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Experimental and natural borna disease virus infections: presence of viral RNA in cells of the peripheral blood

T.W. Vahlenkamp*, H.K. Enbergs, H. Müller

Faculty of Veterinary Medicine, Institute of Virology, University of Leipzig, An den Tierkliniken 29, D-04103 Leipzig, Germany

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Abstract

Cells of the peripheral blood of experimentally and naturally borna disease virus (BDV)-infected animals and of human psychiatric patients and healthy individuals were analyzed for the presence of viral RNA using a BDV-p40-specific nested reverse transcription-polymerase chain reaction (RT-PCR). The assay proved to be highly sensitive as 10 RNA molecules were reproducibly amplified. BDV RNA was detected in blood cells of experimentally infected immunocompetent mice and rats. Mice were persistently infected without showing clinical signs of borna disease (BD), whereas the rats suffered from acute BD. Among 19 horses examined, five were positive for viral RNA in the blood. In a flock of sheep with a history of BD, 1 out of 25 clinically healthy animals was positive. BDV RNA was also detected in cells of the peripheral blood of 10 out of 27 selected humans with psychiatric disorders, and in 2 out of 13 healthy individuals. Remarkably, BDV-specific RNA was present in some cases in the absence of BDV-specific antibodies. Sequence analysis of PCR products confirmed the specificity of the amplification system. The presence of BDV RNA in the blood of naturally and experimentally BDV-infected individuals may point to an incidental but relevant role of blood for the spread of BDV in the infected organism, as well as for the transmission of BDV to other individuals. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Borna disease virus; BDV; PBMC; Horse; Sheep; Rat; Mouse; Man; PCR

1. Introduction

Borna Disease Virus (BDV) is a non-segmented, negative-stranded RNA virus and the etiological agent of a non-purulent meningoencephalomyelitis in a wide range of

* Corresponding author. Tel.: +49-341-9738200; fax: +49-341-9738219.
E-mail address: virology@vetmed.uni-leipzig.de (T.W. Vahlenkamp).

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animal species including horses, sheep, cattle, cats and ostriches (Rott and Becht, 1995; Gonzalez-Dunia et al., 1997; Hatalski et al., 1997). Serological and molecular biological data reveal a world wide distribution of BDV infections (Kao et al., 1993; Kishi et al., 1995b; Malkinson et al., 1995; Bahmani et al., 1996; Nakamura et al., 1996). Most of the clinical cases are recognized in horses and sheep, particularly in certain areas of Germany and Switzerland (Metzler et al., 1976; Lange et al., 1987; Herzog et al., 1994). BDV infections in cats have been reported from Sweden, Austria, Japan and the United Kingdom (Lundgren et al., 1993; Nowotny and Weissenböck, 1995; Nakamura et al., 1996; Reeves et al., 1998). The natural transmission of BDV still remains enigmatic.

The experimental host range of BDV includes rodents, lagomorphs, birds, carnivores, ungulates as well as primates. Infections of rats and mice are animal models to study the BDV-induced T-cell dependent immunopathogenesis (Narayan et al., 1983; Richt et al., 1990; Stitz et al., 1995; Hallensleben et al., 1998; Lewis et al., 1999). BDV replicates in a number of cells of the nervous system including neurons, astrocytes, oligodendrocytes, and ependymal cells. In the infected organism, viral spread depends on the development or the reactivity of the immune system at the time of infection. In newborn or immunosuppressed rats BDV replicates to high titers in the brain after intracerebral infection; viral spread via neuronal pathways is accompanied by extraneuronal replication in various organs, and virus is shed in urine and saliva (Herzog et al., 1984; Morales et al., 1988). In these animals, BDV can also be isolated from cells of the peripheral blood and bone marrow (Sierra-Honigmann et al., 1993; Rubin et al., 1995). In contrast, BDV infections of immunocompetent rats and mice revealed that viral replication remains restricted to the central and the peripheral nervous system (Carbone et al., 1987).

