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# The diagnosis of proventricular dilatation disease: Use of a Western blot assay to detect antibodies against avian Borna virus

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ABSTRACT

Avian Borna virus (ABV) has recently been shown to be the causal agent of proventricular dilatation disease (PDD) a lethal neurologic disease of captive psittacines and other birds. An immunoblot assay was used to detect the presence of antibodies against avian Borna virus in the serum of affected birds. A lysate from ABV-infected duck embryo fibroblasts served as a source of antigen. The assay was used to test for the presence of antibodies to ABV in 117 birds. Thirty of these birds had biopsy or necropsy-confirmed proventricular dilatation disease (PDD), while the remaining 87 birds were apparently healthy or were suffering from diseases other than PDD. Sera from 27 of the 30 PDD cases (90%) contained antibodies to ABV. Seventy-three (84%) of the apparently "healthy" birds were seronegative. Additionally, sera from seven macaws and one parrot trapped in the Peruvian Amazon were seronegative. Positive sera recognized the bornaviral nucleoprotein (N-protein). While the presence of antibodies to ABV largely corresponded with the development of clinical PDD, 14 apparently healthy normal birds possessed detectable antibodies to ABV. The existence of a carrier state was confirmed when 13 of 15 apparently healthy cockatiels were shown by PCR to have detectable ABV RNA in their feces. Western blot assays may be of significant assistance in diagnosing proventricular dilatation disease. Many apparently healthy birds may however be seronegative while, at the same time, shedding ABV in their feces.

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## 1. Introduction

Proventricular dilatation disease (PDD) has caused significant losses in captive birds since the early 1980s when it was first recognized in Europe and North America. PDD affects at least 50 species of psittacine birds as well as other bird species (Clark, 1984; Gregory et al., 1994). The clinical signs of PDD vary between individuals and species, but in general they exhibit two general types of disease. Some develop neurologic signs such as depression, seizures, ataxia, blindness, tremors and incoordination (Steinmetz et al., 2008). A peripheral neuritis has also been reported in some birds involving the sciatic, brachial and vagal nerves (Berhane et al., 2001). Alternatively, birds may develop gastrointestinal problems such as crop stasis, regurgitation, inappetance, and undigested food in feces secondary to damage to the enteric nervous system. This damage leads eventually to starvation and death. Death due to circulatory collapse or food aspiration is also common. Birds may show neurologic signs or gastrointestinal signs or both. It is also suspected that some affected birds may show minor or no clinical signs. Definitive

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diagnosis of PDD is based on detection of a lymphoplasmacytic infiltration in the ganglia and nerve plexus, especially the myenteric plexus of the gastrointestinal tract (Schmidt et al., 2003). This diagnosis is however made difficult by the variable distribution of lesions in birds. For example, in one series of 14 birds (Berhane et al., 2001) lesions were seen in the crop in 43% of cases, proventriculus 36%, ventriculus 93%, duodenum 21%, heart 79%, adrenal gland 50%, spinal cord 69%, brain 46%, sciatic nerve 58%, brachial nerve 46% and vagus nerve 46%. In another series (Shivaprasad et al., 1995), 72% of 61 PDD cases had lesions in the crop. While crop biopsy has been considered the most definitive diagnostic test, it clearly fails to detect a significant portion of the affected population.

PDD has long been considered to have an infectious etiology. Recently, the use of high-throughput viral screens has enabled investigators to identify the presence of a new virus, avian Borna virus (ABV), from several cases of biopsy-confirmed PDD (Honkavouri et al., 2008; Kistler et al., 2008). Subsequent studies using PCR and immunohistochemistry on tissues of affected birds have confirmed the association between ABV infection and PDD (Rinder et al., 2009; Weissenbock et al., 2009). Ouyang et al. (2009) have demonstrated the presence of ABV by immunohistochemistry in the central nervous system of multiple clinical cases of PDD. PDD has been induced experimentally in cockatiels (*Nymphicus hollandicus*) by Gancz et al. (2009) using infected bird brain and in Patagonian conures (*Cyanoliseus patagonus*) by Gray et al. (2009) using ABV4 passed for six passages in duck embryo fibroblasts. We have also reported on the presence of ABV antigen in the tissues of birds with PDD using a Western blot assay (Villanueva et al., 2008).

