OVERVIEW OF THE ROLE AND DIAGNOSIS OF AVIAN BORNAVIRUS RELATED TO PDD IN PSITTACINES [1]

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ABSTRACT

Proventricular dilatation disease (PDD) in parrots (Psittaciformes) is a disease, which can affect the central nervous system as well as the digestive system. The disease causes "wasting" and is recognized primarily by the discharge of undigested seeds in the feces and emaciation of the affected birds. A viral or microbial etiology of the disease has been considered for quite some time, but only recently evidence has been presented that associates a novel avian Borna virus (ABV) with PDD. This conclusion was based on the finding that, tissues from PDD positive birds contained the virus, whereas no ABV was found in PDD-free controls. Now, one and a half year after the discovery of this virus at least two successful transmission studies have fulfilled the trias of Koch.
Investigations have shown that contrary to the mammalian Borna disease virus infecting horses and sheep, where the virus shows a high preference for the central nervous system, ABV may be found in all tissues. Since the discovery of ABV many studies have been initiated to develop diagnostic tests (PCR and serology), and also to demonstrate whether the virus is causing PDD. This review will give an overview on what has been found, our current understanding, and what are still open questions. With respect to diagnostics a main topic addressed will concern the interpretation of a positive serology for p40 nucleoprotein and a positive PCR result for ABV RNA.

INTRODUCTION AND REVIEW

Proventricular dilatation disease (PDD) is a fatal inflammatory disease of predominantly psittacine birds. It primarily affects the autonomic nerves located in the upper and middle digestive tracts. The disease is characterized by lymphoplasmacytic infiltrations, mainly in the ganglia of the central nervous system. These infiltrations lead to central nervous system abnormalities such as ataxia, abnormal gait, proprioceptive defects and gastrointestinal tract dysfunctions including dysphagia, regurgitation and the passage of undigested food in feces (Gregory et al., 1994).
Until recent, birds that were suspected to suffer from PDD were diagnosed using CT-scan or x-ray photography to show a dilated proventriculus, or using crop biopsy to show infiltrates in neural tissues (Bond et al, 1993). The danger of crop biopsy in living patients is the invasiveness of the procedure, which can cause permanent damage to the crop with possible fatal outcome. Frequently, the infiltrates are detected post-mortem at histological

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examination of crop, proventriculus, gizzard, adrenals and brain tissue-sections. Another drawback of both the x-ray diagnostics as well as the crop biopsy is that they only provide useful data when a patient is in a progressed state of PDD and already shows clinical symptoms, and even in these advanced cases false negative, but also false positive results are not uncommon.

Many etiological agents have been proposed for PDD. Reports of the presence of pleomorphic virus-like particles in tissues of PDD affected birds led to the proposal that a paramyxovirus may cause the disease (Grund et al., 2002). This is at this moment considered less likely, because serological data showed that PDD affected birds lacked detectable antibodies against paramyxovirus correlating with PDD cases (Deb et al., 2008). Other potential PDD causing viruses proposed in the past are avian herpes viruses, polyomavirus, equine encephalitis virus, adeno-like viruses, enteroviruses, coronaviruses, reoviruses, and avian encephalitis virus (Ritchie 1994; Gough et al., 2006). In each case, follow-up evidence for reproducible isolation of these viruses specifically from PDD cases or identification of these candidate agents in sufficient detail has not been reported.

A recently discovered avian Borna virus (ABV) has been suggested as a potential etiologic agent for PDD or macaw wasting disease (Kistler et al., 2008; Honkavuori et al., 2008; Rinder et al.2009). This suggestion was mainly based on a relation between PDD and the presence of ABV infection. Tissues from five out of eight (Kistler et al., 2008) and three out of three PDD positive birds (Honkavuori et al., 2008) contained the virus, whereas no ABV was found in PDD free controls.