Recognition of the broad host range and the observed behavioral disorders suggested that BDV might also infect humans. Diagnosis of BDV infection in vivo is currently performed by the demonstration of virus-specific antibodies in serum and cerebrospinal fluid (Herzog and Rott, 1980). Using an indirect immunofluorescence assay (IIFA), BDV-reactive antibodies were detected in 4–7% of sera obtained from more than 5000 psychiatric or neurological patients from Germany, USA, and Japan and in about 1% of sera obtained from healthy individuals (Rott et al., 1991). Subsequent seroepidemiological examinations confirmed a higher prevalence of BDV infections among humans with psychiatric disorders (Bode et al., 1992, 1993; Kishi et al., 1995b; Waltrip et al., 1995). The isolation of BDV from peripheral blood cells of psychiatric patients and the demonstration of viral antigens in the blood was also reported (Bode et al., 1996). Reports on the detection of BDV RNA in peripheral blood mononuclear cells (PBMC) caused considerable controversy because these observations were not confirmed by others (Kubo et al., 1997; Lieb et al., 1997; Richt et al., 1997). In order to verify whether BDV RNA is present in the blood, a sensitive RT-PCR was set up and cells of the peripheral blood of various naturally and experimentally infected animal species and man were analyzed. BDV RNA was detected in PBMC obtained from individuals at different time points of infection and with different immunological status, as well as at acute or chronic stages of the disease.

2. Materials and method

2.1. Collection of blood from experimentally and naturally BDV-infected animals and men

Mice of the inbred strain SJL/J (Charles River) were intracerebrally (i.c.) infected with BDV strain He-80 (Herzog and Rott, 1980). Six to eight months post infection (p.i.), approximately 0.5–0.7 ml of blood were collected using EDTA. Four 4-week-old Lewis rats were infected with the same BDV strain. For immunosuppression, one rat was treated with 860 μl of a 20 mg/ml solution of cyclophosphamide 15 days prior to infection. Age-matched rats served as controls. About 1–1.5 ml of blood were collected by cardiac puncture 4 weeks p.i. using EDTA.

Blood samples were collected from five horses with clinical signs of acute BD, and from 14 asymptomatic animals. Among the latter, four had been in contact with the horses showing signs of BD. Four ml of EDTA blood were collected for subsequent RNA isolation. Blood samples of sheep were derived from a flock with a history of BD. Six animals had developed clinical signs of BD 1 year ago. These BDV infections were confirmed by immunohistopathological examination and by the demonstration of BDV p40 RNA in the brain. In bleedings at a 2-month interval 3 ml of blood were collected from each of the 25 animals by jugular venipuncture using EDTA.

PBMC were obtained and investigated in the framework of a double-blinded multicenter study. Blood samples collected in different geographical regions of Germany included 27 from patients with psychiatric disorders, and 13 samples from healthy individuals as controls.

2.2. RNA isolation and nested RT-PCR

RNA was isolated from PBMC purified by centrifugation in Ficoll-Paque® (Pharmacia Biotech) gradients (horse, man), or from whole blood (mouse, rat, horse, sheep) after erythrocyte lysis using the RNeasy Blood Kit® (Qiagen) according to the manufacturer's instructions. RNA extracted from at least 2×10^6 PBMC (human, horse) or whole blood cells (sheep, horse) was used for RT-PCR amplification.

The primers were derived from a published nucleotide sequence of the BDV genome (Briese et al., 1994). The RT-PCR primers were: p40 s 5′-ACGCCCAGCCTTGT-GTTTCT-3′ (nucleotides 270–289), and p40 as 5′-AATTCTTTACCTGGGGACTCAA-3′ (nucleotides 720–697). These primers generate a 449 bp product. Primers for the nested PCR were p40 nested s 5′-TTACGGGGAAAAGACGA-3′ (nucleotides 407–423), and p40 nested as 5′-TTAGTAGAGACAACACAAAGGAG-3′ (nucleotides 687–666), generating a 281 bp product. Amplification was performed using an MJ Research Peltier Multicycler PTC 200 and the Titan™ One Tube RT-PCR System (Boehringer, Mannheim). Prior to the reaction, RNA ($\leq$1 μg) was heated for 3 min at 72°C and placed on ice immediately. RT-PCR reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 5 mM DTT, 1 μl of the enzyme mix provided, 100 pmol of p40 s and p40 as, and 10 U RNAsin (Promega) were added. The RNA was reverse transcribed in a volume of 50 μl for 45 min at 42°C. Amplification consisted of five cycles with 30 s denaturation at

