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Analysis of Naturally Occurring Avian Bornavirus Infection and Transmission during an Outbreak of Proventricular Dilatation Disease among Captive Psittacine Birds▽† ¶

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A proventricular dilatation disease (PDD) outbreak provided the opportunity to investigate the transmissibility of avian Bornavirus (ABV) and its linkage to PDD under natural conditions. Upon exposure to a bird with a fatal case of PDD, 10 birds became symptomatic and died. ABV2 RNA was recovered from available tissues. Further screening revealed that 12/46 exposed birds were ABV2$^+$. Three chicks boarded at this aviary developed PDD. They harbored the same ABV2 isolate and transmitted it to five of eight chicks in their home aviary. These findings demonstrate that ABV infection precedes the development of PDD. ABV-specific Western blotting and reverse transcription-PCR indicate that ABV2 is not strictly neurotropic.

Proventricular dilation disease (PDD) is a potentially fatal disease of psittacine birds (parrots) and potentially of other species (2, 5, 17) that has been detected throughout the world (3, 5, 11, 14, 16). Although the clinical course of the disease can vary, PDD is generally fatal if left untreated (5) and is considered to be a major threat to psittacine aviculture.

PDD primarily affects the autonomic nerves of the upper and middle digestive tract, including the esophagus, crop, proventriculus, ventriculus, and duodenum. Clinically, birds with PDD present with gastrointestinal tract dysfunction (dysphagia, regurgitation, and passage of undigested food in feces), neurological symptoms (e.g., ataxia, abnormal gait, proprioceptive defects), or both (5, 10, 15). Many birds with PDD develop microbial overgrowth in the dilated and nonmotile proventriculus and can succumb to sepsis. Histologically, PDD is characterized by the presence of lymphoplasmacytic infiltrates within myenteric ganglia and nerves (5, 9, 10). Similar infiltrates are often also present in the brain, spinal cord, peripheral nerves, conductive tissue of the heart, and adrenal glands (1).

Recently, a number of independent case-control studies with psittacines have demonstrated a statistically significant association between PDD and infection with a novel clade of avian bornaviruses (ABV) (7, 8, 11, 16). Moreover, inoculation of cockatiels with ABV$^+$ brain homogenates derived from a fatal case of PDD efficiently transmitted both ABV infection and PDD, strongly implicating ABV in PDD pathogenesis (4). Additionally, a novel ABV isolate has also recently been recovered from a canary with a fatal case of ganglioneuritis and encephalitis, suggesting that ABV may also play a role in similar diseases in other avian species (17).

Despite these advances, many aspects of the natural history of ABV infection and its role in PDD remain to be elucidated. Here, through detailed analysis of an outbreak of PDD in two aviaries, we provide prospective epidemiologic evidence supporting a causal role for ABV in PDD and derive additional inferences about the course and transmission of naturally acquired ABV infections.

Initial outbreak of proventricular dilatation disease. Figure 1 provides a schematic overview of the PDD outbreak (for a detailed chronology, see Table S1 in the supplemental material). The index case corresponded to an ailing African gray hen introduced into the aviary (aviary 1) of a hobbyist breeder. In late June, the breeder noticed undigested seeds in her feces; soon thereafter, the bird began to mutilate her feet. She was brought indoors for topical treatment of the wounds on the same counter where unweaned chicks in the aviary were routinely hand fed. Regular hand washing and disinfection of the counter were not performed after these treatments or before hand feeding of the chicks. On 15 July, the hen died. Histopathology showed characteristic lesions of PDD in the crop, proventriculus, ventriculus, upper small intestine, and brain.