Borna viruses are nonsegmented, negative strand RNA viruses belonging to the family *Bornaviridae*. Unique characteristics of Borna viruses (nuclear localization of transcription, alternative splicing, and a differential use of initiation and termination signals) justified their classification into a separate family in the order *Mononegavirales* (Briese et al., 1992). Until recently, only one member of the *Bornaviridae* was known, Borna disease virus (BDV), the cause of a meningoencephalitis in horses and sheep largely restricted to central Europe (Ludwig and Bode, 2000). However pyrosequencing of cDNA from the brains of parrots with PDD identified two strains of a novel Borna virus (Honkavouri et al., 2008). Using real time PCR, the study confirmed the presence of this virus in brain, proventriculus and adrenal gland in three birds with PDD but not in four unaffected birds. Kistler et al. (2008) used a microarray approach to identify a Borna virus hybridization signature in five of eight PDD cases and none of eight controls. Using high-throughput pyrosequencing in combination with conventional PCR cloning and sequencing, these investigators were able to recover the complete viral genome sequence of one strain and named this virus avian Borna virus. Subsequent investigations have identified seven genotypes of this virus (Weissenbock et al., 2009).

Our ability to culture ABV from cases of PDD provides a convenient source of antigen for serologic assays. We have therefore developed a Western blot assay that can detect antibodies to ABV in the serum of psittacids. We report here on the use of this test as an aid to the diagnosis of PDD and provide further evidence for the linkage between PDD and ABV infection.

## 2. Materials and methods

### 2.1. Sample material

*Schubot Center birds*: The Schubot 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. All birds underwent crop biopsy and blood was taken for serology. Birds that died were necropsied and the cause of death was determined.

*Submitted samples*: A total of 94 serum samples from 33 species of normal psittacines and suspected cases of PDD were submitted by veterinarians who were members of the American Association of Avian veterinarians. A confirmed diagnosis of PDD was based on a positive necropsy or crop biopsy showing the characteristic lymphoplasmacytic infiltration of the neuronal ganglia. A negative diagnosis was based on a histopathologically negative necropsy or biopsy, apparent good health, or clear evidence from necropsy that the bird was suffering from a disease other than PDD. A diagnosis of PDD could not therefore be positively excluded in these birds.

*Cockatiels*: Fifteen apparently healthy cockatiels (*Nymphicus hollandicus*) were purchased from a single breeder.

*Wild birds*: Blood samples were obtained from seven wild scarlet macaws (*Ara macao*) and one mealy Amazon parrot (*Amazona farinosa*). The samples were taken at the Tambopata research center, Peru (−13.138290°, −69.607960°) from birds trapped for radiotelemetry studies, sampled, and released immediately afterwards.

### 2.2. Antigen preparation

The antigen preparation used for immunoblotting was a lysate from duck embryo fibroblast (DEF) cultures infected with ABV strain M24 originally isolated from a yellow-collared macaw (*Primolius auricollis*) and passaged six times in the duck embryo fibroblasts. M24 belongs to ABV genotype 4. Cultures were harvested at 5 days post-infection when the ABV-infected or normal DEF cells were lysed by freezing and thawing. Recombinant ABV N-protein, also used as a source of antigen, was generated as follows: RNA was isolated from DEF inoculated with the brain homogenates of a PDD-positive bird (M14) using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). First strand cDNA was generated using the High Capacity cDNA Reverse Transcription Kit and random primers (Applied Biosytems, Foster City, CA, USA). ABV N sequence was amplified using primers 5′ GCG GTA ACA ACC AAC CAG CAA 3′ and 5′ GTT CAT TAG TTT GCR AAT CCR GTT A 3′. The amplified region was initially cloned into pTOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The N open reading frame was then reamplified using forward primer Borna N attB1 (5′-AAA AAG CAG GCT TCA CCA TGG AAA