94°C, 30 s annealing at 56.5°C, and 30 s extension at 72°C, followed by additional 35 cycles with 30 s denaturation at 94°C, 30 s annealing at 55°C, 30 s extension at 72°C. Five micro liter of the RT-PCR product were used in the nested PCR. Amplification was performed in a 50 μl reaction volume containing 100 pmol of both p40 nested primers, 0.2 mM dNTP, Expand^{TM} High Fidelity buffer with 1.5 mM $MgCl_2$, and 2.6 U Expand^{TM} High Fidelity PCR System enzyme mix (Boehringer, Mannheim), applying the same cycle and temperature profile as used in the first PCR.

2.3. Generation of the competitive BDV p40-specific RNA standard

Plasmids for in vitro transcription were constructed using standard cloning techniques (Sambrook et al., 1989). A 449 bp fragment (nucleotides 191–640) of the BDV p40 gene was amplified using primer p40 s and p40 as and cloned into vector pST73 (Promega) downstream of the T7 promotor to generate pSP73/p40-wt. Plasmid pSP73/p40-comp was constructed with the same genomic region. However, nucleotides 426–485 were deleted by two subsequent amplification reactions using primers p40 as–p40 del s [5′-ACGCGTCCATGGGCGCGCATCATGGTGAGACTGCTAC-3′ (nucleotides 585–601)], and p40 s–p40 del as [5′-ATGCGCGCCCATGGACGCGTATCCTATCACAACCC-CAAT-3′ (nucleotides 504–486)]. Both PCR products were targets in a second PCR using primer p40 s and p40 as. Due to the complementary sequences (underlined) of the p40 del primers, this PCR generated a 389 bp fragment, which was cloned downstream of the T7 promotor into vector pST73 (Promega).

To generate known amounts of the RNA standards BDV p40-wt and BDV p40-comp, 2 μg of linearized pSP73/p40-wt and pSP73/p40-comp DNA were transcribed in vitro for 1 h at 37°C with 40 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM NTPs, 20 U RNAsin, and 10 U T7 RNA polymerase (Boehringer, Mannheim). After incubation with RNAse-free DNAse (1 U per μg DNA), RNA was purified using phenol/chloroform, precipitated and subsequently resuspended in TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The quality of the RNA was evaluated by agarose gel electrophoresis and the amount was determined by four photometrical measurements at 260 nm.

2.4. RT-PCR performance testing and amplification control

RT and PCR were performed using a one tube amplification system to avoid a loss of templates and to exclude contaminations. RNA isolation and PCR mixture preparation were done in rooms outside the institute where cloned or amplified BDV nucleic acids had never been used. The amplification and the analysis of the PCR products were done in separate rooms. All handlings were performed with different sets of pipettes and the exclusive use of filter tips. Each RT-PCR was screened routinely for contaminations using negative reagent controls and RT-dependent amplification controls. In the case of restricted amounts of RNA, e.g. from human and mouse samples, RT-dependent amplification controls could not always be performed. Positive control reactions were performed using 20 copies of the BDV p40-comp RNA standard. The p40INS RNA standard (Sauder and de la Torre, 1998) was amplified using the same protocol.

The specifity of the obtained PCR products was investigated by digestion with *Nco*I and further confirmed by sequence analysis. PCR products derived from the PBMC of the sheep, one clinically healthy, one diseased horse as well as from one of the investigated rats were ligated into PCR 2.1-Toto (Invitrogen) and designated pS, pH(A), pH(B), and pR, respectively. The nucleotide sequences of two clones of each PCR product were determined using an ABI PRISM 377 sequencer (Perkin-Elmer).