On 16 July, crop stasis and feed refusal commenced in a 5-week-old chick, which then died 10 days later. That same day, its sibling and two unrelated nest mates began regurgitating and shaking their heads. Later that day, the sibling regurgitated during a feeding, aspirated, and died. Eight days later, another chick refused to be fed, aspirated, and died. Necropsy showed gross acute aspiration as the cause of death, with moderate breast muscle atrophy and an atrophic ventriculus. The breeder declined histopathologic examination. As this chick was necropsied, two jenday conures in the collection presented with regurgitation and head shaking. Five days later, two more chicks died. The breeder submitted these birds for necropsy and histopathologic testing.

Histopathologic testing confirmed PDD in the two deceased chicks. By the time this diagnosis was made, the jenday conures

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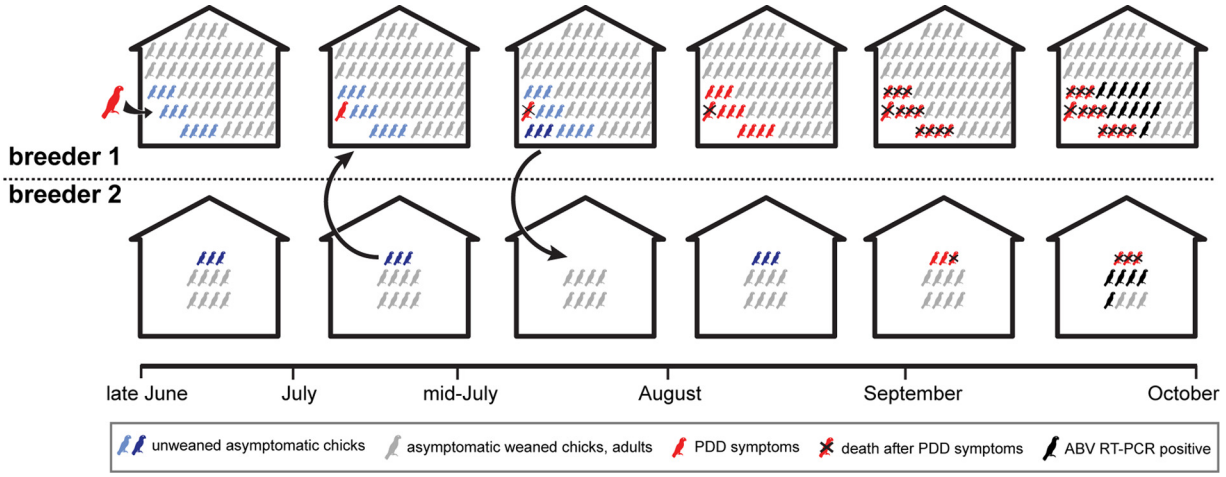

FIG. 1. Timeline of clinical symptoms and deaths documented among birds in a PDD outbreak. Schematic showing bird collections in the homes of breeders 1 and 2. Arrows depict introduction or transfer of birds into and out of the collections, and age categories and changes in clinical status of birds over time in each collection are coded by the size and color of bird symbols as indicated in the box below the timeline.

had died and several more birds had begun showing clinical signs of regurgitation, feed refusal, and crop stasis. An additional chick showed central nervous system (CNS) signs and was euthanized. Necropsy revealed an extremely enlarged, thin-walled proventriculus and atrophic ventriculus diagnostic of PDD. Two more birds in the collection with neurologic signs were euthanized, one of which was provided for necropsy and ABV RNA and protein analyses. This bird also showed a greatly enlarged, thin-walled proventriculus and an atrophic ventriculus. By the end of September, a total of 11 birds in aviary 1 had died or been euthanized, all of which had symptoms suggestive of PDD.

Expansion of the PDD outbreak. Three unweaned chicks from aviary 2, which had never experienced PDD, were boarded at aviary 1 from 10 to 17 July. Two weeks after returning home, all three chicks began showing signs of crop stasis and lethargy. Although the chicks appeared to recover, at the end of August, one chick relapsed and died within 48 h. Three days afterward, the other two chicks began showing the same clinical signs. The breeder elected euthanasia for these chicks, and on necropsy, each harbored an enlarged, thin-walled proventriculus and an atrophic ventriculus, characteristic of PDD.