TGC CAC CCA ARA GAC AAA-3′) and reverse primer N1-V5 attB2 (5′-AGA AAG CTG GGT GTT TGC RAA TCC GST-3′), to generate a V5-tagged N product, or Borna N1 attB2 (5′-AGA AAG CTG GGT TCA TTA GTT TGC RAA TCC G-3′). The amplified products were moved into pcDNA3.2/V5 DEST (Invitrogen, Carlsbad, CA, USA) by recombination-mediated cloning. N expression vectors were transfected into CHO-K1 cells using Lipofectamine 2000 (Invitrogen) and after 36–48 h the cells were collected and lysed with RIPA buffer (150 mM $NaCl_2$, 0.5% DOC, 0.1% SDS, 50 mM Tris–HCl, 1% NP-40) by incubation for 5 min on ice. Expression of V5-tagged N-protein was verified by Western blot with anti-V5 antibody. In addition, cloned histidine-tagged ABV N (strain 1367; 49 kDa, including tag and vector sequence) purified on Ni-NTA-Agarose (Qiagen) was used to confirm the identity of the dominant antigen.

### 2.3. Western blot assay

Western blot analysis was used to test the serum or plasma of birds for the presence of ABV-specific antibodies. Western blots were performed based on methods originally described by Towbin et al. (1979). Infected DEF lysates were standardized to contain 30 μg of protein/lane and electrophoresed on 10% polyacrylamide gels. Proteins were transferred to a polyvinylidene fluoride (PVDF) in transfer buffer at 100 mA for 2 h. Membranes were blocked with PBS pH 7.4 (Sigma, St. Louis, MO, USA) plus 3% non-fat dry milk and 0.05% Tween 20 at room temperature for 2 h. Test sera were diluted 1:100 in PBS plus 1.5% BSA and 0.05% Tween 20 and exposed to the antigen blots at room temperature for 2 h. Membranes were rinsed three times for 5 min with PBS plus 0.05% Tween 20. Membranes were then incubated with alkaline phosphatase labeled goat anti-macaw IgY (Bethyl Laboratories, Montgomery, TX) at a dilution of 1:10,000. The membrane was rinsed again and developed for 10 min with 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT) from Sigma. Membranes were air dried for about 30 min before reading. Positive control serum was obtained from M24, a yellow-collared macaw with clinically, necropsy- and histologically confirmed PDD. For control purposes, an antibody-negative serum from M21, a blue-and-gold macaw (*Ara ararauna*) histopathologically confirmed to be free of PDD lesions, was used.

### 2.4. Fecal PCR assay

Fresh fecal samples were suspended in 500–750 μl of sterile saline on ice. The samples were mixed and the solids were pelleted by brief centrifugation at 5000 rpm. RNA was purified from the supernatants using either the Ambion MagMAX™ Viral RNA Isolation Kit or the QIAamp Viral RNA Mini kit, according to manufacturer's recommendations. Approximately 1 μg of RNA was used for the synthesis of cDNA (Applied Biosystems® High Capacity cDNA Reverse Transcription Kit) using random primers. ABV sequences were amplified using primer sets: forward (5′-CAG ACA GCA CGT CGA GTG AGA-3′) and reverse (5′-GGC TCT TGG TCT GAG ATC ATG GAA-3′). The PCR conditions were as follows: initial denaturation, 94 °C for 3 min, followed by 35 cycles of 94 °C, 30 s, 54 °C, 30 s and 72 °C, 20 s followed by a final extension of 5 min at 72 °C. Samples were analyzed by agarose gel electrophoresis.

