Presence of Avian Bornavirus RNA and Anti-Avian Bornavirus Antibodies in Apparently Healthy Macaws

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SUMMARY. Recently a novel avian bornavirus has been described that has been suggested to be the possible etiological agent for proventricular dilatation disease or macaw wasting disease. This article describes two macaws that shed avian bornaviral RNA sequences and demonstrated anti-avian bornavirus antibodies as revealed by reverse transcriptase polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and Western blot, yet are free of outward clinical signs of the disease.

RESUMEN. Presencia de ARN de un bornavirus aviar y de anticuerpos contra el mismo virus en guacamayas aparentemente sanas.

Recientemente, se describió un nuevo bornavirus aviar que se ha sugerido como el posible agente etiológico de la enfermedad de la dilatación proventricular o enfermedad debilitante de las guacamayas. Este artículo describe el caso de dos guacamayas que presentaban secuencias de ARN de un bornavirus aviar y la presencia de anticuerpos contra el mismo virus tal como se determinó por la transcripción reversa y reacción en cadena de la polimerasa (RT-PCR), también por un ensayo de inmunoabsorción con enzimas ligadas (ELISA) y por la prueba de inmunoelectrotransferencia. Sin embargo, las aves no presentaron signos clínicos de enfermedad.

Key words: proventricular dilatation disease, avian bornavirus, psittaciformes, RT-PCR, ELISA

Abbreviations: ABV = avian bornavirus; bp = base pair; BFDV = beak and feather disease virus; EDTA = ethylene diamine tetra-acetic acid; ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry; MBP = maltose binding protein; NEB = New England Biolabs; NOIVBD = Nederlands Onderzoek Instituut voor Vogels en Bijzondere Dieren; nt(s) = nucleo-tide(s); PBFD = psittacine beak and feather disease; PBS = phosphate-buffered saline; PBST = PBS containing 0.05% Tween 20; PDD = proventricular dilatation disease; POLY = polyomavirus; RT-PCR = reverse transcriptase polymerase chain reaction

Proventricular dilatation disease (PDD) in parrots (Psittaciformes) is a disease that affects the central nervous system as well as the digestive system. The disease causes wasting and is recognized in living birds primarily by outward clinical signs such as the discharge of undigested food in the fecal matter, emaciation, neurological disorders of the affected birds and also by histopathological examination of the crop wall and postmortem by demonstration of lymphocyte infiltration of the proventriculus (8,9). A viral or microbial etiology of the disease has been considered for quite some time and recently evidence has been presented that associates a novel avian bornavirus (ABV) with PDD (6,12,15,20). This conclusion was based on the finding that whereas no bornavirus could be found in PDD-free parrots, tissues from five out of eight (15) and three out of three PDD-positive birds (12) contained the virus. Gancz et al. (6) demonstrated after inoculation of cockatiels (Nymphicus hollandicus) with ABV brain tissue that all three cockatiels inoculated developed full PDD and therefore provided evidence of a causal association between ABV infection and PDD in cockatiels. Investigations by Rinder et al. (20) have shown that contrary to the mammalian bornavirus infecting horses and sheep, where the virus shows a high preference for the central nervous system, avian bornavirus can be found in many tissues.

In the exotic bird collection of one of the authors (S. R. K.) there are four macaws (two military macaws [*Ara militaris*], one blue and yellow macaw [*Ara ararauna*], and one hybrid scarlet macaw [*Ara ararauna*]) which were donated to us in February 2007 from an aviary that suffered from a severe outbreak of PDD [e.g., 100% mortality of a flock of 90 conures (*Aratinga, Pyrrhura* sp.)] as

diagnosed by clinical signs such as emaciation, neurological signs, and undigested seeds in their fecal matter, and postmortem by histopathologic examination of the proventriculus. The macaws were slated to be euthanatized in an attempt to contain the spread of the disease in the aviary. The four birds did not have the disease as judged by their physical condition and the absence of undigested seeds in their fecal matter and have remained free of these outward clinical signs that could indicate PDD during the 2 yr that they have been in our possession. The recent determination of the nucleotide sequence of the avian bornavirus strains associated with the PDD (6,12,15,20) enabled us to investigate whether these birds might be carriers of the virus and/or its antibodies by RT-PCR and serological means.

