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Prevalence of Circulating Antibodies to p10, a Non-structural Protein of the Borna Disease Virus in Cats with Ataxia

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ABSTRACT. Japanese domestic cats were surveyed for circulating antibodies to the p10 and p24 proteins of the Borna disease virus (BDV) by Western blotting. Twenty-four of 52 cats (46.2%) with ataxia and other neurologic symptoms of unknown cause were positive for antibodies to BDV p10 and/or p24. In contrast, cats without neurological symptoms gave a significantly lower prevalence of anti-BDV antibodies to p10 and/or p24 (36 of 152 cats, 23.7%). Thirty specific pathogen-free (SPF) cats tested as controls were uniformly negative to BDV p10 and p24 antigens. These results suggest that BDV may play a role in ataxia in cats. Additionally, our results suggest that it is necessary to use both p10 and p24 as antigens to detect circulating antibodies to BDV in cats.
KEY WORDS: anti-BDV antibody, ataxia, Borna disease virus, feline.

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Borna disease (BD) is a progressive encephalomyelitis of horses and sheep caused by infection with the neurotropic Borna disease virus (BDV) that is an enveloped, non-segmented, negative-stranded (NNS) RNA virus [6, 8, 19, 39]. BDV is the prototype genus of the new family of *Bornaviridae*. Epidemiological studies have documented that diverse species of warm-blooded animals including cats possess circulating antibodies to BDV. In cats, a spontaneous non-suppurative meningoencephalomyelitis with clinical signs of ataxia and behavioral abnormalities, referred to as staggering disease (SD), is thought to be a counterpart of Borna-encephalitis, because the prevalence of BDV-specific antibodies seen in these cats is higher than that seen in randomly selected domestic cats, not having neurological disorders [18, 20, 22, 23, 28, 30, 31, 34]. Moreover, a feline BDV has been isolated from the central nervous system of cats with SD [24]. Cats also developed neurological signs and encephalitis when experimentally infected with this feline BDV isolate [21]. These findings support the hypothesis that BDV may correlate with SD in cats.

Molecular biological studies on BDV have shown that the BDV antigenome consists of at least 6 open reading frames (ORFs), ORF I to ORF V and ORFx1 [5, 9, 10]. ORF I encodes the viral nucleoprotein N (p38/40) of 38/40 kDa [25, 33, 40]. ORF II encodes the phosphoprotein P (p24) of approximately 24 kDa [40, 41], and ORF V encodes for a 190-kDa protein that may be the putative viral polymerase L [43]. Studies on the replication of other NNS-RNA viruses have shown that the minimal replicative and infectious unit for these viruses is the ribonucleoprotein containing the genomic RNA that is tightly associated with the N, P and L proteins, whereas the precise nature of the minimal replicative unit of BDV is unclear. Unlike other NNS-RNA viruses, BDV replicates in the nucleus [6, 8]. We and others have studied the BDV protein p10 of approximately 10 kDa that is encoded by ORFx1, and translated from mRNA representing the second transcription unit of the BDV genome [27, 44]. The p10 are found in brain cells of naturally and experimentally infected animals as well as in persistently BDV-infected cells [27, 44]. Further studies revealed that, in an *in vitro* expression system, p10 can associate with p24 and p40, and the resulting complexes are transported into the nucleus of cells wherein BDV replicates [26, 27, 36, 45]. Although the functions of these BDV proteins are unknown, they may play important roles in the viral replication in the nucleus. The BDV p24 and p40, but not p10, have been used as test-antigens in numerous sero-epidemiological surveys to study the prevalence of BDV-infection [4, 35]. This is the first study to assess if p10, together with p24, also is a suitable "test" antigen to determine serologically the prevalence of BDV in cats with ataxia and other neurologic symptoms.