2.5. Detection of BDV-specific antibodies

To investigate the presence of BDV-specific antibodies, serial dilutions of plasma samples were tested on acetone-fixed uninfected and BDV-infected Madin Darby Canine Kidney (MDCK)-cells by the IIFA (Herzog and Rott, 1980). Antibody titers $\geq$10 were defined positive.

3. Results

3.1. Sensitivity of the nested RT-PCR

BDV p40-wt and BDV p40-comp RNA standards were serially 10-fold diluted in a background of 100 ng of calf liver t-RNA, reverse transcribed and amplified. These titration experiments were repeated three times. Six out of six samples containing 10 BDV p40-wt RNA molecules were positive. Eight out of nine samples containing one RNA molecule were positive, and 2 out of 15 test samples containing 0.1 RNA molecules were positive. The result of one of these titration experiments is shown in Fig. 1A. When RNA standard p40INS was used, in six out of six samples containing 100 RNA molecules, in nine out of nine samples containing 10 RNA molecules, and in 3 out of 15 samples containing one RNA molecule, the generated 281 bp products were present (data not shown). It was assumed that the detection limit of the RT-PCR system was in a range of 1–10 RNA molecules, since samples containing 10 RNA molecules of either RNA standard were positive. A nested RT-PCR using 10 000 copies of BDV p40-wt RNA was negative when the reverse transcriptase was omitted during the RT-reaction, indicating the absence of contaminating plasmid DNA.

Titration experiments were performed to test the amplification efficiency for the wild-type and the competitive template. The results shown in Fig. 1B verify that both primer pairs used in the nested RT-PCR amplified the templates with a similar efficiency. Based on the results of these experiments, 20 molecules of the BDV p40-comp RNA or 150 molecules of p40INS RNA standard were used as reliable standards to control the performance of the amplification system.

3.2. Viral RNA in cells of the peripheral blood of experimentally infected animals

SJL/J mice were investigated for the presence of BDV-specific RNA in the peripheral blood cells 6–8 months after i.c. BDV infection. During this time, the animals had remained clinically healthy and had developed BDV-specific antibody

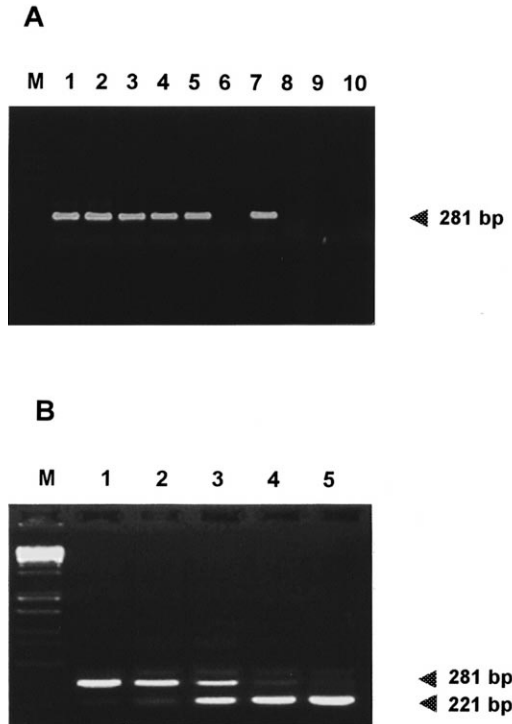

Fig. 1. (A) Sensitivity testing of the BDV p40-specific nested RT-PCR. The BDV p40-wt RNA standard was serially diluted 10-fold. RNA copy numbers added to the reactions were: 10 molecules (lanes 1, 2), 1 molecule (lanes 3 through 5), 0.1 molecule (lanes 6 through 10). The 281 bp products were analyzed on a 2% ethidium bromide-stained agarose gel. (B) Competitive RT-PCR to verify that both BDV RNA standards are amplified with the same efficiency. BDV p40-wt RNA molecules (10^3) were mixed with 10^2 (lane 1), 3×10^2 (lane 2), 10^3 (lane 3), 3×10^3 (lane 4), and 10^4 (lane 5) BDV p40-comp RNA molecules and amplified by nested RT-PCR. The 281 and 221 bp products are indicated by arrows. Molecular size marker (M).