Borna disease virus (BDV) is a negative strand, non-segmented RNA virus known to cause a neurologic syndrome, Borna disease (BD), in horses and sheep (Lipkin and Briese, 2007). Serologic and PCR data (Malkinson et al., 1993; Berg et al., 2001) had previously indicated a possible presence of BDV in ostriches, mallards and jackdaws. However, genomic studies showed a close relationship between these sequences and mammalian BDV, whereas the viruses detected in PDD-affected birds showed less than 70% sequence identity to the known mammalian BDV isolates, suggesting the existence of a previously unrecognized species avian Borna virus (ABV). Investigations by Rinder et al. (2009) indicated that, whereas the mammalian BDV has a general preference for the central nervous system, ABV may be found in many tissues, at least in the later stages of PDD. The same was found by other researchers (Kistler et al, 2010). Viral nucleic acids were found in feces and cloacal swabs of infected birds, suggesting the possibility of virus transmission by a fecal-oronasal route (Rinder et al., 2009; Dorrestein et al, 2009; Lierz et al, 2009, Kistler et al, 2010). Based on these findings cloacal swabs can enable the non-invasive detection of ABV infection.

Until recent at least 5 genotypes of ABV have been described (Honkavuori et al., 2008, Kistler et al., 2008, Rinder et al., 2009). Partial sequences of nucleoprotein (p40) and matrix protein (gp18) genes in braintissues of PDD cases diagnoses between 1999 and 2008 showed that virus in most of cases belonged to the ABV-2 and ABV-4 groups among the 5 genogroups described so far. Viral sequences of 2 birds did not match any of the described sequences and clustered together in a new branch termed ABV-6. (Weissenböck et al., 2009a).

Virus isolation of genotypes 2 and 4 readily established persistent, non-cytolytic infections in quail and chicken cell lines but did not grow in cultured mammalian cells in which classical BDV strains replicate efficiently. ABV antigens were present in both cytoplasm and nuclei of infected cells, suggesting nuclear replication of ABV (Rinder et al., 2009). Researchers at Texas A&M also have successfully cultured ABV from brain tissue harvested from 7 birds with confirmed PDD, whereas no virus was recovered when brain tissue from 4 birds confirmed unaffected from PDD were used to inoculate tissue cultures (Hoppes and Gray 2009).

On the other hand not all ABV infected birds do develop PDD immediately. ABV RNA-positive, clinically healthy birds demonstrated the same serological response as the animal with confirmed PDD. These results indicate that ABV infection may occur without clinical evidence of PDD (de Kloet and Dorrestein 2009, Lierz et al, 2009).

Western blot analysis was used to demonstrate the presence of PDD-specific antigens in tissues of affected birds. Sera from both affected and normal birds were applied to PDD-positive brain tissue extracts to determine whether the birds produce antibodies to PDD-specific antigens (Villannueva et al., 2008). Recently, we also developed enzyme-linked immunosorbent assay (ELISA) and Western blotting protocols for the detection of antibodies against ABV nucleoprotein (N; p40) in bird sera (Dorrestein et al., 2009; De Kloet and Dorrestein 2009). In mammalian BDV the p40 nucleoprotein is a major antigen, which has been used for serological studies (Ludwig et al., 1988, Briese et al., 1995). The nucleoprotein is coded by the first ORF along the viral antigenome and protects the genomic RNA, forming together with the phosphoprotein P the viral nucleocapsid and is involved in nucleocytoplasmic shuttling during virus maturation (Kobayashı et al., 2001, Schneider et al., 2004). Our Western blot analysis using serum from PDD affected birds showed proteins of a molecular size of approximately 40.000 Da; the expected size of the recombinant ABV p40 protein after digestion of the MBP-p40 fusion protein with the protease Factor-Xa (De Kloet and Dorrestein, 2009).

By now a causative relationship between ABV and PDD seems to be very likely. Gancz et al., (2009) inoculated three cockatiels (*Nymphicus hollandicus*) with avian bornavirus-4 (ABV4) brain homogenates. This study showed that all three cockatiels inoculated with ABV4 developed gross and microscopic PDD lesions, and two birds exhibited overt clinical signs. ABV RT-PCR and sequence analysis demonstrated the presence of ABV4 RNA in numerous tissues. ABV was further shown by IHC in the central nervous system of the three ABV-inoculees. This study provides evidence for a causative association between ABV4 infection and PDD in cockatiels. Also at TA&M university successful transmission trial have been performed in ducks, Patagonion conure (*Cyanoliseus patagonus*) and cockatiels (Gray et al, 2009).
In a analysis of naturally occurring avian Bornavirus infection and transmission during an outbreak of proventricular dilatation disease among mixed collection of captive psittacine birds it was concluded that unweaned birds (in this case: umbrella cockatoo (*Cacatua alba*), Moluccan cockatoo (*C. moluccensis*), scarlet macaw (*A. macao*), jenday conures (*Aratinga jandaya*) are extremely sensitive to ABV2 infections. The estimated incubation period in this natural outbreak was extrapolated to be between 2 and 4 weeks (Smith, 2009; Kistler et al, 2010).

