number of identical sites was 61 aa (~69%). Four of the Texas ABV isolates (NM-M14, NM-M15, NM-M20 and NM-AG5) had identical X proteins. The two ABV2 isolates (FJ620690 and EU781967.1), have identical N proteins, but have X proteins that differ by 4%. The NM-CT15 X protein was most closely related to EU781967.1 (86 identical sites) and shared 84 identical sites with FJ620690. NM-M14, NM-M15, NM-M20 and NM-AG5 also had identical X proteins. The NM-06 X protein was most closely related to the NM-01 X protein.

P Protein

The BDV P protein is 203 aa. The ABV P proteins were also predicted to be 203 aa and overall, they shared 116 (57%) identical positions with BDV P. Among the ABVs, the NM-AG-5, NM-M14 and NM-M15 P proteins were identical. The NM-06 and NM-01 P proteins were also identical. NM-CT-15 shared 202 of 203 sites with EU781967.1 and 199 sites with FJ620690, but only 190 sites with the AG-5 group of ABVs. NM-M25 showed 61% identity with BDV, 93.6% identity with NM-CT15, 90.5% identity with the AG-5 group and 90.6% identity with the NM-01/06 isolates.

G Protein

The virion surface glycoprotein (G) is responsible for BDV attachment and fusion (22) and encodes a highly glycosylated 504 aa precursor protein (gp94). Furin cleavage of BDV G yields GP-1 and GP-2 that correspond to the N- and C-terminal regions of gp94. Like the BDV, the ABV G open reading frame predicted a highly

glycosylated 504 aa protein with a furin cleavage site. The furin cleavage site was conserved between ABV and BDV and among different genotypes of ABV (Fig. 12). The ABV G proteins had more predicted N-linked glycosylated sites (NXS/T) than BDV (Fig. 13). The BDV G aa sequence ranges from 65-66% identical to the ABV Gs. Among the ABV G proteins, NM-M25 was the most different sharing 88% aa identity with NM-06 and the most (93%) with NM-CT15.

L Protein

The BDV L protein is 1711 aa in length. It is translated from a spliced mRNA such that the first 5 aa of L are encoded from a region that overlaps the G (15). The ABV L protein sequences were translated in silico using the assumption that a similar splicing event generates the ABV L mRNAs, as nucleotide sequence comparisons show conservation of consensus intron/exon boundaries in the ABV genomes. The ABV L proteins range in size from 1710 to 1713 aa. A comparison of BDV to ten ABV L proteins predicts 63.7% identical sites. BDV L is ~60% identical to ABV L at the aa sequence level. All of the ABV isolates were more closely related to one another than to the BDV L protein. Among the ABV sequences, NM-01, NM-06, NM-M20, NM-AG5, NM-M14 and NM-M15 formed a cluster and NM-CT15 grouped with other ABV2s (FJ620690 and EU781967.1).


**Fig. 12** Conserved furin cleavage sites in BDV (1.NC_0016…) and ABV. The cleavage site is shown by yellow arrow in this figure.

