UPDATE ON AVIAN BORNAVIRUS AND PROVENTRICULAR DILATATION DISEASE

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INTRODUCTION

Proventricular dilatation disease (PDD) is an infectious disease of psittacine birds and perhaps many other bird species that occurs in many different countries, including Australia (Clark, 1984, Doneley, et al., 2007). Its present name is derived from the predominant clinical sign in large parrots, namely the dilatation of the proventriculus by accumulated food as a result of intestinal pseudo-obstruction although the degree of intestinal dysfunction varies greatly between species. This intestinal dysfunction is secondary to damage in the enteric nervous system. Additional nervous system damage may also include the development of a non-suppurative encephalitis resulting in depression, seizures, ataxia, blindness, tremors and incoordination (Steinmetz et al., 2008). The lesions in the enteric nervous system may affect the crop, proventriculus, ventriculus and intestine leading to crop stasis, regurgitation, inappetance, and eventually to starvation and death. Death due to circulatory collapse or food aspiration is common. Affected birds may show some combination of neurologic or gastrointestinal signs. Definitive diagnosis has been based on biopsy or necropsy findings, specifically, the presence of a lymphoplasmacytic infiltration in the ganglia and myenteric plexus of the gastrointestinal tract (Schmidt et al., 2003). Definitive diagnosis of PDD is made more difficult by the inconsistent distribution of lesions. Thus Berhane et al. (2001) found lesions 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%. Similar results have been reported by others (Shivaprasad H. Personal Communication).

PDD has long been considered to have an infectious aetiology. Numerous observations and anecdotes have suggested that it is a transmissible disease and it has generally been assumed to be caused by a virus. Several different viruses have been suggested as its cause. For example, a paramyxovirus related to Newcastle disease was long considered a likely cause (Grund et al., 2002), since it was reported that this virus could be isolated in up to 60% of PDD cases. Likewise a coronavirus has been isolated from a bird with PDD (Gough et al., 2006). Neither of these agents are consistently present in affected parrots and they are almost certainly not a cause of this disease.

In 2008, pyrosequencing of cDNA from the brains of parrots with PDD identified two strains of a novel bornavirus (Honkavuori 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 used a microarray approach to identify a bornavirus hybridization signature in five of eight PDD cases and none of eight controls (Kistler et al., 2008). Using high-throughput pyrosequencing in combination with conventional PCR cloning and sequencing, these investigators were able to recover the complete viral genome sequence and named this virus avian bornavirus (ABV). At the same time, our group succeeded in culturing ABV from the brains of 7 psittacine cases of PDD, providing a source of antigen for serologic assays and nucleic acid for molecular assays.

Bornaviruses are negative strand RNA viruses that belong to the family *Bornaviridae*. Their most unique characteristic is that they undergo transcription within the nucleus. They also undergo alternative splicing, and use different initiation and termination signals (Briese et al., 1992). Until recently, only a single member of the family was known, Borna disease virus (BDV), the cause of a meningoencephalitis in horses and sheep that occurs only in central Europe. BDV has been identified in the feces of wild mallards and corvids in Scandinavia but the extent of avian infections and their epidemiologic significance is unclear (Berg et al., 2001). An outbreak of disease attributed to BDV has been reported in ostriches. This diagnosis was based only on serology and unfortunately those virus isolates have been lost (Malkinson et al., 1993). Mammalian bornavirus is however only distantly related to ABV such that reagents against one do not react with the other. The two diseases do not appear to be directly related.

In this review we provide proof that ABV is the cause of PDD and describe initial studies on its epidemiology as well as some of the unique pathology of this virus infection.

