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NOTES

Novel Avian Bornavirus in a Nonpsittacine Species (Canary; *Serinus canaria*) with Enteric Ganglioneuritis and Encephalitis[▽]

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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. Recently, several genotypes of a novel avian bornavirus have been linked to PDD. In the canary, bornaviral antigen was detected by immunohistochemistry in both neural and extraneural tissues. The widespread viral dissemination was confirmed by reverse transcription-PCR. 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.

Avian bornavirus (ABV) is a recently identified virus species that is considered to be the causative agent of a common and fatal disease of psittacine birds, proventricular dilatation disease (PDD) (5, 6). Currently, six genotypes of ABV are known, all of which seem to be associated with the same clinical disease and pathological lesions (13). Thus far, ABV has been detected in at least 28 different psittacine species from four continents (5, 6, 11, 13). The associated disease in psittacine birds has been recognized since the late 1970s; it is clinically characterized by depression, weight loss, regurgitation, and passage of undigested seeds in the feces. At necropsy, emaciation, dilatation of the crop, proventriculus, and gizzard, and the presence of undigested feed in the lower digestive tract are common (4, 9). These changes can be attributed to the conspicuous histological findings which present as nonsuppurative ganglioneuritis of the vegetative nerve system of the upper digestive tract and nonsuppurative encephalitis (4, 8, 9). Although large and medium-sized parrots appear to be most affected by this disease, there are a few reports on sporadic diseases with similar clinical signs and pathological lesions in nonpsittacine bird species, such as Canada goose (*Branta canadensis*), canary (*Serinus canaria*), greenfinch (*Carduelis chloris*), long-wattled umbrellabird (*Cephalopterus penduliger*), and bearded barbet (*Lybius dubius*) (2, 10). PDD-like diseases in nonpsittacine bird species have only been characterized morphologically, and attempts to identify a causative agent were not successful or were not made.

We report here the immunohistochemical detection and molecular identification of a novel genotype of ABV in a retrospectively analyzed canary with the initial diagnosis of a condition comparable with PDD of psittacine birds. In August 2002 a male canary (*Serinus canaria*) with a 3-day history of apathy and consecutive spontaneous death was necropsied. The bird was emaciated, the proventriculus was severely dilated and impacted with seeds, the gizzard was almost empty, and there were moderate numbers of undigested seeds in the lower digestive tract. Histologically, the brain showed focal and moderate perivascular cuffs of mononuclear cells especially in areas adjacent to the lateral ventricles, i.e., the hippocampus, the fimbria of hippocampus, the parahippocampus, and the mesopallium (Fig. 1A). These inflammatory changes were accompanied by activated and hypertrophic astrocytes. There was also severe infiltration of myenteric and subserous nerve plexus and nerve fibers of proventriculus, gizzard (Fig. 1B), and duodenum with mononuclear inflammatory cells. Frequently, the inflammatory infiltrates also invaded the adjacent connective tissue or smooth muscle tissue. Other organs (liver, lung, and kidney) showed no relevant histological changes.

After elucidation of the potential role of ABV in PDD (5, 6), paraffin-embedded tissue blocks of this case were taken from the archive and investigated further. For screening purposes, an immunohistochemistry assay, which had proved useful for the identification of ABV-infected birds in a previous study, was used. Briefly, a rabbit polyclonal antibody to recombinant phosphoprotein of Borna disease virus (kindly provided by W. I. Lipkin, University of California, Irvine), which had shown cross-reactivity with ABV (13), was used as primary antibody (dilution 1:3,000). The immunohistochemical procedure was performed in a Thermo Autostainer 360-2D System (Histocom, Wr. Neudorf, Austria) using the UltraVision LP detec-