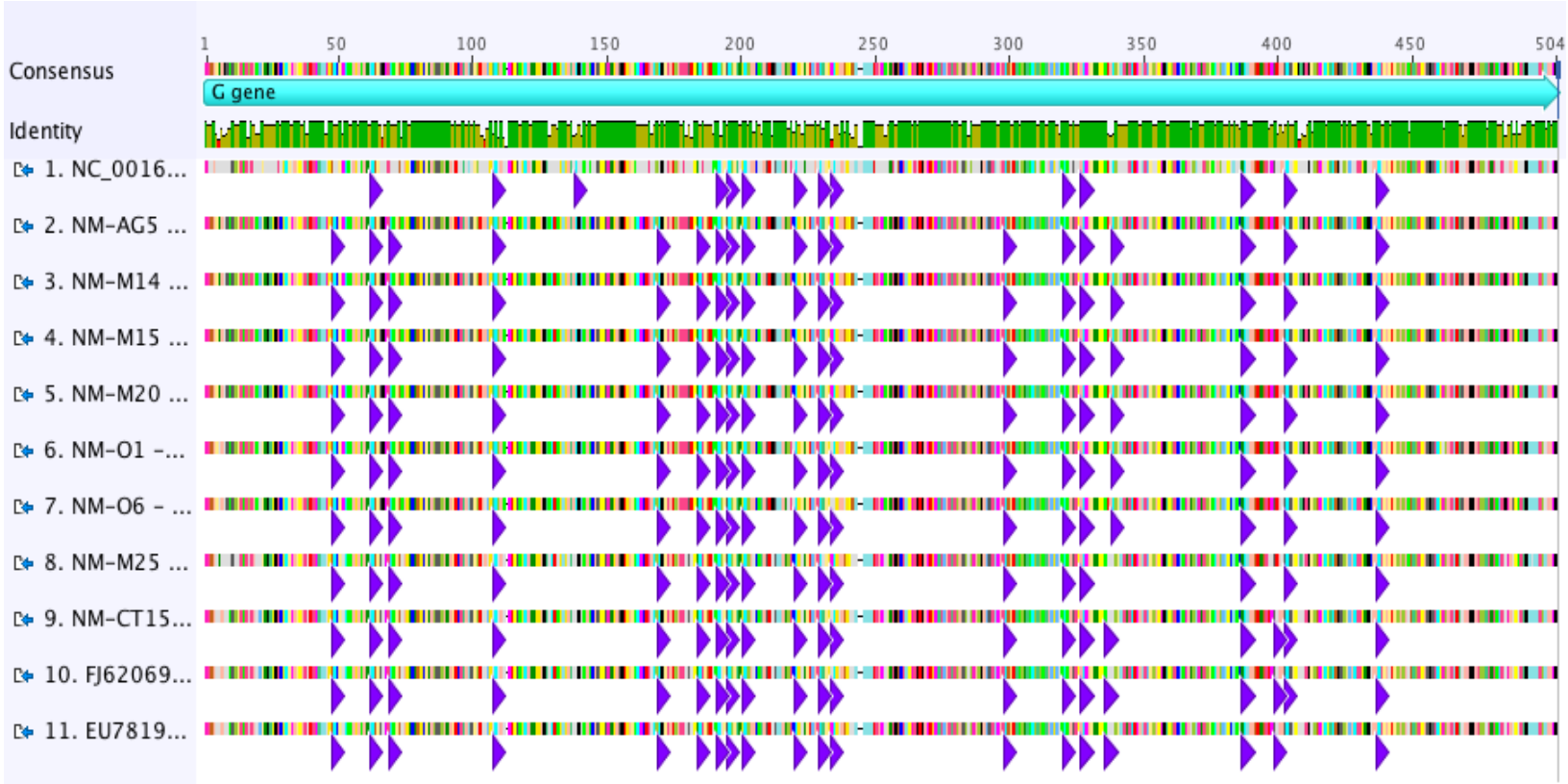


**Fig. 13** G protein showing the locations of predicted N-linked glycosylation sites. The predicted N-linked glycosylation sites (NXS/T) are indicated by purple arrows.

**Discussion**

We identified ABV genotypes 1, 2, and 4 in parrots and cockatiels originating from the United States. ABV4 was the most common genotype. The ABV-infected birds showed either typical clinical signs of PDD or were healthy (or apparently healthy). All the isolates were successfully recovered in DEF but it is important to note that the sequences reported herein were obtained from RNA purified directly from brain tissues. N sequences were also recovered from cultured DEF and these were essentially identical to those from the brain tissue (data not shown).

Comparing the viral genome structures, we confirmed that ABV genome organization is highly similar to BDV genome organization, with the same gene order as reported previously (34, 39, 59). The locations of transcription initiation and transcription termination sites were also conserved although the positions of the TIS were shifted closer to the start sites of the N, X and M ORFs. The most obvious difference was the location of TIS2, located upstream of the X gene. In all of the ABVs this region was approximately 20 nt shorter than in BDV. This region of BDV contains a sequence important for regulating the frequency by which the polymerase terminates transcription after the N gene (55), thus is absence from ABV isolates suggests a different mechanism of regulating gene expression.