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MATERIALS AND METHODS

Cats: One hundred and fifty-two domestic cats without neurological symptoms and 52 cats with ataxia of unknown cause were studied. Additional clinical information such as astasia, paralysis of hindquarter, paralysis of legs, wryneck, tremor, head shaking, circling, epilepsia, depression, sudden excitement, and other individual information such as age, breed, sex, body temperature, the habit of hunting mice, living areas and breeding ambience (indoor or outdoor) in cats with or without neurological symptoms were gleaned from veterinarians, who collected cat blood samples in various

areas of Japan. The cats with or without neurological symptoms were free from known infectious agents such as feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), and feline infectious peritonitis virus (FIPV), which are known to cause encephalitis [11, 17, 37, 38], hydrocephalus and brain tumors in cats. The cat tested were also free from feline panleucopenia virus (FPLV) which is known to case ataxia [7, 14].

Thirty specific pathogen-free (SPF) cats (Harian Spraque Dawley Inc., IN, U.S.A.) were used as controls. The SPF cats were certified free from FIV, FeLV, FPLV, feline calicivirus, feline rhinotracheitis virus, feline enteric coronavirus, FIPV, rabies virus, *Chlamydia psittaci* and feline toxoplasmosis.

Plasmid construction and protein purification: A prokaryotic expression plasmid pGEX-ORFx1 encoding a glutathione-S-transferase-p10 fusion protein (GST-p10) was constructed as previously described [27]. Briefly, cDNA fragment encoding the BDV ORFx1 was amplified from cDNA of MDCK cells persistently infected with BDV (MDCK/BDV) [13] and cloned in-frame to the nucleotide sequence of GST in the pGEX-4T-3 vector (Amersham Pharmacia Biotech, Inc., NJ, U.S.A.). Similarly, a prokaryotic expression plasmid pGST-p24 encoding GST-BDV p24 (GST-p24) was constructed as described [15, 16]. The recombinant constructs were used to transform *E. coli* (BL21 strain) and the expressed fusion proteins were purified by use of glutathione-Sepharose 4B affinity column chromatography [1, 16, 27].

Western blot analysis: Antibodies to BDV p10 and p24 in cat plasma were detected by Western blotting as previously described [1, 28]. Briefly, purified GST-p10 and GST-p24 fusion proteins (40 *n*g/reaction) were used as test antigens. A recombinant GST protein (rGST) alone, expressed in *E. coli* transfected with the pGEX-5X-3 plasmid (Amersham Pharmacia Biotech, Inc.), was used as a control antigen. All of the plasma samples were pre-inactivated at 56°C for 30 min and pre-absorbed with a crude lysate of *E. coli* (60 μg protein for each 10 μ*l* plasma) expressing the rGST protein. The pre-absorbed plasma samples were diluted to 1 in 50 with phosphate-buffered saline (pH 7.2) containing 3% skim milk before testing. A horseradish peroxidase (HRP)-conjugated sheep anti-cat immunoglobulin (IgG) serum (1:1,000 dilution: American Qualex International, Inc., CA, U.S.A.) was used as secondary antibody. For color reactions to detect the positive signals, the HRP-1000 kit (Konica Corporation, Tokyo, Japan) was used according to the manufacturer's instructions.

Western blotting was standardized for day to day variation by use of serum from a rat experimentally infected with BDV as positive control. When cat plasma gave weak color reaction in Western blotting, such plasma was diluted to 1 in 25 and 1 in 100, and retested. In the retest, the plasma giving clear color reaction at 1:25 dilution and no reaction at 1:100 dilution was determined as positive for antibodies to BDV.