ABV transmission during the PDD outbreak. At aviary 1, 46 surviving birds were housed indoors and potentially exposed to the PDD index case (and the subsequently transmitted cases). Cloacal swabs and blood samples were obtained from each bird, and RNA was extracted and screened for the presence of ABV RNA and host glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as previously described (4, 8). Of the 46 birds tested, 12 had ABV$^+$ cloacal swab specimens and 2 also tested positive for ABV RNA in their blood. Sequence analysis of the recovered ABV reverse transcription (RT)-PCR products indicated that all of these birds harbored virtually identical ABV2 isolates (Fig. 2; see Fig. S2 in the supplemental material). At aviary 2, within 4 weeks of exposure to the three

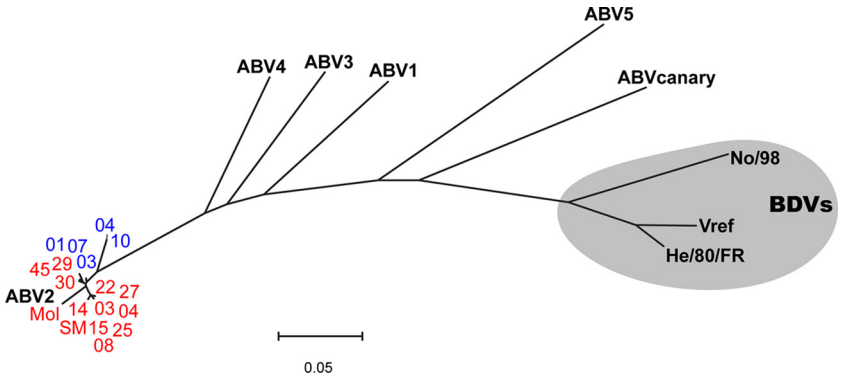

FIG. 2. Relationship of ABVN partial nucleotide sequences isolated in the outbreak specimens relative to a representative set of previously recovered ABV sequences (NCBI accession numbers: ABV1, FJ002326; ABV2, EU781967; ABV3, FJ002315; ABV4, FJ002316; ABV5, FJ002319; ABV canary, GQ161095), as well as mammalian BDV sequences (highlighted in gray) (NCBI accession numbers: No/98, AJ311524; He/80/FR, AJ311522; Vref, U04608). Neighbor-joining phylogenetic tree based on nucleotide alignment of a 345-bp sequence recovered by RT-PCR from cloacal swabs (red and blue numbers) or necropsy brain specimens (Mol and SM) isolated from the PDD outbreak. Red numbers indicate cage identifiers of ABV$^+$ indoor birds sampled from the collection of breeder 1, and blue numbers indicate cage identifiers of ABV$^+$ indoor birds sampled from the collection of breeder 2. For the corresponding alignment of outbreak isolate sequences and NCBI nucleotide accession numbers, see Fig. S2 in the supplemental material. A genetic distance scale bar is provided below tree.

TABLE 1. Necropsy tissues testing positive for ABV by RT-PCR[a]

Tissue	Necropsy 1	Necropsy 2	Necropsy 3
Cerebrum	+++	+++	+++
Cerebellum	+++	++	+++
Optic lobe	NT[b]	NT	+++
Spinal cord	+++	+++	+++
Adrenals	+++	+++	+++
Pectoral muscle	+++	+	+++
Lung	+++	++	+++
Heart	+++	++	+++
Liver	−	−	+
Crop	ND[c]	++	+++
Proventriculus	+++	++	+++
Ventriculus	+++	++	+++
Duodenum	NT	+	+++
Small intestine	+++	+++	+++
Large intestine	+++	+++	+++
Cloaca	+++	+++	+++
Kidney	++	+	+++
Bursa of Fabricus	+++	+	+++
Spleen	+	++	+++
Pancreas	+++	++	+++
Blood cell pellet	−	+	−