ABV CAN BE ISOLATED FROM PDD CASES

We initially spent considerable resources attempting to infect chickens or their eggs with material from PDD cases but without success. When however we inoculated mallard embryo fibroblasts with a fresh brain suspension from a case of PDD in a yellow-collared macaw (*Primolius auricollis*) we succeeded in growing a virus (Gray et al., Submitted). This agent caused no detectable cytopathic effect but we had available serum from a PDD-affected green-winged macaw (*Ara chloroptera*) that recognized specifically ABV N-protein. By performing a western blot on infected duck cell lysates we demonstrated a progressive increase in the quantity of this viral antigen when cultured over five days. Indirect immunofluorescence assays on these infected cells using this same antiserum showed foci of antigen-positive cells demonstrating characteristic speckled intranuclear fluorescence. This speckled pattern is similar of that considered diagnostic of mammalian bornavirus infection: the stained particles are called Joest-Dagen bodies and are believed to be complexes of the viral N and P proteins (Herzog and Rott, 1980). PCR assays conducted on this and additional infected tissue cultures confirmed the presence of ABV growing in the cultures. Subsequently we have succeeded in isolating ABV in duck embryo fibroblasts using freshly harvested brains from 6 additional birds with necropsy-confirmed PDD. Of the 7 isolates, one is genotype 1 (ABV1) while the remainder are genotype 4 (ABV4).

ABV IS PRESENT IN THE TISSUES OF PDD CASES

Initial studies using the western blot assay on the tissues derived from necropsy of about 15 PDD-affected birds demonstrated that most PDD-affected bird serum consistently detected an antigen of 38-40 kDa in their central nervous system (Villanueva et al., 2008). We also occasionally found this antigen, now known to be ABV N-protein, in myocardium but not in other organs of affected birds. We subsequently tested 24 stored avian brain samples, processed for histopathology and retained following their submission for necropsy or histopathology to the Schubot Exotic Bird Center diagnostic laboratory in 1992 - a year selected at random. Thirteen of these samples were from birds diagnosed at that time as suffering from PDD. The remaining 11 were diagnosed as suffering from diseases other than PDD. Immunohistochemistry was performed using the macaw anti–protein serum directed against the ABV nucleoprotein (N-protein) and developed with a peroxidase-labeled anti-macaw serum. Stained slides were read by an investigator unaware of their prior histopathology results. We found cells containing ABV N-protein in the brain and spinal cord of all 13 PDD cases (Ouyang et al. 2009). One bird, not previously diagnosed with PDD also had ABV N-protein-positive cells in its

cerebrum. A review of this bird's necropsy report indicated that it was, most probably, also suffering from PDD. Ten birds submitted for necropsy after dying for reasons other than PDD, had no detectable N-protein in their brains. ABV antigen was found consistently in cerebrum, cerebellum and spinal cord. In the cerebrum it was usually found in scattered neurons and glial cells. In the cerebellum viral antigen was expressed in the Purkinje layer of the cerebellum although Purkinje cells were never observed to contain the antigen. The cells containing the antigen were located adjacent to the Purkinje cells. Similar lesions have been observed in mammalian bornavirus infections (Eisenman et al., 1999.) All levels of affected spinal cord contained ABV antigen positive neurons and glia.

In a single separate case of PDD in an Eclectus parrot, eye fluid (vitreous plus aqueous fluid) was collected on necropsy. This fluid contained so much virus that an N protein band was visible in stained electrophoresis gels. This protein band was excised and sequenced by mass spectroscopy and confirmed to be the ABV N-protein. It was from this fluid that the first particles with a morphology consistent with that of a bornavirus were observed by transmission electron microscopy (Figure 1).