In a retrospective study 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 were evaluated using mmunohistochemistry with an antiserum directed against the ABV nucleoprotein (N-protein). Cells containing ABV N-protein were present in the nervous tissues of all 13 originally diagnosed PDD cases. One bird not previously diagnosed with PDD also had ABV N-protein in its brain. A review of this bird's necropsy report indicated that it was, most probably, also suffering from PDD. The remaining 10 non-PDD birds had no detectable N-protein in their brains (Ouyang et al., 2009). Also in tissue samples collected from 1999 through 2008 in Austria, Switzerland, Hungary, and Australia viral antigen of ABVs was demonstrated (Weissenböck et al., 2009a).

Although most research is concentrated in psittacine birds, the first demonatrion of ABV in a non-psittacine birds has been published. A canary bird (*Serinus canaria*) died with nonsuppurative ganglioneuritis of the proventriculus and gizzard and encephalitis, lesions comparable to proventricular dilatation disease (PDD) of psittacine birds. In the canary, bornaviral antigen was detected in both neural and extraneural tissues. Sequence analysis revealed a unique genotype of avian bornavirus. This observation suggests that bornaviruses are natural pathogens of several avian species and that the family Bornaviridae comprises more viral genotypes (or viral species) than previously assumed (Weissenböck et al., 2009b).

With the identification of this novel etiological candidate for PDD, detection of ABV RNA or serum antibodies can be used as a diagnostic marker for PDD. An advantage over classical crop biopsy and x-ray diagnostics is that the virus is detectable before clinical signs appear. Therefore, ABV rna detection on cloacal swabs or anti-ABV antibodies in serum would be a valuable tool for detecting infected birds, screening birds when taking them into shelters or monitoring breeding programs and preventing spread and future outbreaks of PDD.

In this presentation we report methods for detection and amplification of ABV specific genes from PDD affected brain tissues, blood, cloacal swabs and fecal samples as used in our laboratory. We report the development of a serological screening method using p40 (N) nucleoprotein. We will also present some epidemiological findings based on these results and discuss our current understanding and still unresolved questions.

METHODS FOR ABV RNA DETECTION

Birds and tissue sample preparation
For the development of the different PCR assays we used brain tissue of 4 birds (2 *P. erithacus*, 1 *Poicephalus senegalus* and 1 *P. gulielmi),* euthanized in the final stage of PDD. PDD was confirmed postmortem by histological examination. Brain material was immediately placed in Trizol reagent (Roche Diagnostics, Almere, The Netherlands), buffered 4 M guanidinium isothiocyanate, or 96% ethanol and tested with the validated assays at Columbia University and Animal Genetics Inc. In addition, blood, cloacal swabs, and fecal samples of these birds were analyzed.

A second set of specimens included serial collection over 2 weeks of cloacal swabs and fecal samples obtained from 4 live *Poicephalus* sp. of one breeder. In addition, we also obtained samples of an *Ara chloroptera*, *A. severa, A, ararauna* and *Cacatua ducorpsii* from a different source. All birds were either suffering from PDD or being the partner of a bird that died of a histologically confirmed case of PDD. The brains and cloacal swabs of these partners were included in this second study.

Finally, we repeated the sampling of the second experiment but placing the samples in 96% ethanol as well as in buffered 4 M guanidinium isothiocyanate, a "new" different fixation and transport buffer.

RNA extraction. Total RNA was extracted from ~80 mg brain tissue per 1 ml lysing solution. Samples were homogenized using a rotor-stator homogenizer. RNA was isolated from 400 µl lysed sample using the High Pure RNA tissue Kit (Roche Diagnostics) according to manufacturer's instructions. RNA was eluted in 100 µl elution buffer. Quality and quantity of the eluted RNA was analyzed by using gel-electrophoresis, spectrophotometric analysis (Nanodrop, ND1000, isogen-lifescience, De Meern, The Netherlands), and a RNA 6000 Nano Chip kit (Agilent Technologies, Cheshire, UK). Eluted RNA samples were stored at -80°C.