MATERIALS AND METHODS

Sources of materials. The four macaws (two military macaws [Ara militaris], one blue and yellow macaw [Ara ararauna] and one hybrid scarlet macaw [Ara ararauna|Ara macao] are housed in outdoor, openair aviaries of one of the authors (S. R. K.), as were the other birds included in this study. (These were 6 African grey parrots [Psittacus erithacus] randomly selected out of a group of 12, one sulfur-crested cockatoo [Cacatua sulfurensis], one chattering lory [Lorius garrulous], four peach-faced lovebirds [Agapornis roseicollis], and 2 grey peacock pheasants [Polyplectron bicalcaratum]. The latter two birds were housed in the same aviary as the A. militaris.) The birds had never had PDD as judged by the outward clinical signs of the disease; the author has never had this disease in his aviaries, again as judged by outward clinical signs. Access to these aviaries is limited to the owner (S. R. K.) and his direct relatives. The birds were maintained on a diet of sunflower seeds, peanuts, apples, oranges, and a small amount of pelleted food (Kaytee®, Kaytee Products Inc., Chilton, WI).

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Species ^A	Gene	Primers	Sequence	Location 3' terminus	Accession no.
ABV	P40	PDDFS1	5'-CACAGATCTATGCCACCCAAAAGGCAAAGAAGTC-3'	80	EU781967
ABV	P40	PDDRS2	5'-CACGTCGACTCATTAGTTTGCAAATCCACTTCAG-3'	1004	
BFDV	COAT	COAR	5'-GGGTCTAGATTCWCAATAGAATTTACACWCTC-3'	1256	AY521237
BFDV	COAT	COAF1	5'-GGGAAGCTTTTAAGTACTGGGATTGTTRGG-3'	1830	
POLY	CAPSID	VP1AG	5'-CCCTCTAGAATGTCCCAAAAAGGAAAAGGAAAGGAAG-3'	1921	AF118150
POLY	CAPSID	VP2AG	5'-GGCATATAAAAAGCTTCGTCTAGTCTATTTAGC-3'	2925	

Table 1. Names of genes or gene fragments, sequence, and location of the 3' terminus of the primers used for amplification and GenBank accession numbers of the sequences used for the production of the recombinant antigens described in this study.

^AABV = avian bornavirus; BFDV = beak and feather disease virus; POLY = polyomavirus.

In addition, two frozen serum samples were available from one of the military macaws (*A. militaris* 11) and the above-mentioned blue and yellow macaw (*A. ararauna*) collected in 2007. Also one sample was available of a *P. erithacus* with clinical signs of PDD. This *P. erithacus* currently lives in the same aviary where the macaws described in this paper had come from in February 2007, which had been repopulated with birds since.

Cloacal swabs and small amounts of fecal samples (<100 mg) were collected in 0.5 ml of 4 M guanidinium isothiocyanate, containing 50 mM Tris-HCl buffer (pH 7.5) and 25 mm ethylene diamine tetra-acetic acid (EDTA) and stored for at most 24 hr at ambient temperature (approximately 25 C) prior to processing in the laboratory. Blood was taken from the bracheal vein and diluted tenfold in 0.15 M NaCl containing 0.02 M EDTA (pH 8.0). Blood cells were collected by centrifugation and the supernatant was used as (10-fold diluted) serum. Samples were taken three times over a period of 8 wk at approximately 4-wk intervals. Brain tissue of other psittaciformes positively identified with PDD was obtained from the Nederlands Onderzoek Instituut voor Vogels en Bijzondere Dieren (NOIVBD), Veldhoven, the Netherlands. Brain tissue was transported to us as small fragments (approximately 100 mg) in 1–2 ml of 96% ethanol.