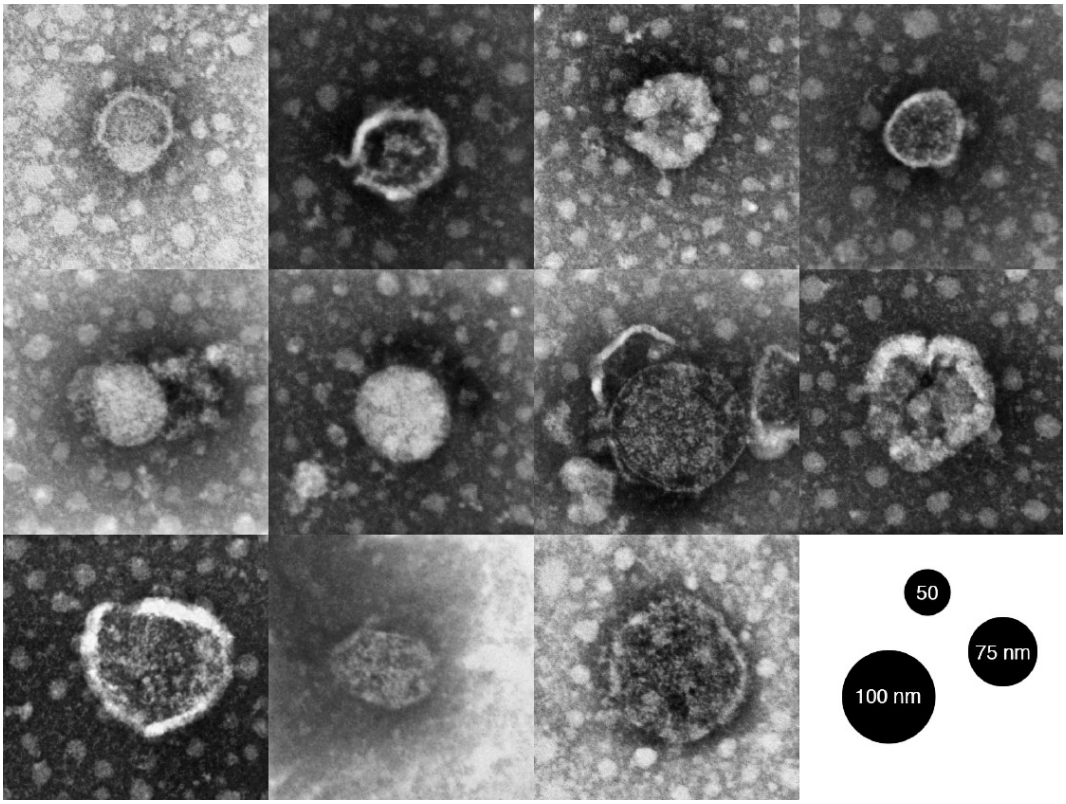

Figure 1. Negative-stained electron micrograph of virus-like particles from the eye fluid of an Eclectus parrot (*Eclectus roratus*) with necropsy-confirmed PDD. Photo courtesy of Dr Ross Payne.

BIRDS WITH PDD MAKE ANTIBODIES TO ABV

An immunoblot assay (Towbin et al. 1979) was used to detect the presence of antibodies against avian bornavirus (ABV) in the serum of 117 psittacine birds. A lysate from ABV-infected duck embryo

fibroblasts served as the source of antigen. The test was used to detect antibodies against the 38 kDa N-protein. 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 N-protein. Seventy three (84%) of the 87 apparently "healthy" birds were seronegative. Additionally, sera from 7 macaws and one parrot trapped in the Peruvian Amazon were negative. Most of the positive sera recognized only the N-protein in infected DEF cells. These positive sera also reacted strongly with two different preparations of recombinant N-protein. One clone was generated in an *E. coli* vector, the other was generated in Chinese hamster ovary (CHO-K1) cells. Some of the positive sera could also recognize cloned bornaviral P protein generated in an *E. coli* vector. While the presence of antibodies to ABV largely corresponded with the development of clinical PDD, it must be pointed out that 14 apparently healthy normal birds possessed detectable antibodies to ABV. We suggest that these birds are infected by ABV but have yet to develop disease. We suggest that western blot assays may be of assistance in diagnosing proventricular dilatation disease. Their sensitivity in diseased birds is as high as 90% and their specificity may be as high as 100%. However, many apparently healthy birds may be seronegative while, at the same time, shedding ABV in their feces so serology is of limited usefulness in any disease eradication programs. It is important to point out however that the western blot assay is slow and expensive. We believe that an ELISA employing purified, cloned ABV N-protein as the test antigen has a greater potential as a practical and economic diagnostic test. Such a test is under development.