Reverse-transcriptase reaction. cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). 1 µg of total RNA and 60µM random hexamer primers were heated for 10 minutes at 65ºC. 1x Reverse transcriptase reaction buffer (50 mM Tris/HCl, 30 mM KCl, 8 mM $MgCl_2$, pH 8,5), 10 U of Protector RNAse inhibitor, 10 mM of each deoxynucleotide and 10 Units of Transcriptor Reverse Transcriptase were added to the reaction mixture to a final volume of 20µl. The reaction mixtures were incubated for 10 minutes at 25ºC, and 60 minutes at 50ºC, followed by enzyme inactivation for 5 minutes at 85ºC.

(Real Time) PCR primer design. Genomic sequences of ABV, recovered from the NCBI database, were aligned using ClustalW software. (Sequence accession numbers available on request). Regions found to have a high similarity across different strains were used to select primers. Degenerate bases were incorporated into the primer sequences to overcome possible sequence variation in the targeted genes. Previously published primers based on research at Columbia University were also tested (Honkavuori et al., 2008).

PCR amplification and analysis. ABV gene specific primers of the polymerase (L), nucleoprotein (N) and matrix protein (M) gene (Honkavuori et al., 2008), and control primers

for psittacine Glyceraldehyde 3-phosphate dehydrogenase (Kistler et.al, 2008) were used in the PCR assay. The obtained cDNA's were amplified in a Mastercycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation for 5 min at 94 ºC, followed by PCR amplification 30 sec , 90ºC; 30 sec, 50ºC and 30 sec 72ºC for 35 cycles and a final extension cycle of 10 min at 72 ºC. Amplified samples were analyzed on a 2% agarose gel stained with ethidium bromide and photographed using a UV-transluminator/camera combination (Ultra-Violet Products Ltd, Cambridge, UK).

Real time PCR analysis. cDNA was amplified using with the newly selected primers or primers from Columbia University and control primers for psittacine Glyceraldehyde 3-phosphate dehydrogenase gene. 2 μl of cDNA was amplified in a mixture containing 1x LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics), 0.5 μM of each primer, and 3 mM $MgCl_2$ in a Lightcycler 1.2 (Roche Diagnostics) using a initial denaturation of 10 min at 95 ºC followed by 40 cycles of amplification (10 sec, 95 ºC; 5 sec, 47 ºC; 5 sec, 72 ºC). Primers obtained from HONKAVUORI et al.(2008) were used as described before, however the annealing temperature was increased to 60ºC. Data was collected and analyzed using the Lightcycler Data Analysis Software (Version: 3.5.2.8). For the detection of PCR inhibitors the Spud assay was used (Nolan et.al., 2006).

METHODS FOR p40 ANTIBODY DEMONSTRATION

Synthesis of recombinant ABV p40 (N) and other proteins in E. coli (De Kloet and Dorrestein, 2009). Relevant DNA fragments coding for the bornavirus p40 nucleoprotein, which is a major bornaviral antigen in mammals were obtained by RT-PCR using primers with suitable restriction sites for cloning in the pMal 2CX expression vector (NEB). After ligation into the vector, the ligation products were used to transform E. coli DH5α. Recombinant plasmids were isolated and sequenced for verification using dideoxynucleotide chemistry (Sanger et al., 1977). The plasmids were then used for transformation of E. coli Rosetta 2 (DE3)pLysS (Novagen, Madison, Wi, USA). The p40-MBP fusion proteins were used as antigens in the serological investigations.

ELISA and Western blotting for detecting the presence of serum antibodies against p40 ABV protein. The protocol used for the ELISA for the detection of ABV p40-specific antibodies was carried out as described before (Harlow and Lane, 1988). Briefly, p40-MBP fusion protein is applied to the wells of a medium binding polystyrene ELISA plate and incubated with shaking at room temperature for 1 hr. The maltose binding protein (MBP) fusion partner was used as a negative control. After washing with PBST (phosphate buffered saline, containing 0.05% Tween 20) the wells were blocked for 2 hours at roomtemperature with 5% instant nonfat dry milk in PBST (PBSTM). After washing again with PBST the wells were subsequently incubated for one hour at room temperature with dilutions of bird serum in PBSTM, washed with PBST, incubated for 1 hr at room temperature with goat antibird antibody conjugated to horse radish peroxidase (SRdK) or alkaline phospatase (GMD) in PBSTM. The horse radish peroxidase activity was measured at 450nm using TMB1 (3,3',5.5'-tetramethylbenzidine, Promega) and the alkaline phospatase at 405nm using pNPP (*p*-Nitrophenyl Phosphate, Disodium Salt, Sigma) as the substrate.