Isolation of RNA from brain, cloacal swabs, fecal samples, and blood. Total RNA was isolated from 100 mg brain tissue with the use of a mini RNA isolation kit (Zymo Research, Orange, CA) and the protocol provided by the manufacturer, or by homogenization in 4 M guanidinium isothiocyanate. The tissue was homogenized in these solutions with a minihomogenizer consisting of a Teflon pestle fitting in an Eppendorf tube. Prior to binding to the minicolumn the solution was centrifuged for 5 min in a minifuge to remove debris. RNA was similarly isolated from 100 µl blood with the use of a Zymo Research mini RNA isolation kit. RNA was isolated from white blood cells prepared from 100 µl blood by the procedure described by Swaggerty et al. (26). RNA was isolated from cloacal swabs and fecal samples by a modification of the procedure described for the preservation and isolation of RNA from feces of wild birds (5) or gorillas (30). In short, cloacal swabs or small amounts of fecal matter (<100 mg) were immersed in 0.5 ml of 4 M guanidinium isothiocyanate containing 50 mM Tris-HCl buffer (pH 7.5) and 25 mM EDTA for about 24 hr (the time between collecting the swabs or fecal matter and processing in the laboratory). RNA was isolated from these solutions by spin column technology with the use of spin columns provided by Zymo Research or Epochbiolabs (Houston, TX). Prior to binding to the column, the brain and the fecal samples were spun for 2 min in a microfuge to remove particulate matter. The swabs were put in the lower half of a microfuge tube with a small hole in the bottom placed on top of another microfuge tube and spun in a minifuge for 2 min at 12,000 revolutions/minute to collect material that had been taken up by the swab. After the columns were washed with 70% ethanol as recommended by the Zymo Research protocol, the RNA was eluted in 15 μl (swabs or fecal matter) to 30 μl (brain, blood, and serum) of nuclease-free water.

RT-PCR for the detection of avian bornavirus-specific sequences. RT-PCR for the detection of bornavirus was carried out with the use of the consensus primers described by Kistler *et al.* (15). These primers (from Integrated DNA Technologies, Coralville, IA) were ABVNF (5'-CCHCATGAGGCTATWGATTGGATTAACG-3') and ABVNR (5'-GCMCGGTAGCCNGCCATTGTDGG-3) for a segment of the P40 (N) gene, and ABVMF (5'-GGRCAAGGTAATYGTYCCTGGATGGCC-3' and ABVPR 5'-(CCAACACCAATGTTCCGAAGMCG-3') for a segment covering part of the P40 and the P24 gene. As an internal control primers GAPDHF (5'-AGTCATCCCTGAGCTSAAYGGGAAGC-3') and GAPDHR (5'-ACCATCAAGTCCACAACACGG-3') amplifying a segment of the host glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The incubation mixture for the RT-PCR was the following: 2 µl of the RNA extracts were incubated in 20 µl of 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.1% Triton X-100, 5% DMSO, 5 mM dithiothreitol, 5% polyethylene glycol 6000, bromocresol red as a tracking dye, 0.5 µM primers, 0.5 U Taq DNA polymerase [New England Biolabs (NEB), Ipswich, MA], 4 U RNAase inhibitor (Promega, Madison, WI) and 2.5 U AMV reverse transcriptase (NEB). The thermocycling conditions were as follows: after a 30-min incubation at 45 C, the samples were heated for 2 min at 94 C followed by 30 cycles of 20 sec at 93 C, 30 sec at 55 C, and 80 sec at 68 C, and a final extension step of 10 min at 72 C. The amplification products were analyzed by gel electrophoresis with the use of 1.5% agarose gels (Taiwan Agar Agar Industries, Chia Yi, Taiwan) in tris-borate-EDTA buffer (pH 8.4; 19), containing 0.1 µg of ethidium bromide per milliliter The sensitivity of the procedure was approximately 10.000 molecules of viral RNA. For sequence analysis the RT-PCR products were cloned in the TopoTA vector for sequencing (InVitrogen, Carlsbad, CA) and sequenced with the use of the dideoxynucleotide procedure (22) and a Dye Terminator kit (Applied Biosystems, Foster City, CA).

Synthesis of recombinant bornavirus P40 (N) and other proteins in Escherichia coli. Relevant DNA fragment coding for the proteins used in this study were obtained by RT-PCR and PCR with the use of primers with suitable restriction sites for cloning in the pMal 2CX expression vector (NEB). This vector was used because of its superior solubilization properties of recombinant proteins, which otherwise might be sequestered as insoluble inclusion bodies in E. coli (14). These proteins were: 1) bornavirus P40 nucleoprotein, which is a major bornaviral antigen in mammals (28); 2) beak and feather disease truncated coat protein (13); 3) beak and feather disease replicase protein; and 4) budgerigar polyomavirus capsid protein. The names of the proteins, the sequences of the primers, the locations of their 3' termini and the accession numbers of the sequences they were derived from are given in Table 1. After ligation into the vector, the ligation products were used to transform *E. coli* DH5 α and the recombinant plasmids isolated and sequenced for verification with the use of dideoxynucleotide chemistry (22) and a Dye Terminator kit (Applied Biosystems). Sequence alignment was carried out with the use of Clustal X (27). The plasmids were than used for transformation of E. coli Rosetta 2 (DE3)pLysS (Novagen, Madison, WI). The cells were induced with 0.5 mM isopropyl-\beta-thiogalactoside and the maltose-binding protein (MBP) fusion proteins isolated by affinity chromatography with the use of amylose according to the protocols provided by the manufacturer (NEB). The fusion proteins were used as antigens in the serological investigations.