Comparisons of predicted proteins reveal some motifs that are well conserved between ABV and BDV. Conserved motifs in the G protein included the furin cleavage site and most of potential N-linked glycosylation sites. However, there were additional predicted potential N-linked glycosylation sites in the ABV G proteins, suggesting that

in ABV, G may be even more highly glycosylated than in BDV. Alteration of a glycosylation site can have dramatic consequences for a virus as it may impact protein folding or cause conformational changes (73). Although increases in glycosylation can sterically cover epitopes, the loss of a glycosylation site could result in tighter packing of the amino acid backbone. In regions containing neutralization epitopes this could reduce accessibility and also facilitate immune escape (72). Moreover, it has been shown that alteration in patterns of glycosylation sites in human immunodeficiency virus (HIV) and hepatitis C virus can be a factor in escape from T cell responses (2, 64). It has been shown in HIV and influenza virus that small changes in glycoproteins can influence receptor binding and phenotypic properties of viruses (37, 46). Therefore the differences in the glycoproteins of ABV and BDV may be the reason for difference in the cell tropism of two viruses.

The M protein is the most highly conserved protein among the ABVs and BDV overall. Since M is a structural protein that assembles as homotetramers, (44) this may constrain sequence variability of M.

The X proteins showed the least similarity between BDV and ABV. X is a regulatory protein which may play a role in negative regulation of BDV polymerase activity (54). Moreover, it has been recently shown that X represents the first mitochondrion-localized protein of an RNA virus, and that it inhibits apoptosis in cultured cells and infected animals (53). The role(s) or function(s) of the ABV X protein are not yet known, but amino acid sequence variation in this region is likely to have an impact on virus replication.

While the overall genome organization for the *Bornaviridae* was quite conserved, our ABV isolates showed much greater variability than observed among BDV strains from mammals. It is possible that the currently known BDV strains do not reflect the complete genetic repertoire of this virus and currently known BDV strains might only represent a small fraction of virus variants which are able to cross the between natural hosts of BDV and horse or sheep (65).

This is the first report of complete genome sequences for ABV genotypes 4 and 1. We failed to find any correlation between ABV genotypes and the bird species from which they were isolated. We isolated an ABV2 from a healthy cockatiel, while this genotype previously was isolated by others from macaws with PDD (39, 59). We isolated ABV4 from different species of birds such as macaws, African gray parrots and healthy cockatiels. Thus different genotypes of ABV appear to be able to infect the same species of birds.

Hopefully, the data we presented here will provide a better understanding of structure of ABV. The sequence data are also critical for future studies, such as constructing a reverse genetic system as well as for vaccine development. There are also many questions that remain to be addressed, such as the importance of the differences between ABV and BDV genomes, or even among different ABV strains and the effects these differences have on virus transmission, replication and disease.

# CHAPTER IV

# AVIAN BORNAVIRUS, GENOTYPE 2 CAUSES PROVENTRICULAR DILATATION DISEASE IN COCKATIELS *(NYMPHICUS HOLLANDICUS*)

## Introduction

ABV is the cause of PDD in psittacines and some passerines and waterfowl (21, 26, 35, 49) The clinical presentations of PDD vary and may be predominately neurological (weakness, ataxia, proprioceptive deficits, seizures, blindness), gastrointestinal (weight loss, passage of undigested food, regurgitation, delayed crop emptying), or a combination thereof (66). The gastrointestinal signs, especially proventricular dilatation, are secondary to damage to the enteric nervous system. Typical PDD is characterized by the presence of a lymphoplasmacytic inflammation in peripheral, central and autonomic nervous tissues (1).

Epidemiological studies have shown however that ABV is carried by many apparently healthy birds (49). This appears to be especially true of cockatiels where a high percentage of birds may be shedding the virus in their feces (26). Although not unexpected in a RNA virus, ABV is genetically diverse with 7 distinct genotypes identified to date. All 7 genotypes have been isolated from psittacine cases of PDD but is of interest to note however that both in Europe and North America, ABV4 is by far the predominant genotype isolated (65). This may be due either to a true predominance of genotype 4 among birds, or alternatively, because ABV4 is more virulent than other

genotypes and is thus more likely to be encountered when investigating deaths due to PDD.

No formal investigations into the relative virulence of the different genotypes of ABV have yet been undertaken. Given that all genotypes identified to date have been identified in diseased birds, it is likely that all are capable of causing disease. Recently however, we isolated ABV genotype 2 (ABV2) from a cockatiel that, while not healthy, did not appear to be suffering from PDD, and indeed appeared to be a carrier of the virus. Because the infected cockatiel was relatively healthy and did not have gross lesions suggestive of PDD, it was hypothesized that this particular strain of ABV2 was either avirulent or of low virulence. In order to test this hypothesis the isolate was cultured and used to challenge two healthy cockatiels.