The ELISA was used to test sera collected from birds euthanized in the final stages of PDD as well as sera sampled from live birds with clinically confirmed PDD and from clinically healthy birds. In all euthanized cases the presence of ABV RNA was tested using RT-PCR. In a second study, sera and cloacal swabs were collected once a month for a period of 6 months from a collection of birds housed at the NOIVBD. These birds had a history of losing a partner due to PDD or were added to this collection because of the shedding of ABV RNA in the PCR development studies above.

In a 3rd study collection of breeders with a history of PDD were serologically tested.

Western blotting was carried out according to the Texas A&M protocol (VILLANNUEVA et al., 2009) or (SRdK) using nitrocellulose filters, wet transfer using BioRad (Hercules, CA, USA)

equipment, and detection by chemiluminescence using an Amersham ECLTM advance Western blotting detection kit (GE Healthcare BioSciences, Piscathaway, NJ, USA) and rabbit antibird antibody conjugated to horseradish peroxidase (Bethyl Laboratories). The western blotting was used to demonstrate presence of antibodies against ABV using brain homogenate as well as to prove the specificity of the recombinant bornavirus p40 protein.

RESULTS AND DISCUSSION

The results will be presented and discussed at the conference.

SOME PRELIMINARY CONCLUSIONS

- There is evidence of a causal association between ABV4/ABV2 infection and PDD in cockatiels and Patagonian conures.
- Degenerate and non-degenerate ABV primers can be used in a PCR analysis to detect ABV.
- In our hands previously published primers (Honkavuori et al., 2008) gave better results than newly developed primers.
- The collection method and transport medium can influence the results of PCR assays.
- Clinically healthy birds can shed ABV, but not all contact birds appear to become shedders.
- Fecal samples were often negative in a PCR assay, whereas the cloacal swab taken at the same time was positive for ABV.
- Blood is an unreliable sample for detection of ABV RNA.
- Partners of a breeding couple and birds housed in same indoor aviary where ABV-positive birds died of PDD, are frequently, but not always positive for ABV.
- DNA analysis of the first series of 4 birds yielded sequences that mapped to 2 different genotypes with 2 different sequences from a collection of one breeder.
- Although ABV has been detected in the feces of birds with PDD, little is known about the significance and timing of viral shedding at this point.
- ELISA using the p40 nucleoprotein can be used to detect serologically positive birds.
- Clinically healthy parrots may carry high levels of anti-p40 ABV antibodies and contain ABV RNA (De Kloet and Dorrestein 2009).
- With further study, serology will likely provide a reliable and non-invasive screening method for PDD infection status.
- Results show that despite the presence of ABV in the infected birds, the other birds in a collection have remained free of the virus for more than 2 years, suggesting that avian bornavirus may not be highly contagious (De Kloet and Dorrestein 2009).
- The practical diagnostic result of our study is that if ABV is indeed the cause of PDD, a virtually noninvasive way of testing for this disease has become available through RT-PCR examination of cloacal swabs and serological testing for anti ABV antibodies instead of the current diagnosis of PDD, which is based on histopathological tissue examination (De Kloet and Dorrestein 2009).

Based on the current knowledge our policy is:
- Is a healthy bird repeatedly serologically negative than the bird has had no contact with ABV and the bird is considered negative for PDD.
- Is a bird ill and shows clinical symptoms that could indicate PDD and the bird is serologically positive, than the bird is considered to have PDD, which should be confirmed by a PCR.

- When a bird is ill and suspected of PDD, but the serology is negative, than this bird is considered not to be infected with ABV Most probably this bird has not PDD and it should also be negative in the ABV RT-PCR.
- Is a bird clinically healthy, but serologically positive, than it is considered to be carrier of ABV if PCR positive, but "clean" if PCR negative. Future research will show if and when a serologically positive but PCR negative has become a "clean" bird.

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