ELISA and Western blotting for detecting the presence of serum antibodies against P40 bornavirus protein. ELISA for the detection of ABV P40 specific antibodies was carried out as described by Harlow and Lane (10). Briefly, 50 μ l of a solution containing 20 μ g of the MBP fusion proteins in 1 ml of 0.05 M Na₂CO₃ (pH 9.6) was applied to the wells of a medium binding polystyrene ELISA plate (Costar, Corning,



Fig. 1. (A) Identification of bornavirus RNA by RT-PCR in cloacal swabs of macaws. Lane 1–5, products of the control GAPDHF \times GAPDHR RT-PCR; lanes 6–9, products of the ABVMF \times ABVPR RT-PCR. Lanes 1: no RNA, control; lanes 2 and 6: *A. militaris* 1; lanes 3 and 7: *A. militaris* 2; lanes 4 and 8: *A. ararauna*; lanes 5 and 9: *A. ararauna/A. macao* hybrid; lane 10: 100–base-pair (bp) molecular weight standard. (B) Identification of avian bornavirus RNA in cloacal swabs by RT-PCR with primers ABVNF \times ABVNFR. Lane 1: 100-bp size standard, lane 2: *A. militaris* 1, lane 3: *A. militaris* 2, lane 4: *A. ararauna*. (C) Identification of avian bornavirus by RT-PCR with primers ABVNF \times ABVNFR in fecal matter. Lane 1: 100-bp molecular weight standard, lane 2: no RNA control, lane 3: *A. militaris* 2, lane 5: *A. ararauna*.

NY) and incubated with shaking at room temperature for 2 hr. The maltose-binding protein fusion partner was used as a control. After washing with PBST (phosphate-buffered saline containing 0.05% Tween 20) with an automated microplate washer (MTX, Vienna, VA) the wells were blocked overnight at 4 C with 300 µl of 5% instant nonfat dry milk in PBST containing 0.02% sodium azide. After being washed again with PBS, the wells were subsequently incubated for 1 hr at room temperature with 50 µl of different dilutions of bird serum in 5% nonfat dry milk in PBST, washed with PBST, incubated for 1 hr at room temperature with goat anti-bird antibody conjugated to horseradish peroxidase (Catalog No. A140-110P, Bethyl Laboratories, Montgomery, TX) at a 1/4000 dilution. The horseradish peroxidase activity was measured at 450 nm with the use of 40 µl of TMB1 (3,3',5,5'-tetramethylbenzidine, Promega) as the substrate and stopping the reaction with 40 µl of 0.5 N HCl. Measurements were carried out with the use of a Genios microplate reader (Tecan, Mannedorf, Switzerland). Serum titers were determined from twofold serial dilutions of the serum samples.

Western blotting was carried out with nitrocellulose filters, wet transfer with BioRad (Hercules, CA) equipment, and detection by chemiluminescence with the use of an Amersham $\mathrm{ECL}^{\mathrm{TM}}$ advance Western-blotting detection kit (GE Healthcare BioSciences, Piscataway, NJ) and rabbit anti-bird antibody conjugated to horseradish peroxidase (Bethyl Laboratories).

RESULTS

Detection of avian bornavirus by RT-PCR. The four macaws included in this study had no outward clinical signs of PDD. There were no undigested seeds in their fecal matter, the birds had no outward neurological signs, were not depressed or emaciated, and weighed (1020 g for A. ararauna, 1150 g for the A. ararauna/A. macao hybrid, and 900 and 930 g for the two A. militaris specimens) within the ranges given for these species in captivity (16). When blood or serum samples of the four macaws were examined (three times in a 2-mo period) by RT-PCR for the presence of bornaviralspecific RNA the results were negative. This result was also obtained when white blood cells where used instead of the whole blood. The same analysis applied to cloacal swabs, and fecal samples revealed a variable-strength positive signal in the samples obtained from the two A. militaris specimens, whereas the samples from the two other macaws were always negative (Fig. 1A-C). Sequencing of the 380nt-long P40-specific fragment showed nine (silent) nucleotide differences (98% identity) with the most similar published sequence of a bornavirus found in an Aratinga solstitalis (accession number EU781967; Fig. 2), which was almost identical to the sequence of the bornavirus from the brain of an infected cockatoo (Cacatua alba [umbrella cockatoo] PA08-826) from the Netherlands (accession number FJ792853) used for the synthesis of the recombinant P40 protein (see below). Amplification with the primers ABVMF and AVBPR revealed components of approximately 180 nucleotides (nts) in all samples as well as material with a molecular size of approximately 360 nts, the expected size of the fragment as it occurs in avian bornavirus. Sequence analysis of the two fragments showed that the 360-nt-long fragment was 98% similar to the homologous avian bornavirus sequence in the database (EU781967), whereas the 180-nt-long fragment was identical to a fragment of A. ararauna 12 S mitochondrial ribosomal RNA (accession number U70739.2).