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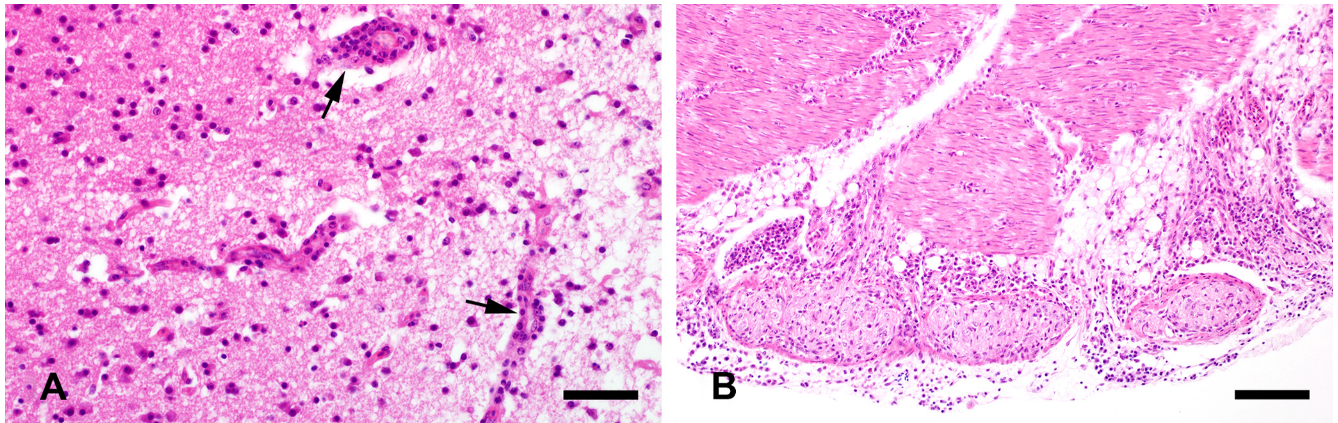

FIG. 1. Histological changes in a canary with ABV infection. Hematoxylin-and-eosin-stained sections of nervous system are shown. (A) Brain, showing nonsuppurative encephalitis characterized by perivascular infiltrates (arrows). Bar, 40 μm. (B) Gizzard, showing severe nonsuppurative infiltration of subserous vegetative nerve fibers and ganglia and of surrounding connective tissue. Bar, 80 μm.

tion system (Thermo Fisher Scientific, Fremont, CA) and DAB Plus (Thermo Fisher Scientific) as a chromogen.

Viral RNA was extracted from a pool of three 10-μm-thick scrolls from the three paraffin blocks available (brain; proventriculus and gizzard; liver, lung, and kidney) according to a previously described method (13). Briefly, the samples were deparaffinized in xylene and pelleted by centrifugation. The pellets were washed in ethanol, dried, and treated with proteinase K for 16 h. Viral RNA was extracted by using a QIAamp Viral RNA minikit (Qiagen, Hilden, Germany). For reverse transcription-PCR (RT-PCR), previously published bornavirus-specific universal oligonucleotide primer pairs were used, which anneal to putative nucleoprotein (N) (forward primer, 5′-CATGAGGCTATWGATTGGATTA-3′; reverse primer, 5′-TAGCCNGCCMKTGTWGGRTTY T-3′) and matrix protein (M) gene regions (forward primer, 5′-CAAGGTAATYGTYCCTGGATGG-3′; reverse primer, 5′-ACCAATGTTCCGAAGMCGAWAY-3′) of ABVs. RT-PCR and nucleotide sequencing were performed according to previously described protocols (3, 13). The nucleotide sequences were identified by the basic local alignment search tool (BLAST) (1) and were aligned using the AlignPlus program (v4.1; Scientific and Educational Software, Cary, NC). Multiple alignments for phylogenetic analyses were created with the help of the CLUSTAL X program (12). Phylogenetic analyses were conducted by the neighbor-joining algorithm. Bootstrap resampling analyses of the phylogenetic trees were performed on 1,000 replicates. Trees were established with the help of the TreeView 1.6.6. software. In addition to the nucleotide sequence obtained in the present study, representatives of five genotypes of ABV recovered from psittacine birds were also included in the sequence alignments and phylogenetic analyses. These sequences were obtained from different countries and are available in the GenBank database. Reference mammalian Borna disease virus (BDV) strains were also included in the phylogenetic analysis. The ABV sequence described in the present study was submitted to GenBank database under accession number GQ161095. The nucleotide sequences were translated into putative amino acid sequences, and phylogenetic analysis was performed on them as well.