SOME PDD BIRDS MAKE AUTOANTIBODIES

During the course of developing the western blot assay we originally used brain tissue from PDD-affected birds as an antigen source. For control purposes we used brain tissue from a bird that had died from causes other than PDD and which was both seronegative and PCR-negative. We found that occasionally the serum of a positive bird would react with antigens in the normal brain tissue. In one case, serum from a "healthy" golden conure (*Guaruba guarouba*) reacted very strongly with a protein migrating in the 18-20 kDa region. Since this is the approximate molecular weight of myelin, we investigated further. We found that this serum also reacted with a similar sized protein in normal chicken brain and with purified myelin basic protein (MBP) derived from that chicken brain. This bird, although a seropositive PDD contact, remains healthy after one year. Serum taken seven months later reacted only weakly with MBP. We interpret this result to suggest that the bird mounted a transient autoimmune response to myelin. During a survey of twelve ABV-positive sera for reactions with normal macaw brain we also found that three reacted with a 40kDa protein. We have not characterized that protein. It has been suggested that PDD results from an autoimmune response to brain gangliosides following viral infection in a manner similar to the induction of Guillain-Barre syndrome (Rossi et al. 2008). We have no evidence to support this suggestion but as described above, transient autoimmune responses do indeed occur in some PDD cases. We believe that they are probably not clinically significant and are not central to the pathogenesis of PDD.

ABV IS SHED IN THE FAECES OF PDD CASES

The polymerase chain reaction (PCR) using sequences from the N-gene as a primer readily detects ABV sequences in faecal samples from ABV-infected birds. Fresh faecal samples were suspended in sterile saline, on ice. The samples were mixed and the solids pelleted by brief centrifugation. RNA was purified from the supernatants using either the Ambion MagMAX™ Viral RNA Isolation Kit or the QIAamp Viral RNA Mini kit. Samples were analyzed by agarose gel electrophoresis. We examined samples from both faecal and cloacal swabs. Any PCR products detected were confirmed by

sequencing. In a group of 13 birds with biopsy confirmed PDD or housed with PDD birds, 77% were positive by faecal PCR.

THERE ARE MANY HEALTHY CARRIERS OF ABV

A group of 16 "healthy" cockatiels from a single aviary, with no history of PDD or exposure to other birds, was screened by faecal PCR. Six were positive on first testing, four more on testing a week later and two more on a third test. Thus 75% of these birds were eventually ABV positive; two (12.5%) were also positive by western blot. The presence of so many PCR positive birds in a "healthy" colony of cockatiels prompted us to screen the cockatiel colonies. A second colony of about 50 birds that practices no biosecurity and purchases birds at random from dealers was tested and 14/15 faecal samples were PCR positive. The owner claimed that he had no significant health problems having lost only 5 birds this year. We consider a 10% loss of some significance. A third cockatiel colony tested practiced fairly rigorous biosecurity and purchased very few birds from outside. From this colony, 0/15 faecal samples were PCR positive. This study is ongoing but it is clear that many "healthy" cockatiels are shedding ABV.

PDD CAN BE INDUCED WITH CULTURED ABV

There is abundant evidence now available to show that infection with ABV is associated with clinical PDD and recently, Gancz et al. (2009) were able to induce PDD in cockatiels with infected brain homogenates. Koch's postulates remain however the standard by which an infectious agent can be proven to cause a specific disease (Koch 1890). When we succeeded in culturing ABV4 in duck embryo fibroblasts, we immediately infected 15, 4-day old, SPF mallard chicks by the oral, intraocular and intramuscular routes. Over a period of six weeks, no consistent clinical signs attributable to ABV infection occurred. The mallards were however infected with this virus and shed feces that were positive for ABV by PCR when tested at weekly intervals. Likewise these birds were seropositive for antibodies to ABV N-protein after three weeks.

Our attempts to induce PDD in 6 cockatiels by infecting them with ABV4 by the intramuscular and oral routes were confounded when we determined that these birds were already healthy shedders of ABV2. One such inoculated cockatiel did however develop classical PDD at day 96. The others remain healthy so it is too early to draw conclusions from this study.