Avian bornavirus in apparently healthy macaws

A. militaris EU781967 FJ792853	NT 610 Q P W V G S F V L A L L T T D F E S P CACAACCATGGGTTGGCTCATTGTCCTTGCTTGCTTGTTAACTACCGACTTTGAAAGCCCTG CGCAACCATGGGTTGGTTCATTGTCCTTGCTTGCTAACTACCGACTTTGAAAGCCCTG CGCAACCATGGGTTGGTTCATTGTCCTTGCTTGTTAACTACCGACTTTGAAAGCCCTG * ***********************************
A. militaris EU781967 FJ792853	G K E F M D Q I K L V A G F A Q M T T Y GCAAAGAGTTTATGGATCAGATCAAATTAGTGGCAGGGTTTGCACAAATGACCACATACA GCAAAGAGTTTATGGATCAGATCA
A. militaris EU781967 FJ792853	T T I K E Y L N E C M D A T L T I P A V CAACAATCAAAGAGTATCTCAACGAGTGCATGGATGCCACACTAACAATCCCGGCCGTGG CAACAATCAAAGAGTATCTCAACGAGTGTATGGATGCCACATTGACAATCCCGGCCGTAG CAACAATCAAAGAGTATCTCAACGAGTGCCATGGATGCCACATTGACAATCCCGGCCGTAG ***********************************
A. <i>militaris</i> EU781967 FJ792853	A L E I K E F L D V T T K L K A E H G D CACTTGAAATTAAGGAGTTTCTAGATGTAACTACAAAGCTAAAGGCAGAGCATGGGGACA CACTTGAAATTAAGGAGTTCCTAGATGTAACTACAAAGCTAAAGGCAGAGCATGGGGACA CACTTGAAATTAAGGAGTTCCTAGATGTAACTACAAAGCTAAAGGCAGAGCATGGGGACA
A. <i>militaris</i> EU761967 FJ792853	$ \begin{array}{llllllllllllllllllllllllllllllllllll$
A. <i>militaris</i> EU781967 FJ792853	F P N L A S A A F Y W S K K E N TTCCAAATTTAGCATCAGCTGCTTTTTATTGGAGTAAGAAGGAGAAT TTCCAAATTTAGCATCAGCTGCTTTTTATTGGAGCAAGAAGGAGAAT TTCCAAATTTAGCATCAGCTGCTTTTTATTGGAGCAAGAAGAAGGAGAAT

Fig. 2. Nucleotide sequence similarity between a RT-PCR product obtained with primers ABVNF \times ABVNR of avian Borna disease virus obtained from fecal matter of a chronically infected *A. militaris* with its closest counterpart (accession number EU781967) in the Genbank database and the sequence of the P40 gene (accession number FJ792853) used for constructing the recombinant P40 protein in this study.

Testing of other birds in our collection (six African grey parrots, randomly selected from a group of 12, one sulfur-crested cockatoo, one chattering lory, four peach-faced lovebirds) showed that all these birds were free of ABV RNA in their cloacal swabs and fecal samples, as were two grey peacock pheasants, which were housed in the same aviary as the *A. militaris*.