At immunohistochemistry, the polyclonal anti-BDV antibody showed reactivity with numerous cells in the brain. Especially in areas which had shown histological changes a large number of cells were positive (Fig. 2A). Most of them were neurons, of which either the nuclei, both the nuclei and cytoplasm, or only the cytoplasm were positive. Cytoplasmic staining frequently extended to neuronal processes, and in areas with many positive cells a diffuse reactivity of the neuropil could be seen. Many hypertrophic astrocytes especially in subependymal locations showed distinct cytoplasmic staining. Several vegetative nerve plexus and nerve fibers of proventriculus and gizzard were also positive. In addition, there was immunostaining of extraneural tissues, such as hepatocytes and bile duct epithelia of liver (Fig. 2C), renal tubule epithelia of kidney (Fig. 2E), and tertiary bronchial epithelium of the lung. In order to exclude possible cross-reactivity of the antibody with cellular proteins, tissues of three canaries without PDD-like pathology were subjected to immunohistochemical analysis. All organs—in particular the brain, vegetative nerve fibers, and plexus of the digestive tract, liver, kidney, and lung—were without immunoreactivity (Fig. 2B, D, and F).

RT-PCR on the N protein coding region of ABV amplified a specific DNA product from all three canary samples, whereas repeated amplification attempts on the M protein coding region remained negative. The 345-nucleotide-long sequences of the amplification products of the different tissues were identical to each other. A BLAST search identified the highest (76%) nucleotide identity with the mammalian (equine) Borna disease virus isolate H3950 (AY374531) (7) and other BDV isolates. The highest identity with a psittacine bornavirus was found with an ABV-5 isolate (000-824-070, GenBank accession no. FJ002319) (6). The probable phylogenetic relationships between the N protein nucleotide sequences of the canary-derived bornavirus and other avian and mammalian bornaviruses are demonstrated in a phylogram (Fig. 3). The canary sequence forms a separate branch of the tree, between the known five avian (psittacine) bornavirus clusters, and the Borna disease virus isolates obtained from horses. The phylogram of the deduced amino acid sequences shows similar topology (data not shown); however, in this tree the canary-derived sequence is