The specificities of the GST-p10 and GST-p24 as test antigens were verified by Western blotting against naturally occurring anti-BDV antibodies in a serum (used at 1:1,000 dilution) from a rat experimentally infected with BDV (kindly provided by Dr. K. M. Carbone, FDA, U.S.A.). Serum (used at 1:10 dilution) from an uninfected rat was used as a negative control serum. An HRP-conjugated goat anti-rat IgG serum (at 1:1,000 dilution) (ICN Pharmaceuticals, Inc., CA, U.S.A.) was used as secondary antibody. Similarly, rabbit antisera raised against the GST-p10 or the GST-p24 fusion protein were used as positive controls (each serum used at 1:5,000 dilution) after absorption with the crude lysate of *E. coli* expressing the rGST protein. Sera (tested at 1:10 dilution) from pre-immune rabbits were used as negative control sera. An HRP-conjugated donkey anti-rabbit IgG serum (used at 1:1,000 dilution) (Amersham Pharmacia Biotech, Inc.) was used as secondary antibody. Additional control includes a goat anti-GST serum (Amersham Pharmacia Biotech, Inc.) used at 1:1,000 dilution to interact with the GST-p10 and GST-p24 test antigens. In this case, an HRP-conjugated rabbit anti-goat IgG (at 1:1,000 dilution) (ICN Pharmaceuticals, Inc.) was used as secondary antibody.

Statistical analysis: The presence or absence of significant correlation between anti-BDV antibodies and clinical symptoms or other individual information was statistically analyzed by use of the Fisher's exact test and the chi-square test.

RESULTS

Specificities of test antigens to anti-BDV antibodies: Prior to use for detecting anti-BDV antibodies in cat plasma, the antigenic specificities of the GST-p10 and GST-p24 fusion proteins were verified by Western blotting against naturally occurring anti-BDV antibodies in a serum from a BDV-infected rat. As shown in Fig. 1A, GST-p10 (lane 1) and GST-p24 (lane 3) were specifically recognized by the infected rat antiserum but not by a serum from an uninfected rat (lanes 2 and 4). Preabsorption of the serum from this BDV-infected rat with the GST-p10 and the GST-p24 fusion protein abrogated these positive reactions whereas preabsorption with rGST protein did not (data not shown).

Rabbit antisera raised against GST-p10 (lane 1) and GST-p24 (lane 3) specifically detected naturally occurring BDV p10 and p24, respectively, in the cell-free lysate of MDCK/BDV cells (Fig.1B). The pre-immunized sera from these rabbits (lanes 2 and 4) did not interact with the MDCK/BDV cell lysate (Fig. 1B). None of the rabbit sera reacted with the cell-free lysate of MDCK cells (data not shown). Figure 1C shows that these rabbit antisera raised against the GST-p10 and the GST-p24 fusion protein specifically recognized the purified GST-p10 (lane 1) and GST-p24 (lane 3), respectively, whereas the pre-immunized sera (lanes 2 and 4) did not. Taken together, these results indicate that the GST-p10 and GST-p24 fusion proteins can be used as test antigens to specifically detect naturally occurring antibodies to BDV p10 and p24 in sera by Western blotting.