Detection of avian bornavirus P40 protein-specific antibodies by ELISA and Western blot. In mammalian bornavirus the P40 nucleoprotein is a major antigen that has been used for serological studies (28). It is coded for by ORF1 and is involved in viral replication (23). An ELISA test for the presence of avian bornavirus P40-specific antibodies (Fig. 3) in the serum of the birds showed that whereas the two A. militaris sera (Fig. 3-3, 3-4) contained relatively large amounts of these antibodies (titers of about 1:4000), the two other macaws (Fig. 3-1, 3-2) tested negative. That the demonstrated serum antibodies were specific for the P40 proteins is shown in the Western blot in Fig. 4, where the antibodies detected material with molecular size of approximately 40,000, the size of the recombinant bornavirus P40 protein after digestion of the MBP-P40 fusion protein with the protease Factor-Xa. The data in Fig. 3 show also that these birds tested negative or had low levels of antibodies against the beak and feather disease truncated coat protein, beak and feather disease replicase protein, and polyomavirus capsid protein, antigens of the most common disease-causing viruses in captive Psittaciformes (21) and further support that the ELISA is indeed specific for avian bornavirus P40 nucleoprotein. Because the birds had been obtained from an aviary with a history of psittacine disorders, such as beak and feather disease and avian polyoma, serum samples had been taken for possible future serological examination of one A. militaris (A. militaris 11, Fig. 3-8) and the A. ararauna (A. *ararauna* 1, Fig. 3-7) when the birds had been received in February 2007. The results show (Fig. 3) that the *A. militaris* 11 already had antibodies against bornavirus P40 protein in 2007, at the time it was obtained, whereas the *A. ararauna* 1 was free at that time. An analysis of the sera of the other birds, mentioned above, and illustrated by the *P. erithacus* in Fig. 3-5, showed that all were free of anti-P40 antibodies. However, a *P. erithacus* (Fig. 3-6) with clinical signs of PDD which currently lives in the same aviary where the macaws described in this paper had come from in February 2007, and which had since been repopulated, showed high levels of anti-P40 antibodies.

DISCUSSION

Proventricular dilatation disease (PDD), originally described as macaw wasting disease, is one of a number of diseases frequently affecting captive Psittaciformes. Evidence for its occurrence in wild Psittaciformes or other avian families is limited and is mainly based on suggestive lesions of the suspected birds (8,9). In captive collections of Psittaciformes the disease is rather rare in wellmanaged collections and is more often associated with poor hygienic conditions (4). Recent investigations (6,12,15,20) have shown that tissues of birds suffering from PDD may contain a novel bornavirus. Because this virus was found in a majority of affected birds and not in birds that were free of the disease, the possibility has been raised that PDD is caused by this novel avian bornavirus. To address this question, the impact of avian bornavirus-4 (ABV4) inoculation on the cockatiel (Nymphicus hollandicus) was studied (6). The result was that all three cockatiels inoculated with ABV4(+) brain homogenate developed gross and microscopic PDD lesions. Two of the birds



Fig. 3. ELISA of parrot serum samples for the detection of antiavian bornavirus P40 antibodies. (1) *Ara ararauna*, (2) *A. ararauna*/*A. macao* hybrid, (3) *A. militaris* 1, (4) *A. militaris* 2, (5) *Psittacus erithacus* 1, (6) *P. erithacus* 2, (7) *A. ararauna* 11, (8) *A. militaris* 11. Serum samples (1)–(6) were collected in December 2008; serum samples (7) and (8) were collected in February 2007.

exhibited overt clinical signs. In numerous tissues, ABV RT-PCR and sequence analysis demonstrated the presence of ABV4 RNA. ABV was detected in the central nervous system of the three ABV-inoculates by IHC. This study provides evidence of a causal association between ABV4 infection and PDD in cockatiels.

In wild birds bornavirus has been found in fecal matter of mallards (*Anas platyrhynchos*) and jackdaws (*Corvus monedula*) in Sweden (1). A data bank search revealed that the segments of the RNA of that virus that were sequenced have a high degree of similarity to the strains that infect mammals and only 75% with the avian bornavirus fragment described here. This shows that the virus carried by the birds in Sweden was different from the strains infecting captive psittacines in Europe and the United States, including the two military macaws in our own collection.

In mammals bornavirus is known to cause Borna disease, a meningoencephalitis, in sheep and horses (17) and staggering disease in cats (18,29). In man, anti-bornavirus antibodies have been found in patients with psychiatric disorders, consequently bornavirus has been thought to be involved in these disorders, but more extensive studies suggest that the evidence is inconclusive (3,24). In animals, the disease was first described in central Europe, but has a worldwide distribution. Bornavirus is the prototype of the family Bornaviridae (order Mononegavirales). It is a noncytolytic, nonsegmented negative-strand RNA virus, with a nuclear localization of its replication, transcription, and RNA processing (2). Whereas in birds the virus shows a broad tissue preference (20), in mammals Borna disease virus is highly neurotrophic (7), yet the clinical symptoms of the disease in mammals do apparently not directly result from the virus itself, but rather from lymphocyte infiltration of the infected central nervous system (11). Despite having heavily infected brains and apparently no efficient antiviral immune response to prevent such infections, many animals remain healthy with only minor behavioral symptoms (24). Studies by Stitz et al. (25) suggest that the neutralizing antibodies prevent a generalized infection with bornavirus in mammals.