## Materials and Methods

### *Experimental Infections*

Experimental infections were carried out under Animal Use Protocol #2009-033B approved by the Texas A&M University Institutional Animal Care and Use Committee. Two (No. 1 and No. 13) adult male and female Cockatiels (*Nymphicus hollandicus*) shown to be intermittent shedders of ABV4 by fecal RT-PCR, but otherwise in good health, were placed in isolation and inoculated by intramuscular injection with infected DEFs (cell culture passage 6) containing $8x10^4$ focus forming units (FFU) of an ABV2 isolate from cockatiel 15 (this isolate was sequenced and was designated NM-CT15). Additionally $4x10^5$ FFU were administered by oral gavage. A

third cockatiel (No. 10) was retained as a negative control bird. During inoculation, $1.4x10^5$ FFU were retained for culture to assure that birds had received viable virus. The birds were housed in flight cages and provided food and water *ad libitum*. Control birds were housed in a separate aviary.

***Challenge Virus***

ABV2 was cultured for 6 passages in DEF monolayers as described previously (26). A piece of frozen CT15 brain was homogenized in 4 ml of LM (containing 5% NBC serum, 1µg/ml Fungizone (antimycotic), and 50 µg/ml penicillin-streptomycin using a 5 CC syringe and 20 GA gauge needle. The homogenates were used to inoculate two confluent T12.5 flasks of DEF. After overnight incubation at 37 °C with 5% CO2, the supernatants were replaced with fresh 2% LM and incubated for 5 to 7 days. The inoculated cells were then harvested using 0.25% trypsin and transferred to a larger flask. The cells were passaged 6 times, collected and frozen at -80ºC using frozen media (LM+10% DMSO, 50% NBC serum). Virus was titered by plating serial dilutions of infected DEF onto uninfected monolayers in 6 well-plate and incubating for 5 to 7 days. The infected cell foci were detected by immunohistochemistry using serum from bird M10 (a PDD positive bird) and secondary antibody (goat anti-macaw conjugated with HRP) and developed using Sigma Fast DAB. Foci were counted using an optical microscope.

***Sample Testing***

Fecal samples were taken at weekly intervals from challenged and control birds for PCR analysis. Briefly, fecal drops were collected with a clean swab and suspended in about 500–750 µl of sterile phosphate buffered saline (PBS). Vials containing fecal material were transported on ice and either immediately processed for RNA isolation or frozen at -80 ºC until RNA processing. RNA was purified from the supernatants using QIAamp Viral RNA Mini kit (Qiagen), according to the manufacturer's recommendations. First strand cDNA was generated using the Applied Biosystems® High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), with 1µg RNA and random primers. cDNA was amplified using primer sets: Forward (5' CAGACAGCACGTCGAGTGAGA 3') and Reverse (5' GGCTCTTGGTCT GAGATCATGGAA 3'). The PCR conditions were as follows: Initial denaturation, 94 °C for 3 min, followed by 35 cycles of 94 °C, 30 sec, 54 °C, 30 sec and 72 °C, 20 sec followed by a final extension of 5 min at 72 °C. Samples were electrophoresed in 0.9% agarose gels containing 0.002% ethidium bromide and were visualized under UV light.

***Pathology***

Immediately upon euthanasia, complete necropsies were performed on all birds. Tissue samples from brain, spinal cord, peripheral nerves, eyes, lungs, heart, liver, pancreas, spleen, kidneys, ovary, oviduct, adrenal gland, skeletal muscles, esophagus, crop, proventriculus, ventriculus, intestine and cloacal were placed in 10% buffered formalin, embedded in paraffin, sectioned at four micrometers, stained with hematoxylin and eosin (H & E), and examined by bright field microscopy.

***Detection of ABV by PCR***

During necropsy of infected and control cockatiels, small portions of brain, lungs, heart, liver, pancreas, spleen, kidneys, and adrenal glands, were snap frozen at -80 °C. Total RNA was isolated from these frozen tissues or from passaged DEF using Qiagen RNeasy Mini kit (Qiagen,Valencia, CA). First strand cDNA was generated using the Applied Biosystems® High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), with 1µg RNA and random primers. For tissue samples, PCR of a region of the ABV N-gene was performed using 1-2 µl cDNA and forward (5F: 5'GCGGTAACAACCAACCAGCAA3') and reverse (1212R: 5'GTTCATTAGTTTGCRAATCCRGTTA3') primers designed based on previously reported ABV N-protein gene sequences (68). Amplification conditions were as follows: 1 cycle at 94 °C for 2 min; 35 cycles at 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 80 sec; final extension at 72 °C for 7 minutes.