## 3. Results

### 3.1. Sera from PDD-affected birds recognize ABV N-protein

Western blot assays using antigen from infected DEF and serum from PDD confirmed birds showed that most birds reacted strongly with a single 38–40 kDa protein (Fig. 1). This antigen is also found in infected tissues, especially the central nervous system of PDD cases (Villanueva et al., 2008). It is expressed in ABV-infected DEF where its quantity increases over time. Its molecular weight is compatible with the predicted size of ABV N-protein. Antisera reacting with this band reacted with cloned ABV N-protein derived by two different procedures (Fig. 2). The specificity of the reaction to N-protein in tissue cultures was verified by preabsorption; antibodies to the N-protein were absorbed out using an infected DEF lysate but not an uninfected DEF lysate (not shown).

### 3.2. Western blot assay

Of the 23 birds from the Schubot Center aviary at Texas A&M University that were crop biopsied or necropsied, 11 had characteristic lymphoplasmacytic infiltrates in their ganglia and thus were considered to have confirmed PDD. Of these 11 biopsy/necropsy-positive birds, nine (82%) were also seropositive (Table 1). Three of these positive birds were African grey parrots in apparent good health. Twelve birds in the aviary were healthy and had no detectable crop lesions so their disease status was unclear. Of these 12 birds, four (33%) were also seropositive. Since it has been determined that crop biopsy is positive in only 40–70% of PDD cases (Berhane et al., 2001; Shivaprasad et al., 1995), it is entirely possible that these four seropositive, biopsy-negative birds were subclinical PDD cases.

Ninety-four serum samples were submitted by veterinarians together with clinical and histopathological documentation of their PDD status. Of 19 birds with necropsy or biopsy-confirmed PDD, 18 (94%) were seropositive. Of 75 "healthy" birds that did not appear

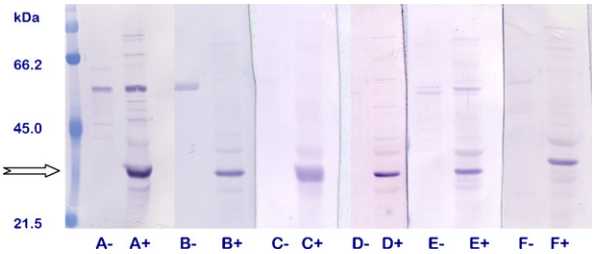


**Fig. 1.** The predominant Western blot patterns observed using a standard tissue culture-derived antigen preparation from ABV-infected (+) or non-infected (−) cultures and exposed to six positive avian sera. Each serum was diluted 1:100. (A) Nanday conure, (B) Cockatiel, (C) African grey, (D) White cockatoo, (E) DuCorp's Cockatoo, and (F) Golden Conure. The arrow denotes the location of ABV N-protein.

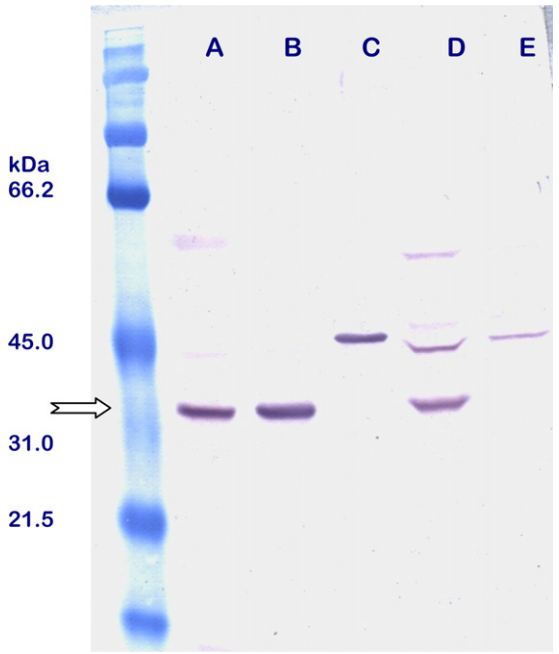


**Fig. 2.** Comparison of reactivity between lane A, crude brain-derived antigen from a confirmed case of PDD; lane B, cell lysate from ABV-infected tissue culture-derived; and two cloned ABV N-protein preparations, lanes C and D using a single positive macaw serum (M24). The cloned N-protein in lane C carries a 7 kDa histidine-tag. The cloned N-protein in lane D is unpurified and grown in Chinese hamster ovary (CHO) cells. Lane E consists of a lysate of normal CHO cells. The positive serum (M24) used in this immunoblot was absorbed once with a CHO cell lysate in order to reduce the background in lanes D and E. The arrow denotes the location of the ABV N-protein.

to have PDD, 65 (85%) were seronegative, while 10 were seropositive. Based on veterinarian's reports, at least nine of these 10 had been in "contact" with known PDD cases.