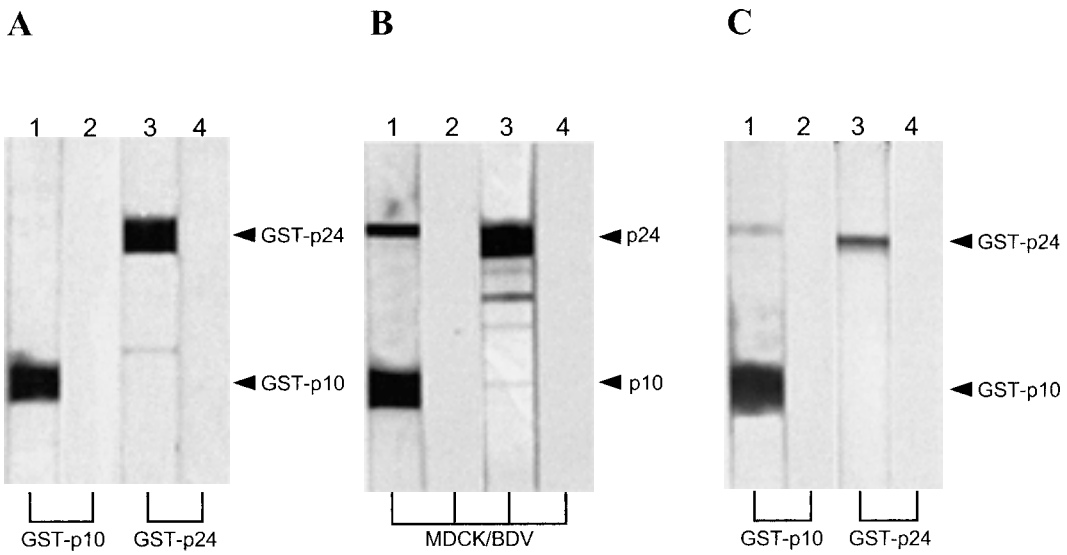

Fig. 1. Specificities of Western blots for detecting anti-BDV antibodies. (A) The fusion proteins GST-p10 (lanes 1 and 2) and GST-p24 (lanes 3 and 4) were used to test a serum collected from a rat experimentally infected with BDV (lanes 1 and 3). Serum from a non-infected rat was used as control (lanes 2 and 4). (B) Cell-free soluble lysate of MDCK/BDV cells were used as antigen, and tested against rabbit antisera raised against GST-p10 (lane 1) and GST-p24 (lane 3). The respective pre-immune sera were used as controls (lanes 2 and 4). (C) The fusion proteins GST-10 (lanes 1 and 2) and GST-p24 (lanes 3 and 4) were used to test rabbit antisera raised against GST-p10 (lane 1) and GST-p24 (lane 3). The respective pre-immune sera were used as controls (lanes 2 and 4).

Prevalence of plasma antibodies to the BDV p10 protein in cats: The prevalence of anti-BDV p10 antibodies not only in cats, but also in horses, sheep and man, in which anti-BDV p24 and p40 antibodies have been detected so far, has never been examined. In this study, anti-BDV p10 and p24 antibodies in plasma from 152 domestic cats without neurological symptoms and 52 domestic cats with ataxia and other neurological symptoms were surveyed by Western blotting with recombinant GST-p10 and GST-p24, respectively. Plasma samples from 30 SPF cats were used as control. As shown in Table 1, 11 of the 52 cats (21.2%) with neurological symptoms had anti-p10 antibodies. This prevalence was lower than that detected for anti-p24 antibodies, i.e., 20 of the 52 cats (38.5%). In contrast, only 19 of the 152 cats (12.5%) without neurological symptoms had antibodies to BDV p10, as compared to 25 of the 152 cats (16.4%) with anti-p24 antibodies (Table 1). The 30 SPF cats tested uniformly negative to both BDV antigens (data not shown). None of the plasma samples tested in this study reacted with the control rGST protein (data not shown). Moreover, pre-absorption of each cat plasma with BDV p10 or p24 prior to Western blotting, abrogated the positive signal to the respective test antigen (data not shown). These results indicated that the positive reactions seen in Western blotting were specific. Representative positive and negative results are shown in Fig. 2.

Prevalence of plasma antibodies to BDV in cats: The present study showed that 24 of the 52 cats (46.2%) with neurological symptoms had antibodies to BDV p10 and/or p24. This seroprevalence was significantly higher than that seen in the 152 cats without neurological symptoms (36 of 152; 23.7%; double-sided p-value=0.0044, Fisher's exact test; p-value after Yates' continuity correction = 0.0038, chi-square test). Of the 24 seropositive cats with ataxia and other neurological symptoms, seven had antibodies to p10 and to p24 (29.2% concordance). Among the remaining 17 cats, four (23.5%) had antibodies to p10, but not to p24, and 13 (76.5%) had antibodies to p24 but not to p10. Hence, p24-seropositive cats are more likely to be in discordance than cats that were seropositive to p10. Likewise, among the 36 seropositive cats without neurologic symptoms, eight (22.2%) had antibodies to both BDV antigens. Among the remaining 28 cats, 11 (39.3%) had anti-p10 but not anti-p24, and 17 (60.7%) had anti-p24 but not anti-p10.