***Genotyping***

PCR products were cloned into TOPO-TA vectors (Invitrogen, Carlsbad CA) and individual clones were sequenced after transformation into *E. coli*. Alternatively, PCR products were sequenced directly. DNA sequencing reactions were performed using the ABI BigDye Terminator Cycle Sequencing Kit, and sequences were generated with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Sequences were assembled and aligned using Sequencher 4.8 (http://www.idmb.tamu.edu/gtl/sequencher.htm) and Geneious Pro 4.6.2 software (www.geneious.com).

**Results**

In order to investigate the virulence of CT15 a genotype 2 ABV, a small challenge study was conducted. This ABV2 isolate was recovered from the brain of an infected cockatiel (CT15) that had presented with lesions not suggestive of PDD (data not shown). After experimental infection, CT15 infected and control birds were checked daily. There were no obvious immediate responses to inoculation. No PCR-positive fecal samples were detected from these birds at any time. However on day 33 one bird (No. 13) was observed to be depressed. Over the next few days the bird lost weight, became weak, and on day 36 was observed clinging to the cage wall by its beak. This bird was euthanized for ethical reasons. On necropsy, the bird was thin with a moderate loss of pectoral muscle mass. Its gastrointestinal tract was empty. The liver was slightly enlarged and the proventriculus slightly dilated. The second challenged bird (No. 1) remained in apparent good health until day 41 when it also was observed to be depressed. It gradually became inactive and it was clearly losing weight. This bird was euthanized on day 45. It too had moderate pectoral muscle wastage. It had a slightly enlarged liver and spleen, an empty and somewhat enlarged small intestine, and a slightly dilated proventriculus. No other gross lesions were observed in either bird.

Histopathological examination of Bird No. 13 identified typical lesions of PDD with a moderate multifocal encephalitis and mild myocarditis (Fig. 14), adrenalitis and intestinal ganglioneuritis. The brain lesions were more significant than in other organs. Tissues of the second cockatiel, bird No. 1 also showed lesions of PDD. It had mild encephalomyelitis, adrenalitis, and intestinal ganglioneuritis. It also had very severe ganglioneuritis of the epicardial ganglia (Fig. 14).

In order to study the sites of viral replication in infected birds, PCR was performed using primer mix 5 or N (Table 2) on pieces of forebrain, spinal cord, heart, lung, spleen, kidney, crop, proventriculus, ventriculus and adrenal gland. In infected-cockatiel No. 1 (CTI1) the virus was detected in brain and CNS, as well as heart, lung, liver, spleen, kidney, crop, proventriculus, and ventriculus (Fig. 15). In infected-cockatiel No. 13 (CTI13), no virus sequence was found in the forebrain, however, the virus was detected in spinal cord, spleen, kidney, proventriculus and adrenal gland. Only the liver was negative for both infected birds. There was no virus detected in the uninfected cockatiel (CT10).