The results obtained in this study show that parrots may carry high levels of anti-P40 avian bornaviral antibodies and contain avian bornaviral RNA. This demonstrates that, as in mammals, birds can coexist with the virus for a long time, at least 2 yr (the length of time the birds were in our collection) without showing any outward clinical signs of disease. We did not find ABV RNA in blood, which may be



Fig. 4. Western blot analysis of parrot serum for the detection of the presence of anti-avian bornavirus P40 antibodies by probing with serum of *A. militaris* 1. Lane 1: MBP2-P40 fusion protein, lane 2: MBP2-P40 fusion protein digested with factorXa, lane 3: MBP1.

the result of the high anti-ABV antibody level in the serum of the macaws. The presence of ABV RNA in cloacal swabs agrees with the findings of Rinder *et al.* (20) who, although they did not examine cloacal swabs found high levels of avian bornavirus in the intestinal villi of the affected birds. The results obtained here show also that despite the presence of avian bornavirus in the infected birds, the other birds in our collection have remained free of the virus, suggesting that avian bornavirus may not be highly contagious. Because it is very suggestive that avian bornavirus may be involved in proventricular dilatation disease (6,12,15,20) it is interesting that others have found that cage mates of birds diagnosed with PDD are frequently unaffected (4). This may reflect a resistance of the birds in our collection, but also that avian bornavirus may be unstable in fecal matter.

The practical diagnostic result of this study is that if indeed avian bornavirus is 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. Serological testing is important because it can detect past exposure to ABV when the RT-PCR diagnosis is negative or inconclusive.

REFERENCES

1. Berg, M., M. Johansson, H. Montell, and A. L. Berg. Wild birds as a possible natural reservoir of Borna disease. Epidemiol. Infect. 127:173–178. 2001.

2. Briese, T., J. C. de la Torre, A. Lewis, Y. S. Park, S. Kim, and W. I. Lipkin. Borna disease virus, a negative strand RNA virus, transcribes in the nucleus of infected cells. Proc. Natl. Acad. Sci. U. S. A. 91:4362–4366. 1992.

3. Chalmers, R. M., D. R. Thomas, and R. L. Salmon. Borna disease virus and the evidence for human pathogenicity: a systematic review. QJM 98:255–574. 2005.

4. Doneley, R. J. T., R. I. Miller, and T. E. Fanning. Proventricular dilatation disease: an emerging exotic disease of parrots in Australia. Aust. Vet. J. 85:119–123. 2007.

5. Evers, D. L., R. D. Slemons, and J. K. Taubenberger. Effect of preservative on recoverable RT-PCR amplicon length from influenza RNA virus in bird feces. Avian Dis. 51:965–968. 2007.

6. Gancz, A. Y., A. L. Kistler, A. L. Greninger, Y. Farnoushi, S. Mechani, S. Perl, A. Berkowitz, N. Perez, S. Clubb, J. L. Derisi, D. Ganem, and A. Lublin. Experimental induction of proventricular dilatation disease in cockatiels (*Nymphicus hollandicus*) inoculated with brain homogenates containing avian bornavirus 4. Virol. J. 6:100. 2009. [Internet].

7. Gonzalez-Dunia, D., C. Sauder, and J. C. De la Torre. Bornavirus and the brain. Brain Res. Bull. 44:647-664. 1997.

8. Gregory, C. R., K. S. Latimer, R. P. Campagnoli, and B. W. Ritchie. Histologic evaluation of crop for diagnosis of proventricular syndrome in psittacine birds. J. Vet. Diagn. Invest. 8:76–80. 1996.

9. Gregory, C. R., B. W. Ritchie, K. S. Latimer, W. I. Steffens, and D. Pesti *et al.* Progress in understanding proventricular dilatation disease. In: Proc. Annual Conference of the Association of Avian Veterinarians. pp. 269–275. 2000.