Clinical features of cats positive for anti-BDV antibodies: The clinical symptoms, age and sex of the 52 seropositive and seronegative cats with neurological symptoms are summarized in Tables 2 and 3, respectively. Ataxia is common to both seropositive and seronegative cats. As shown, no specific neurological symptom can be seen to be associated with seropositivity to BDV. Moreover, an association between seropositivity, a habit of hunting mice, living area, and breeding ambience (indoor or outdoor) was not found (data not shown).

DISCUSSION

Previous studies have shown that feline ataxia due to non-

Table 1. Prevalence of anti-BDV antibodies in domestic cats

Cats wih neurological symptoms (n=52)			Cats without neurological symptoms (n=152)		
Cat No.	Anti-BDV antibodies		Cat No.	Anti-BDV antibodies	
	p10	p24		p10	p24
A-1	–[b)]	+[a)]	N-4	+	–
A-5	+	–	N-5	+	–
A-6	+	–	N-37	+	–
A-9	–	+	N-41	+	–
A-10	+	+	N-47	+	–
A-16	–	+	N-49	+	–
A-17	–	+	N-55	+	–
A-18	+	–	N-69	+	+
A-20	+	+	N-71	–	+
A-21	+	+	N-73	–	+
A-22	+	–	N-74	–	+
A-23	+	+	N-77	–	+
A-24	+	+	N-78	–	+
A-27	+	+	N-80	+	–
A-29	–	+	N-81	+	+
A-32	–	+	N-82	+	–
A-33	–	+	N-84	–	+
A-36	–	+	N-85	+	–
A-37	–	+	N-87	–	+
A-38	–	+	N-92	+	–
A-39	–	+	N-95	+	+
A-45	–	+	N-96	–	+
A-49	–	+	N-98	+	+
A-52	+	+	N-101	–	+
			N-103	–	+
			N-121	–	+
			N-124	–	+
			N-130	–	+
			N-134	–	+
			N-143	–	+
			N-145	–	+
			N-146	–	+
			N-147	+	+
			N-148	+	+
			N-149	+	+
			N-151	+	+
	11/52 (21.2%)	20/52 (38.5%)		19/152 (12.5%)	25/152 (16.4%)
	Total: 24/52(46.2%)			Total: 36/152(23.7%)	

a) Positive. b) Negative.

suppurative meningoencephalomyelitis of unknown aetiology [18] or SD [31] may have an association with BDV infection [2, 3, 21, 23, 24, 29, 30, 34]. Experimental infection of cats with BDV gave neurologic disease and encephalitis comparable to SD [21]. Cats with naturally occurring neurologic symptoms also have a higher seroprevalence of antibodies to BDV antigens than controls without neurologic disease [23, 29, 34]. These studies had used the structural viral proteins p24 and/or p40 as test antigens to assess the seroprevalence of antibodies to BDV infection. Here, we examined for the first time the seroprevalence of antibodies to p10, a putative nonstructural protein of BDV, in cats with or without neurologic disease. We found that cats with ataxia and other neurologic symptoms had higher prevalence of anti-p10 antibodies in plasma than cats without neurologic symptoms (21% against 13%). Our results lend further support to the hypothesis that BDV may play a role in ataxia in cats.

Berg *et al.* have reported that cats with the habit of hunting mice were likely to be infected with BDV at high rate, when compared with cats without such habit, and that male cats might be more frequently exposed to BDV than female cats [3]. However, anti-BDV antibody has not been detected in wild rats in Hokkaido, Japan [42], where anti-BDV antibodies were frequently detected in horses, cats and sheep [12, 28, 42]. Hence, it is not surprising that no signif-