A

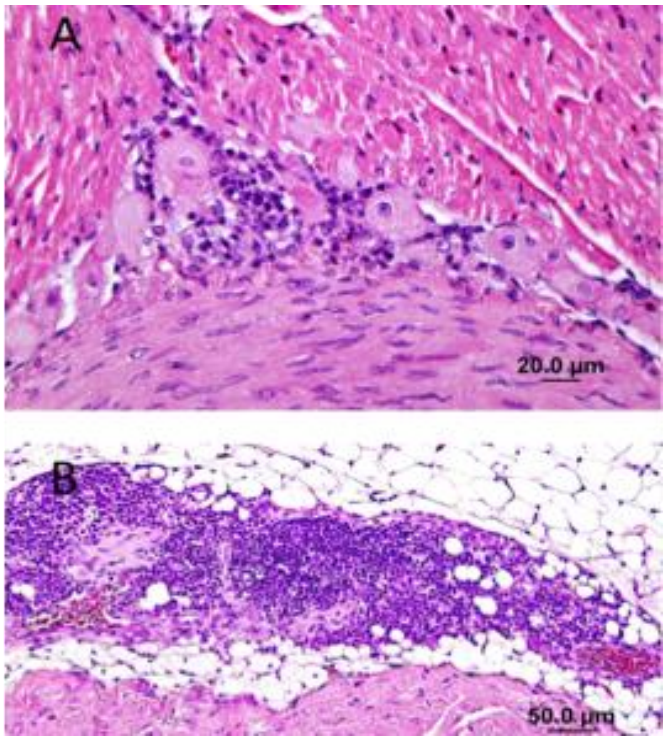


**Fig. 14** Histological examination of ABV2 infected cockatiels. **a** Bird No. 13 showing mild infiltration of lymphocytes and a few plasma cells in and around Purkinje cells of the myocardium. **b** Bird No. 1 showing severe lymphoplasmacytic infiltration in the epicardial ganglia of the heart. (We thank and acknowledge Dr. H.L. Shivaprasad for performing microscopic examination of tissues from ABV2 infected cockatiels and for providing these figures.)

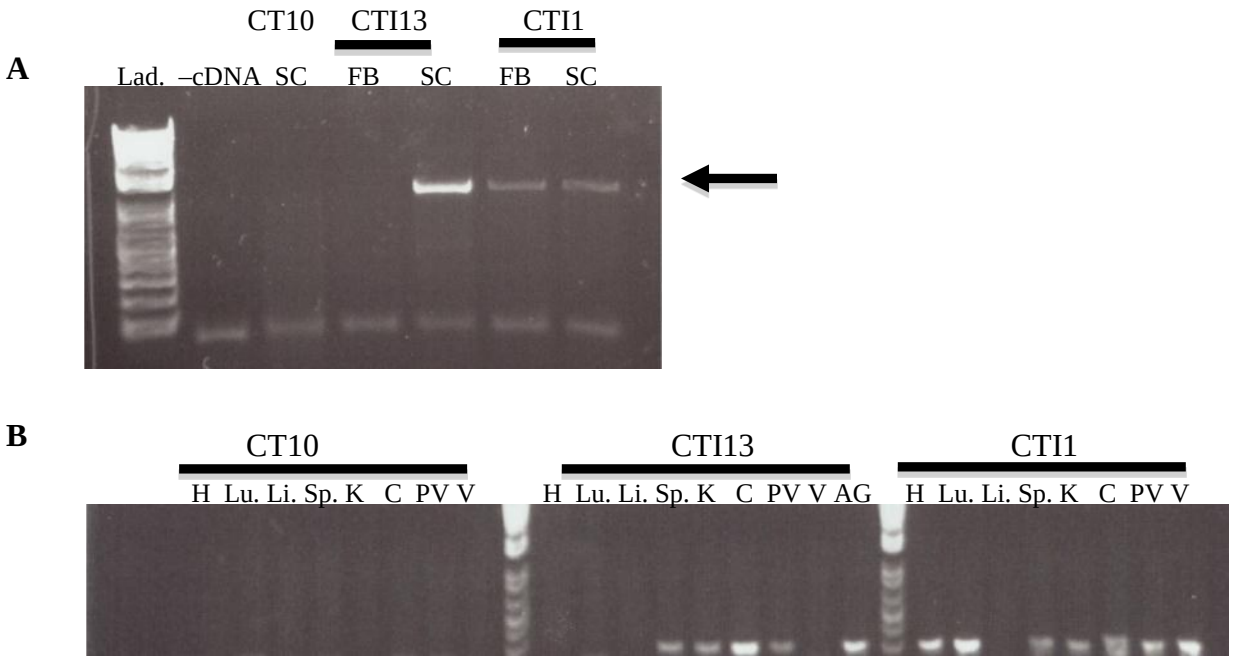


**Fig. 15** ABV in tissues of infected (CTI) or uninfected (CT) cockatiels. **a** Using primer mix N, a single band was observed in spinal cord (SC) of CTI1 and CTI13 and also in forebrain (FB) of CTI1. The lower band is a non-specific primer-dimer product. **b** Using primer mix 5, ABV was detected in kidney (K), crop (C), and proventriculus (PV) of both infected cockatiels. The virus also detected in spleen (Sp.) and adrenal gland (AG) of CTI13 as well as heart (H), lung (Lu) and ventriculus (V) of CTI1. Virus was not detected in liver (L). No spsecific PCR products were seen in any tissues from the infectd control bird, CT10. **C.** Loading control (β-actin).