We had however three Patagonian conures (*Cyanoliseus patagonus*) available in our aviary. All three were at least 15 years old, shedding psittacine herpesvirus but otherwise healthy. These birds were seronegative by western blotting and their feces were PCR negative for ABV. Two were placed in isolation and given cultured ABV4 by both the oral route and intramuscular injection. The third, uninfected bird was housed in a separate aviary and inoculated with uninfected DEFs. The two infected birds seroconverted by about 3 weeks and became faecal shedders by 5 weeks. Aviary workers reported that they were eating but losing weight. At day 64, one of these birds was found dead in its cage. Necropsy showed a very thin bird with gross lesions typical of PDD. The next day, the second infected bird was examined closely, it too was very thin and a decision was made to euthanize it on humane grounds. Necropsy again showed typical gross PDD lesions. Fixed tissues were subjected to histopathologic examination and showed typical PDD lesions - lymphocytic ganglioneuritis throughout the intestine as well as a lymphoplasmacytic encephalitis and myocarditis. PCR on the brain of these birds was strongly positive for ABV. The fragments were sequenced and shown to be identical to the challenge strain, M24. The control bird remained healthy but was euthanized at day 77 after receiving uninfected tissue culture. Necropsy and histopathological

examination showed no evidence of PDD. PCR of four separated brain samples was negative for ABV. There remains no doubt that avian bornavirus is the cause of proventricular dilatation disease.

FUTURE DIRECTIONS OF PDD RESEARCH, CONTROL, TREATMENT AND PREVENTION

The next steps include the development of methods of disease control, treatment and prevention. As far as treatment is concerned, we have in our collection a group of clinically healthy, seropositive, ABV shedding African grey parrots (*Psittacus erithacus*). We have treated them with amantadine for six weeks with no apparent effect on faecal viral shedding. We tested amantadine since the literature suggested that seropositive humans with mental illness showed clinical improvement following amantadine treatment (Dietrich et al. 2000). Biochemically it is unclear just how amantadine would interfere with bornavirus replication and some investigators believe that any effects may have been a result of the drug having a direct effect on brain function. However we plan to test a series of antiviral compounds and use virus shedding as a marker of efficacy. Treatment protocols will have to be developed although experience with mammalian encephalitides suggests that it may be difficult to identify an effective antiviral therapy.

It is generally believed that Borna disease in mammals is in large part immunologically mediated (Stitz et al., 1995, Morimoto et al., 1996. Schwemmie and Lipkin, 2004). Immunosuppressive or antiinflammatory treatment such as corticosteroids appear to reduce the severity of disease while immune stimulation appears to increase its severity. Thus Borna disease is believed to belong to that group of infections where immune responses increase severity and vaccination may be contraindicated. It is certainly true that most birds suffering from PDD are strongly seropositive implying that antibodies to the immunodominant N-protein are not protective. In addition, there are obvious economic issues involved in the commercial production of a vaccine for use in a small, specialised market. I have no doubt that these issues will eventually be overcome but we have a long way to go before this disease is conquered.

The control of viral infections, in many cases depends upon appropriate management practices and infection control. While we have yet to determine the sensitivity of ABV to routine disinfectants, its overall structure is similar to that of Newcastle disease virus (NDV) - both are enveloped Mononegavirales. ABV would be expected therefore to show a sensitivity profile similar to that observed in NDV. Disinfectants that are effective in controlling NDV may also reasonably be expected to be effective in controlling ABV.

With the availability of a faecal PCR, it should also be possible to control the admittance of ABV-infected birds to aviaries. It must be pointed out however that not all birds are constant ABV shedders. We have shown that three African grey parrots had detectable virus in their feces on two out of three occasions tested. Likewise, it took three tests at weekly intervals to demonstrate that 14/16 cockatiels were infected. It is likely that the remaining two birds were also infected but not shedding when sampled. Thus as with psittacine herpesviruses, repeated testing may be required to exclude any specific bird as an ABV carrier.

In conclusion, all available evidence supports the contention that ABV is the sole etiologic agent of PDD. Diagnostic tests such as western blots or faecal PCR can identify many, but not all ABV-infected birds and should be employed to control the spread of this disease. Such tests may be very useful in diagnosis and in epidemiologic studies. Neither however is 100% sensitive and must be interpreted in this light.

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