10. Harlow, E., and D. L. Lane. Antibodies, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1988.

11. Hausmann, J., W. Hallensleben, J. C. De la Torre, A. Pagenstecher, C. Zimmermann, H. Pitcher, and P. Staeheli. T cell ignorance in mice to Borna disease virus can be overcome by peripheral expression of the viral nucleoprotein. Proc. Natl. Acad. Sci. U. S. A. 96:9769–9774. 1999.

12. Honkavuori, K. S., H. L. Shivaprasad, B. L. Williams, P. L. Quan, M. Hornig, C. Street, G. Palacios, S. K. Hutchison, M. France, M. Egholm, T. Briese, and W. I. Lipkin. Novel bornavirus in psittacine birds with proventricular dilatation disease. Emerg. Infect. Dis. 14:1883–1886. 2008.

13. Johne, R., R. Raue, C. Grund, E. F. Kaleta, and H. Muller. Recombinant expression of a truncated capsid protein of beak and feather disease virus and its application in serological tests. Avian Pathol. 33:328– 336. 2004.

14. Kapust, R. B., and D. S. Waugh. *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Sci. 8:1668–1674. 1999.

15. Kistler, A. L., A. Gancz, S. Clubb, P. Skewes-Cox, K. Fischer, K. Sorber, C. Y. Chiu, A. Lublin, S. Mechani, Y. Famoushi, A. Greninger, C. C. Wen, S. B. Karlene, D. Ganem, and J. L. DeRisi. Recovery of divergent avian bornaviruses from cases of proventricular dilatation disease: identification of a candidate etiologic agent. Virol. J. 5:88. 2008.

16. Low, R. Parrots in aviculture. Silvio Mattachione Press. 1992.

17. Ludwig, H., and L. Bode. Borna disease virus; new aspects on infection, disease, diagnosis and epidemiology. Rev. Sci. Tech. 19:259–288. 2000.

18. Lundgren, A. L., and H. Ludwig. Clinically diseases: cats with nonsuppurative meningoencephalomyelitis have Borna disease virus-specific antibodies. Acta Vet. Scand. 34:101–103. 1993. 19. Maniatis, T., E. F. Fritsch, and J. Sambrook. Molecular cloning: a laboratory manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1982.

20. Rinder, M., A. Ackermann, H. Kempf, B. Kaspers, R. Korbel, and P. Staeheli. Broad tissue and cell tropism of avian bornavirus in parrots with proventricular dilatation disease. J. Virol. 83:5401–5407. 2009.

21. Ritchie, B. W. Avian viruses, function and control. Wingers Publ: Lake Worth, FL. 1995.

22. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain terminating nucleotides. Proc. Natl. Acad. Sci. U. S. A. 74:5463–5467. 1997.

23. Schwemmle, M., M. Salvatore, L. Sho, J. Richt, C. H. Lee, and W. I. Lipkin. Interactions of the borna disease virus P. N. and X proteins and their functional implications. J. Biol. Chem. 273:9007–9012. 1998.

24. Staeheli, P. Bornaviruses. Virus Res. 82:55-59. 2002.

25. Stitz, L., K. Noske, O. Planz, E. Furrer, W. Lipkin, and T. Bilzer. A functional role for neutralizing antibodies in Borna disease; influence on virus tropism outside the central nervous system. J. Virol. 72:8884–8893. 1998.

26. Swaggerty, C. L., I. Y. Pevzner, P. Kaiser, and M. H. Kogut. Profiling pro-inflammatory cytokine and chemokine mRNA expression levels as a novel method for selection of increased innate immune responses. Vet. Immunol. Immunopathol. 126:35–47. 2008.

27. Thompson, J. D., T. J. Gibson, F. Plowniak, F. Jeanmougin, and D. C. Higgins. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876–4882. 1997.

28. Watanabe, M., Q. Zhong, T. Kobayashi, K. Tomonega, and K. Ikuta. Molecular ratio between Borna disease viral-P40 and P-24 proteins in infected cells determined by quantitative antigen capture ELISA. Microbiol. Immunol. 44:765–772. 2000.

29. Wensman, J. J., P. Thoren, M. Hakhverdjan, S. Belak, and M. Berg. Development of a real-time RT-PCR assay for improved detection of Borna disease virus. J. Virol. Methods 143:1–10. 2007.

30. Whittier, A. A., W. Horne, B. Slenning, M. Loomis, and M. K. Stoskopf. Comparison of storage methods for reverse transcriptase PCR amplification of rotavirus RNA from gorilla (*Gorilla g. gorilla*) fecal samples. J. Immunol. Methods 116:11–17. 2004.

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