Table 2. Clinical symptoms in seropositive cats with neurological disorders

Cat	Sex	Age (years)	Antibodies to p10	Antibodies to p24	Major symptoms
A1	Male	3	–[b)]	+[a)]	Ataxia, Collapse of whole body
A-5	Female	1.17	+	–	Ataxia (Hindquater), Depression, Muscle spasm, Chronic fever, Sudden excitment
A-6	Male	4	+	–	Ataxia (Hindquater), Muscle spasm
A-9	Male	11	–	+	Ataxia (Hindquater), FIV, FeLV
A-10	Male	9	+	+	Ataxia (Hindquater), Depression, Head shaking
A-16	Male	2.83	–	+	Ataxia (Hindquater), Depression, Lumbosacral pain, Astasia
A-17	Male	1.5	–	+	Ataxia (Hind legs), Chronic fever
A-18	Female	12	+	–	Ataxia, Depression, Collapse, Astasia
A-20	Male	Unknown	+	+	Atasiz (Hind legs), Chronic fever
A-21	Male	0.5	+	+	Ataxia (Hindquater), Astasia, Head shaking
A-22	Male	5~6	+	–	Ataxia, Wryneck
A-23	Male	3	+	+	Ataxia, Astasia
A-24	Female	0.66	+	+	Ataxia, Periodic attack
A-27	Female	4~5	+	+	Ataxia, Wryneck, Circling, Sudden excitement
A-29	Female	4	–	+	Ataxia (Hind legs), Depression, Wryneck, FeLV
A-32	Male	7	–	+	Ataxia Depression, Circling, Sporadic fever
A-33	Male	2	–	+	Ataxia (Hind legs), Muscle spasm, Astasia
A-36	Female	1	–	+	Ataxia (Hind legs)
A-37	Male	5	–	+	Ataxia (Hind legs), Depression, Epilepsy, Chronic fever, Sudden excitement
A-38	Female	6	–	+	Ataxia (Hind legs), Depression, Chronic fever, Sudden excitement
A-39	Female	9	–	+	Ataxia, Tonic paralysis of whole body, Muscle spasm
A-45	Female	1.75	–	+	Ataxia, Depression, Sudden excitement
A-49	Male	1	–	+	Ataxia (Front/hind legs), Depression, Head shaking, Sudden excitement
A-52	Male	Unknown	+	+	Ataxia (Front/hind legs), Loss of proprioception (Hind legs)

a) Positive. b) Negative.

Table 3. Clinical symptoms in seronegative cats with neurological disorders

Cat	Sex	Age (years)	Major symptoms
A-2	Male	6.33	Ataxia (Front/hind legs)
A-3	Male	11	Ataxia, Sudden excitement
A-4	Female	2	Ataxia, Depression, Severe weight loss
A-7	Female	6.3	Ataxia (Hindquarter), Depression, Wryneck, Circling
A-8	Male	5.7	Ataxia (Front/hind legs), Hunting mice
A-11	Female	3	Ataxia, Epilepsia
A-12	Male	12.5	Ataxia (Front/hind legs)
A-13	Female	1	Ataxia (Front legs)
A-14	Female	13	Ataxia (Hindquarter)
A-15	Male	0.6	Ataxia
A-19	Male	1.66	Ataxia (Hindquarter), Depression
A-25	Female	0.33	Ataxia (Hind legs)
A-26	Female	21	Ataxia, Epilepsia, Renal failure
A-28	Male	1	Ataxia,
A-30	Male	1	Ataxia, Trembling
A-31	Male	0.5	Ataxia (Front/hind legs), Depression, Sudden excitement
A-34	Male	14	Ataxia,
A-35	Female	1	Ataxia, Depression, Convulsive seizure
A-40	Male	0.25	Ataxia
A-41	Female	Unknown	Ataxis, Depression, Wryneck, Encephalatrophy
A-42	Female	0.66	Ataxia (Hind legs)
A-43	Male	2.25	Ataxia (Front/hind legs), Epilepsia
A-44	Male	3	Ataxia (Hind legs)
A-46	Male	13	Ataxia, Depression, Wryneck
A-47	Male	7.17	Ataxia (Hindquarter)
A-48	Male	3	Ataxia (Hindquarter), Hypergammaglobulinaemia, Sporadic fever
A-50	Female	9	Ataxia
A-51	Male	2.67	Ataxia, Ventricular enlargement