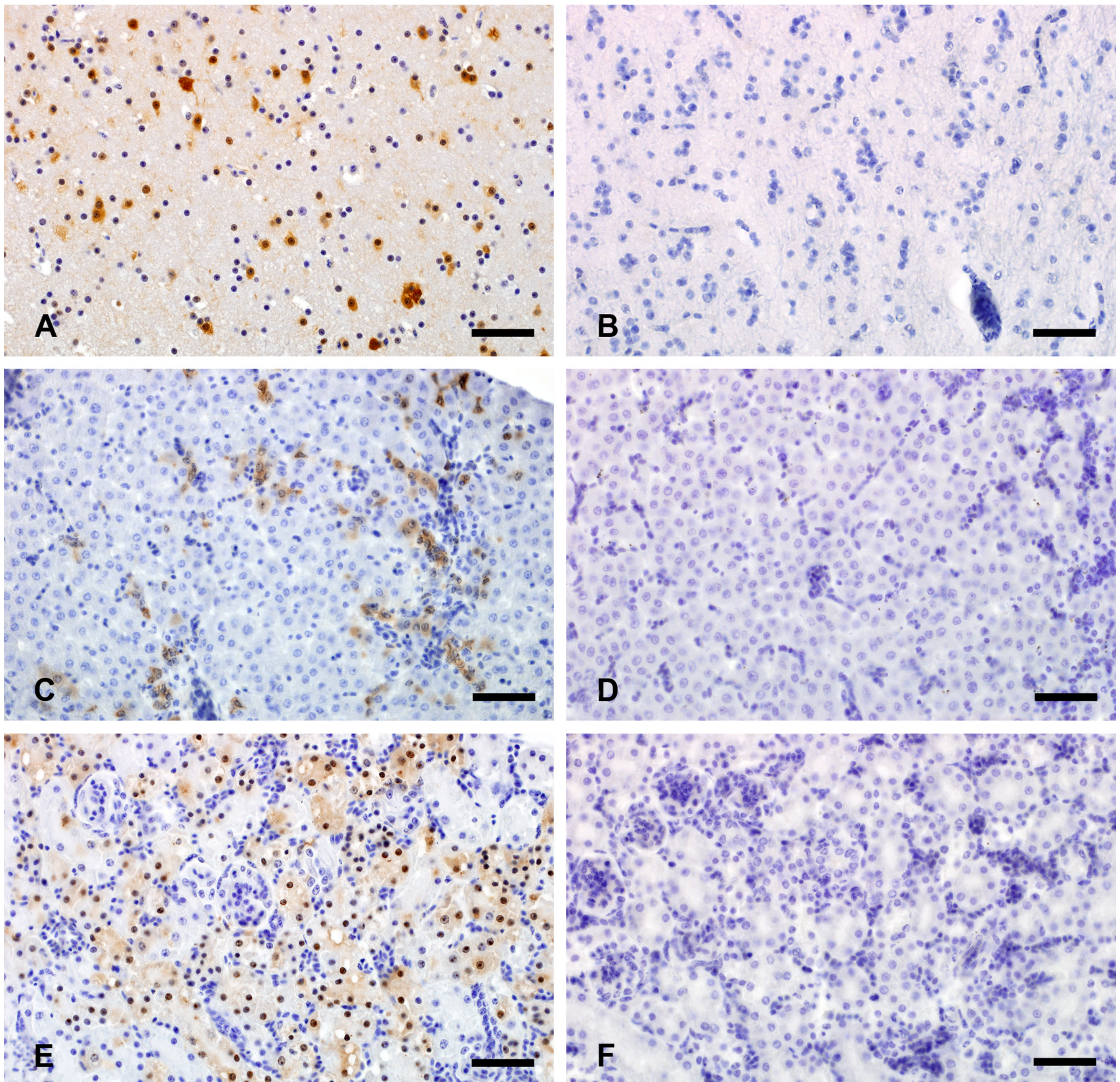

FIG. 2. Positive immunostaining of different tissues of the ABV-infected canary in comparison to matching tissue locations of a canary without ABV infection. (A) Brain. Immunoreactivity is present in numerous neurons and possibly also in glial cells. (B) Brain (negative control). (C) Liver. The immunoreactivity of hepatocytes and groups of bile duct epithelia is evident. (D) Liver (negative control). (E) Kidney. The image shows the immunostaining of tubular epithelia. (F) Kidney (negative control). Bars, 40 μm.

closer to the psittacine ABV sequences than to the BDVs. The clustering of the canary-derived sequences is statistically supported in both trees (100 and 99.9% bootstrap values, respectively). Also, the canary-derived ABV partial sequence is only very distantly related to sequences of other *Mononegavirales*.

We describe here for the first time the presence of an ABV in a nonpsittacine species with a pathological condition closely resembling PDD, a disease designation that is currently reserved for psittacine birds. This finding is even more remarkable since the recovered viral nucleotide sequence is unique and adds another genotype or viral species to the family *Bornaviridae*. A more detailed characterization of the detected canary bornavirus genome, as well as isolation of the virus would be necessary to clarify this issue. Unfortunately, repeated attempts to amplify further genomic regions were unsuccessful thus far and isolation of the virus from the available sample material (paraffin-embedded tissue) was not applicable. The distribution of ABV antigen in nervous tissue closely resembles the picture described in psittacine birds (13). In addition, positive immunostaining of a number of extraneural