[a] All samples were assayed using a one-step RT-PCR for ABVN fragment as previously described (8). Results shown are qualitative values based on RT-PCR product signal intensity on agarose gel (+++, strongly positive; ++, clearly positive; +, faintly positive; −, negative). All three necropsied chicks were exposed to a bird with a histologically confirmed case of PDD while being handfed at breeder 1's home: necropsy 1, breeder 1's scarlet macaw chick (severe clinical signs of PDD at necropsy); necropsy 2, breeder 2's umbrella cockatoo chick (milder clinical signs of PDD at necropsy); necropsy 3, breeder 2's umbrella cockatoo chick (severe clinical signs of PDD at necropsy).

[b] NT, not tested.

[c] ND, not determined; RT-PCR results for both control (GAPDH mRNA) and ABVN RNA were negative.

chicks transferred from aviary 1, cloacal swab specimens of five of eight birds were ABV$^+$. ABV RNA was undetectable in all of their blood specimens, and in both the blood and cloacal swab specimens from two unexposed (outdoor) birds tested in parallel. All ABV RT-PCR products recovered from birds of aviary 2 showed >95% nucleotide sequence identity to the ABV2 isolate found in aviary 1 (Fig. 2; see Fig. S2 in the supplemental material).

These findings illustrate the high potential for transmission of ABV among captive birds. The routes of transmission are still unknown, but the high level of detection in cloacal swabs and the associated spread in aviary 1 with exposure to a common feeding area point to the possibility that fecal-oral spread is involved. These data also indicate that in unweaned chicks, signs of PDD can develop as early as 2 to 4 weeks after exposure to ABV. It is notable that at necropsy, some apparently asymptomatic ABV$^+$ birds nonetheless had pathological evidence of early PDD. This suggests that many seemingly asymptomatic infected birds may ultimately develop PDD. Careful long-term prospective follow-up studies of sizeable cohorts of infected birds are required to determine if an asymptomatic ABV carrier state exists.

Tissue distribution of ABV in necropsied birds with PDD. To investigate the tissue distribution of ABV in birds with acute cases of clinical PDD, specimens obtained at necropsy from the three euthanized chicks were probed for the presence of both ABV RNA and protein.

RNA was extracted from each tissue specimen and assayed by RT-PCR described above (4, 8). ABV2 RNA was detected in a wide variety of tissues in each of the necropsied birds (Table 1; Fig. 3A); only liver and blood cells showed little or no signal (Table 1; Fig. 3A). These results augment the mounting evidence of broad tissue tropism for ABV reported in PDD case-control (7, 11, 16) and experimental ABV inoculation (4) studies.

The necropsy specimens were also probed for ABV protein expression using ABV-specific polyclonal antisera raised against purified recombinant ABV nucleoprotein (ABVN) (4) and ABV phosphoprotein (ABVP) (see methods in the supple-