Serum samples obtained from eight wild psittacids captured at the Tambopata Research Center, Peru were negative for antibodies to ABV.

### 3.3. Detection of ABV by virus culture or PCR

PCR was performed on fresh droppings and/or cloacal swabs from the 15 healthy cockatiels. On initial testing, fecal PCR was positive in seven of these birds. Two birds were seropositive, one fecal PCR-negative and one positive. Subsequent weekly testing of the eight remaining negative birds by fecal PCR over the next 4 weeks eventually identified an additional six PCR-positive birds for a total of 13/15 positive cockatiels. None of these birds changed their serologic status during the month-long observation period. In addition, fecal samples from 13 birds housed at the Schubot Center aviary were also tested and five were found to be PCR-positive. Thus of 28 fecal samples tested, 18 (64%) were positive for fecal ABV (Fig. 3). Six of these 18 birds with positive PCR were also seropositive; three of the 10 PCR-negative birds were also seropositive (Table 2).

### 3.4. Sensitivity and specificity of the serological assay

When the results of testing serum from both Schubot Center and submitted samples are combined, 27 of 30 confirmed PDD cases were seropositive giving a sensitivity of 90%. Of 87 apparently healthy or non-PDD cases, 14 were seropositive, a specificity of 82% (Table 1). In contrast however, in those birds where fecal PCRs were performed (Table 2), only six of 18 PCR-positive birds were seropositive while three of 10 PCR-negative birds were seropositive giving the Western blot procedure an apparent sensitivity of 33% and a specificity of 30%.

## 4. Discussion

Serology has been widely employed in the diagnosis of mammalian Borna virus infection although its interpretation has been controversial (Ludwig and Bode, 2000). The most common tests employed have been immunofluorescence, ELISA and Western blot assays using persistently infected cells, lysates of such cells, or cloned recombinant proteins (Wagner et al., 1968; Rott et al., 1991; Thiedemann et al., 1992; Hsu et al., 1994; Briese et al., 1995). Much of the controversy regarding the significance of serologic assays has centered on the proposed association between human neuropsychiatric illness and the presence of antibodies to Borna disease virus. This association has generally been disproven, serologic results obtained have not been reproducible and are probably low-titred false positive reactions or a result of laboratory contamination (Staeheli et al., 2000; Durrwald et al., 2007). It is perhaps appropriate to consider the significance of all bornaviral serology with some caution based on this human experience.

Nevertheless, sera from mammals with confirmed BDV infection and tested by Western blotting usually recognize the viral N, and P antigens. Triggered by the susceptibility of chickens to experimental infection (Ludwig et al., 1985), Borna virus infection in birds has been investigated previously (Malkinson et al., 1993; Berg et al., 2001). For example, Borna disease virus has been detected in the feces of wild birds, notably mallards (*Anas platyrhyncos*) and jackdaws (*Corvus monedula*) by PCR (Berg et al., 2001). However the analyzed viral sequences were a match for

**Table 1**
Summary of Western blot results in serum samples from birds housed in the Schubot Center aviary and samples submitted by participating veterinarians. Confirmed PDD-positive birds had the characteristic histopathological lesions as determine by biopsy or necropsy.

| | Schubot Center confirmed positive | Schubot Center confirmed negative | Submitted confirmed positive | Submitted believed "healthy" | Total |
|---|---|---|---|---|---|
| Seropositive | 9 | 4 | 18 | 10 | 41 |
| Seronegative | 2 | 8 | 1 | 65 | 76 |
| Total | 11 | 12 | 19 | 75 | 117 |