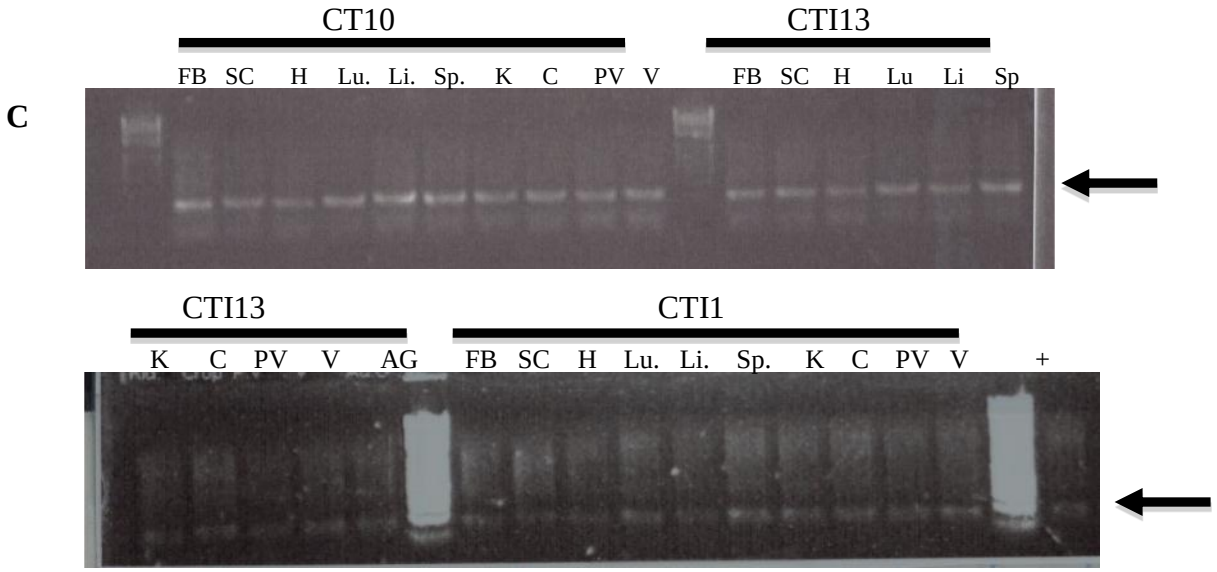


**Fig. 15,** continued

**Discussion**

We isolated a genotype 2 ABV from a cockatiel (CT15) with no evidence of PDD. The cockatiel, from which the isolate originated was euthanized because of a lipoma on its wing and it was not believed to be a clinical case of PDD. Previously, we had demonstrated that an ABV isolate of genotype 4 (M24) causes typical PDD in Patagonian conures (26) and cockatiels but that isolate was recovered from a bird with frank signs of PDD. We hypothesized that the ABV2 isolate, from a bird without clinical PDD, might be avirulent. Identification of a non-pathogenic strain of ABV would be helpful by providing a potential starting point for the development of a vaccine. However, the deaths of two CT15-challenged birds, at days 35 and 44, indicated that ABV2 is fully virulent. The onset of disease was more rapid (44 days compared to 64 to 111 days) than previously observed in experimental challenges with ABV4 isolates (21, 49).

It is interesting to compare the lesions induced in challenged cockatiels given similar doses of ABV4 and ABV2 in our laboratory. The two cockatiels challenged with ABV4 M24 died in about 100 days and had unusually florid and severe lesions involving primarily the gastrointestinal tract (49). In contrast, following ABV2 challenge the lesions were relatively mild, although sufficiently intense as to cause severe clinical disease. The observed differences in survival and lesions may be due to the involvement of the immune response. As the CT15-challenged birds died at 35-44 days post-challenge, perhaps there was not sufficient time for development of the severe

inflammatory lesions seen in the M24-challenged birds at 95-111 days post-challenge. That would imply that the inflammatory responses pre se do not drive disease development. In fact the prolonged survival of the M24-challenged birds may well be a reflection of prior immunity, as this specific group of cockatiels was shown to be naturally infected with ABV4 a year prior to this study despite being apparently healthy (21).