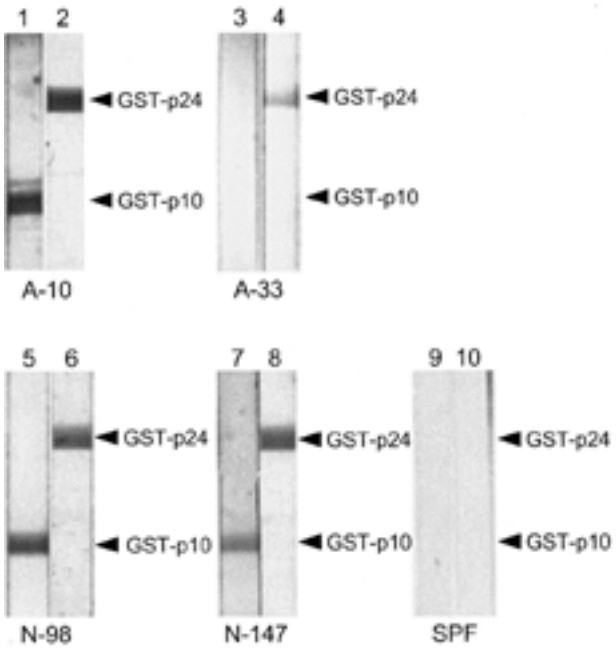

Fig. 2. Representative positive and negative results of anti-BDV p10 and p24 antibodies tests in cat plasma. Representative positive and negative results in plasma from cats with neurological symptoms, A-10 and A-33, without neurological symptoms, N-98 and N-147, and an SPF cat, SPF, in Western blots with each of GST-p10 and GST-p24 were shown. Positive results of anti-p10 antibodies are lanes 1, 5 and 7. Positive results of anti-p24 antibodies are lanes 2, 4, 6, and 8.

icant correlation was observed between the presence of anti-BDV antibodies, the habit of hunting mice, the sex and age of the cats in the present study. The seroprevalence of antibodies to p10 and/or p24 in cats with ataxia and other neurological symptoms was high (46.2%). This finding is consistent with that reported earlier in cats with neurologic diseases in Japan [29], Sweden [23] and the United Kingdom [34].

We showed that 38.5% of the 52 cats with ataxia and other neurological symptoms, and 16.4% of the 152 cats without neurologic signs had antibodies to BDV p24 antigen. These results are consistent with the seroprevalence reported earlier for 15 cats (46.7%) with neurologic disorders [29] and for 32 cats (15.6%) without neurologic signs [30] tested against BDV p24. Whereas the seroprevalence of anti-p10 antibodies in the 152 cats without neurological symptoms (12.5%) is comparable to the seroprevalence of anti-p24 antibodies (16.4%) in this study, the prevalence of antibodies to p10 in the 52 cats with neurologic disorders (21.2%) is lower than that seen against p24 (38.5%). It is not clear why the cats with neurologic symptoms are more likely to have antibodies to p24 than to p10. Nowotny *et al.* [32] have reported that, in contrast to p24 and p40 proteins that are relatively conserved, the p10 of a new BDV subtype exhibited about 81% identity to its counterparts in reference strains such as strains V and He/80. This raises the possibility that infection with specific BDV subtypes may be pathogenic in cats. In this study, we used p10 from the BDV He/80 strain to study the prevalence of antibodies to BDV. It will be important to perform additional seroepidemiologic studies by use of p24, p40 and p10 from different virus subtypes to investigate the association of SD with BDV. In addition, there were many cats positive for antibodies to either one of p10 or p24, suggesting the necessity to use both p10 and p24 as test antigens for detecting circulating antibodies to BDV in cats.

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