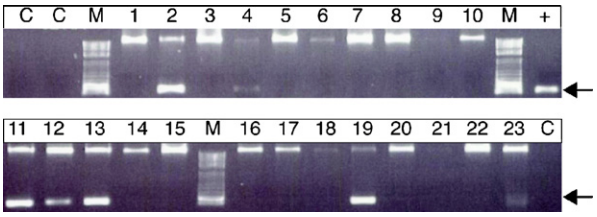


**Fig. 3.** Use of PCR for the detection of ABV sequences in bird feces. Lanes 1–10 and 16–23: PCR products from fecal samples from healthy cockatiels. Lanes 11–15: PCR products from fecal samples from healthy African gray parrots. Lanes 2, 4, 11, 12, 13, 19 and 23 show the presence of a band corresponding to the expected 131 bp fragment. 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.

**Table 2**
Summary of Western blot results for birds from the Schubot Aviary tested by fecal RT-PCR for the presence of ABV.

| | Fecal PCR-positive | Fecal PCR-negative | Total |
|---|---|---|---|
| Seropositive | 6 | 3 | 9 |
| Seronegative | 12 | 7 | 19 |
| Total | 18 | 10 | 28 |

those of mammalian BDV and different from those of the recently characterized ABV.

In this study, sera from PDD-affected birds reacted strongly with cloned N-protein indicating that the immunodominant antigen detected in Western blot assays is the 38 kDa N-protein of ABV. A comparison of Western blotting results with fecal PCR indicates that the Western blot assay does not detect all birds infected with ABV. The survey of cockatiels showed that almost all the birds tested had positive fecal PCRs yet were seronegative. The birds appeared to be carriers of ABV that showed no clinical symptoms. Repeated testing of these birds also indicated that seroconversion does not necessarily occur rapidly after the appearance of ABV sequence in feces samples and may not even develop months after ABV is detectable by PCR (Unpublished observations).

Given the absence of a satisfactory antemortem diagnostic test for PDD, it is very difficult, if not impossible, to certify any bird free of this disease. Crop biopsy is not only a highly insensitive procedure but also a significant surgical procedure not undertaken lightly. Furthermore crop biopsy is only positive in 40–70% of PDD cases. It is difficult to justify this procedure in clinically healthy, normal birds just to confirm the absence of PDD. Thus a "healthy" bird cannot be guaranteed to be free of PDD. As a result, it is very difficult to determine the true sensitivity of the Western blot assay. Seropositivity in "healthy" birds is also difficult to interpret. As in most other infectious diseases, a population of seropositive healthy birds can be expected to exist that may include infected animals about to develop disease or recovered animals that are immune to the disease. The relatively few seropositive healthy birds observed in this study (16% or less) is compatible with two hypotheses that reflect what we know about PDD. First, few birds may survive an infection to become seropositive. Second, seropositivity may develop only immediately prior to disease onset–a situation analogous to mammalian Borna disease where clinical disease is a result of immunopathologic mechanisms (Ludwig and Bode, 2000). Although seropositivity may therefore serve as a useful non-invasive diagnostic marker in general, in some cases a more complex scenario may need to be considered. Three of our African Grey parrots were crop biopsy positive, seropositive and fecal PCR-positive but remained clinically healthy for at least 1 year. However our finding that all eight healthy, wild-caught birds from Tambopata, Peru were seronegative provides some assurance that ABV is not present in that specific region of the Amazon basin.

Western blotting represents a significantly improved diagnostic test for PDD when compared to the currently available crop biopsy. Given the finding that seronegative birds that appear to be shedding the virus as detected by PCR, it is however not feasible to eliminate infection from an aviary by the use of Western blotting alone. Western blotting requires only a small blood sample and offers in combination with fecal PCR a practical tool for *intra-vitam* diagnosis and surveillance.

## Conflict of interest statement

None of the authors has a conflict of interest that could inappropriately influence this study.

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