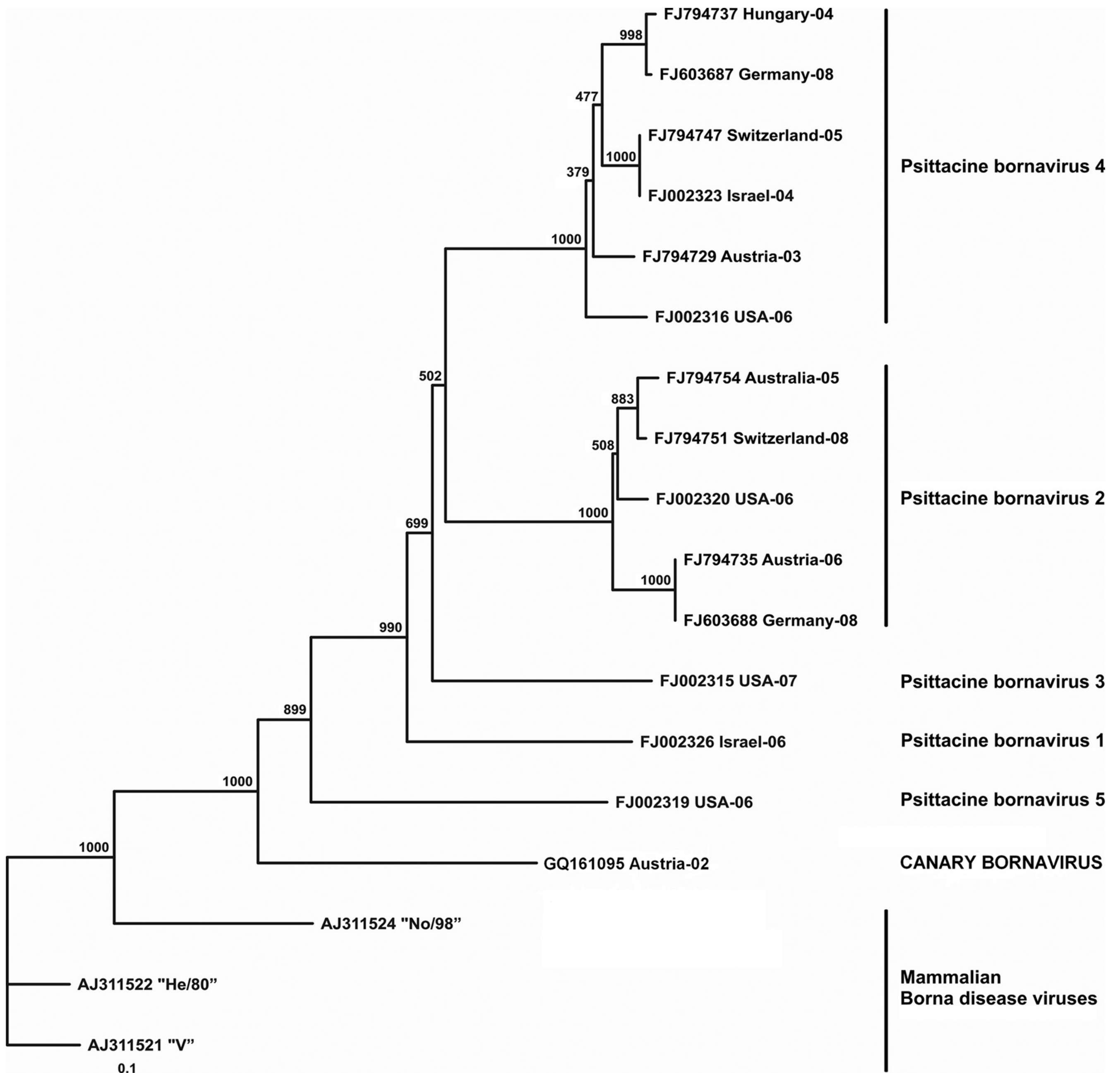

FIG. 3. Phylogram illustrating the genetic relationships between the canary bornavirus (GQ161095, Austria-02) and other bornavirus genotypes, based on a partial nucleotide sequence of the putative nucleoprotein gene. Representatives of the known five ABV genotypes from different countries and three representatives of the BDV are indicated as follows: GenBank accession number and country of collection-year of collection. The reference BDV "V" strain's sequence (AJ311521) was used as an outgroup. The scale bar indicates the genetic distance; the bootstrap support values of 1,000 replicates are shown at the nodes. The main ABV genogroups are indicated.

cells suggests a broad tissue tropism of the present virus, also including extraneural cells. This immunostaining result has to be interpreted with caution, since cross-reactivity of the used antibody with cellular proteins cannot be entirely excluded. However, the detection of viral sequences by RT-PCR in extraneural tissue adds further support to the notion of ABV replication in extraneural cells. The finding is also in line with the observation of Rinder et al. (11) in a psittacine bird with PDD. Concerning epidemiology and pathogenesis, the broad tissue distribution of ABV would facilitate direct transmission between birds via infected body fluids and feces and probably induce functional impairments of extraneural tissues and organs. The discovery of an increasing number of bornaviruses in birds also opens the door to comparative research approaches in bornavirology, the knowledge of which is based on a single virus species thus far. It is not unlikely that currently only the tip of the iceberg has been seen and that many more viral genotypes or species belonging to the *Bornaviridae* family will

be detected in the future. It might be worth the effort to systematically look for bornaviruses in different avian species with neurological diseases, even those which do not exactly match the PDD criteria.

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