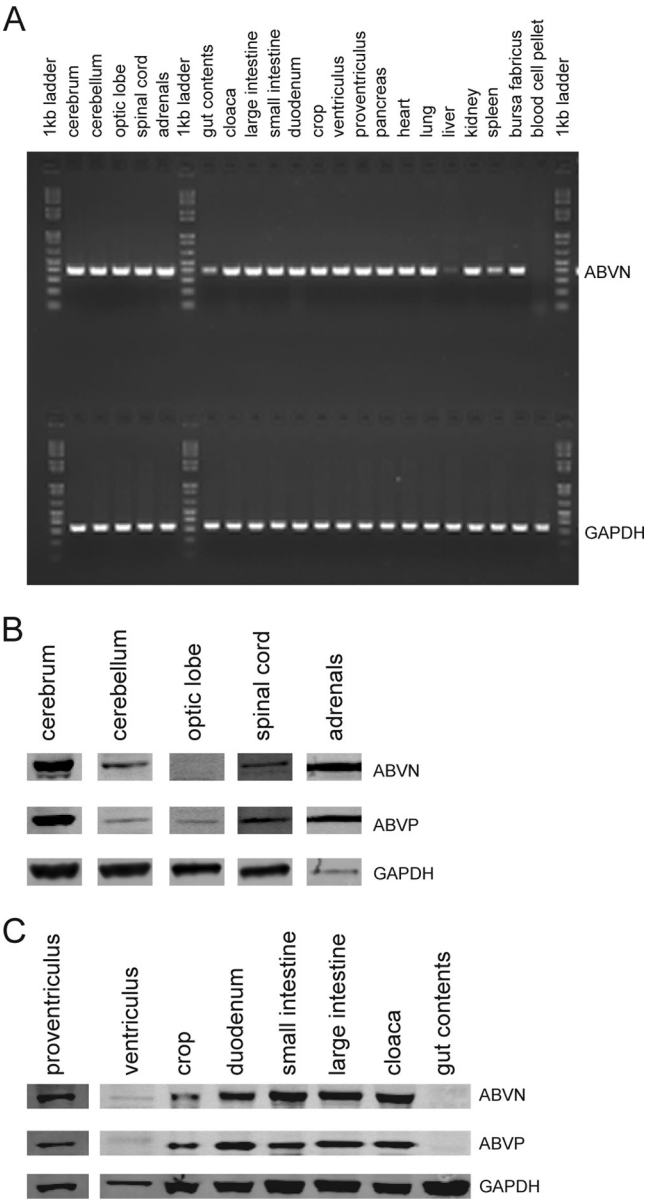

FIG. 3. ABV RNA and protein detected in tissues of a bird with a fatal case of PDD. (A) RT-PCR for ABVN RNA and for a GAPDH mRNA control on a panel of RNA extracted from tissues harvested from necropsied bird 3. The tissue types examined are indicated above the top gel lanes. (B and C) Western blot assay with ABVN, ABVP, and GAPDH antisera from tissues of the nervous and gastrointestinal systems, respectively, of necropsied bird 3.

mental material). For each specimen, 30 μg of soluble protein was assayed by Western blot analysis with both antisera (and with commercially available monoclonal antibodies to GAPDH). For the two birds that exhibited more severe clinical signs of PDD (necropsied birds 1 and 3), ABVN and ABVP were detected primarily in tissues of the CNS and gastrointestinal tract (Fig. 3B and C); in other tissues, the protein levels of these two viral antigens were either undetectable or indeterminate due to a lack of signal in our parallel GAPDH immunoblot assays (data not shown). In contrast, ABVN and ABVP antigens went undetected in the necropsy tissues derived from a chick that had outwardly recovered from PDD but exhibited gross pathology on necropsy (necropsied bird 2), despite the presence of intact GAPDH protein and detectable ABV RNA in numerous tissues (Table 1). While we suspect that this discrepancy may simply reflect a lower viral copy number in the necropsy 2 samples, it is noteworthy that, where positive, the ABV Western blot results more closely track the sites of clinical pathology than do the ABV RT-PCR results.

Our outbreak investigation suggests that the age of the host may influence the outcome of ABV infection. The course of the disease was particularly dramatic in very young birds, with many developing CNS signs within 48 h of initial feed refusal and rapid deterioration to death within 3 days. These results are reminiscent of those observed upon experimental Borna disease virus (BDV) infection of neonates and animals treated with cyclosporine (13), where more severe disease and, in some species, broader tissue tropism have been detected compared to the characteristic neurotropism of BDV observed in immunocompetent adults (6, 12). Although recent studies indicate a broad tissue tropism for ABV in adult psittacines as well (4, 7, 11, 16), more work is required to understand if similar patterns of ABV tropism are detectable in other populations naturally infected with ABV2 or other isolates of ABV and the role that host factors such as age and species play in susceptibility to ABV infection and disease progression.

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