In several prior challenge studies we have employed multiple control birds that received uninfected DEFs. Experience with such birds indicated that no lesions or disease developed as a result of receiving these cells, thus additional DEF control birds were not sacrificed for this study. In summary, although this was a small pilot study, designed to obtain a preliminary assessment of the virulence of the ABV2 isolate CT15, it is clear that at the dose administered, this virus is fully virulent. Additional studies using alternative methods of virus delivery and/or doses that more closely reflect natural infection may demonstrate differences in the pathogenicity of ABV genotypes or isolates.

# CHAPTER V

# CONCLUSIONS

Using PCR, we were able to identify ABV in brain tissue from most suspected PDD cases. However PCR was less useful in detecting ABV infection among birds suspected of being clinically healthy carriers. For example, PCR failed to detect ABV shedding in 33% of ABV seropositive birds housed in the Schubot aviary. The failure of PCR in identifying ABV in those samples can be attributed to factors such as sample collection and handling, the frequency and levels of virus shedding and sequence divergence. Virus shedding was not limited to fecal samples and virus could be detected from other body sites such as nares, the choana, and the feathers. However sampling these sites requires training of both the birds and handlers so the frequency of virus shedding from these sites was not determined in this study, We can conclude that PCR testing of fecal samples alone is inadequate for ABV diagnosis or surveillance in individual birds. However, as the technique is sensitive, non-invasive, inexpensive, rapid and generally easy to perform, PCR screening is a good method for ABV detection amongst flocks, where a positive result is a strong indicator of the presence of the virus among members of the flock.

In a study where the results of PCR and serology were compared, it was clear that neither assay alone is ideal because the results were discordant. Among 18 PCR positive birds, 12 (66%) were seronegative. Serology did detect ABV infection in 3 of 10 (30%) PCR negative birds. The lack of a single clear diagnostic test for ABV

infection is unfortunate as aviculturists and pet owners fear the disease and would like to eliminate infected birds from their flocks, particularly as the birds in question are often threatened or endangered species. Much more research is needed to determine the best methods for detecting healthy carriers as well as for protecting non-infected birds.

The studies described herein also clearly demonstrate that multiple ABV genotypes can be found in psittacines. We identified ABV genotypes 1, 2, and 4 in parrots and cockatiels originating from the United States. ABV4 was the most common genotype. The ABV-infected birds showed either typical clinical signs of PDD or were healthy (or apparently healthy).

Comparing the viral genome structures, we confirmed that ABV genome organization is highly similar to BDV genome organization, The locations of transcription initiation and transcription termination sites were also conserved although the positions of the TIS were shifted closer to the start sites of the N, X and M ORFs. The most obvious difference was the location of TIS2, located upstream of the X gene. Comparisons of predicted proteins reveal some motifs that are well conserved between ABV and BDV. Conserved motifs in the G protein included the furin cleavage site and the majority of potential N-linked glycosylation sites. However, there were additional predicted potential N-linked glycosylation sites in the ABV G proteins, suggesting that in ABV, G may be even more highly glycosylated than in BDV. The M protein is the most highly conserved protein among the ABVs and BDV overall. The X proteins shows the least similarity between BDV and ABV.

While the overall genome organization for the *Bornaviridae* was quite conserved,

our ABV isolates showed much greater variability than observed among BDV strains from mammals. We failed to find any correlation between ABV genotypes and the bird species from which they were isolated. We isolated ABV4 from different species of birds such as macaws, African gray parrots and healthy cockatiels. Thus different genotypes of ABV appear to be able to infect the same species of birds.

Finally, we conclude that ABV recovered from a healthy carrier can show the same virulence, in experimental infections, as an ABV isolate recovered from a bird with clinical PDD. We isolated a genotype 2 ABV from a cockatiel (CT15) with no evidence of PDD, however, the deaths of two CT15-challenged birds, at days 35 and 44, indicated that ABV2 is fully virulent under our challenge conditions. The onset of disease was more rapid (44 days compared to 64 to 111 days) than previously observed in experimental challenges with ABV4 isolates. Thus the observed differences in survival and lesions among ABV infected birds (both naturally and experimentally infected) are unlikely to be related simply to virus genotype, and may involve immune responses. There is clearly significant work to be done before we fully understand ABV-infection of psittacines.

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