titers of 25 000–50 000. Age-matched mock-infected animals were included as negative controls. Using the nested set of BDV p40-specific primers, one out of three mice proved to be positive for BDV p40-specific RNA. The peripheral blood cells of the uninfected animals as well as the included reagent control samples were negative (data not shown).

Four experimentally BDV-infected adult Lewis rats were investigated. One of these animals had been immunosuppressed by cyclophosphamide 15 days prior to infection. Four weeks p.i. the animals were euthanized. The immunocompromised and the immunocompetent rats showed BDV-specific antibody titers of 100 and 12 800, respectively. BDV isolated from the brain of these animals revealed titers of $10^{7.5}$ $TCID_{50}$ per ml brain homogenates. The immunocompetent rats showed signs of an acute BDV infection, involving paralysis of the hind limbs, tremor, and incoordination, whereas the immunocompromised rat remained clinically healthy. Blood cells of all infected animals were positive for BDV p40-specific RNA. Blood of one immunocompetent rat

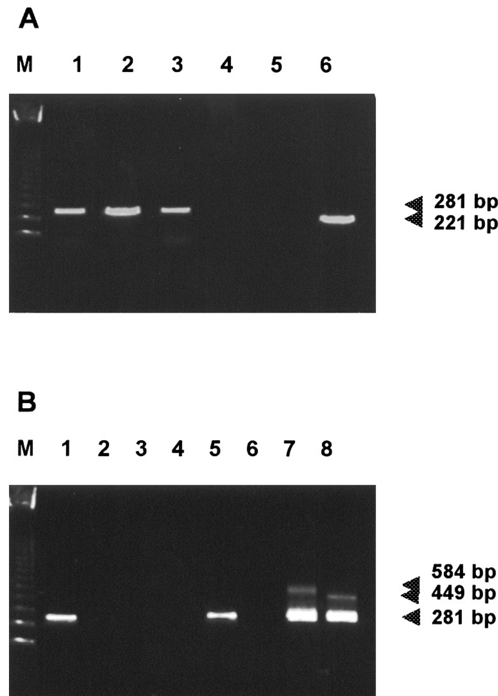

Fig. 2. Detection of BDV p40 RNA in cells of the peripheral blood by nested RT-PCR. Representative results of samples derived from experimentally BDV-infected rats (A); and psychiatric patients and healthy individuals (B). The 281 bp product derived from the BDV p40-wt RNA and the 221 bp product from the BDV p40-comp RNA templates are indicated. Analyzed blood samples shown in (A) were derived from: acute diseased rat #1 (lane 1), immunosuppressed rat (lane 2), acute diseased rat #1 (lane 3), uninfected rat (lane 4), reagent control [nested RT-PCR] (lane 5), positive control [20 BDV p40-comp RNA molecules] (lane 6). Analyzed blood samples shown in (B) were derived from: schizophrenic patient #1 (lane 1), healthy volunteer #1 (lane 2), schizophrenic patient #2 (lane 3), schizophrenic patient #3 (lane 4), healthy volunteer #2 (lane 5), reagent control [nested RT-PCR] (lane 6), positive control [150 p40INS RNA molecules] (lane 7), positive control [wild-type] (lane 8). The 584 and 449 bp products represent the PCR products of the first PCR. Molecular size marker (M).

had been divided into two aliquots which had been processed separately. As shown in Fig. 2A, both of these samples were positive. RNA samples of two uninfected controls were negative.

3.3. Viral RNA in cells of the peripheral blood of naturally infected animal species

The presence of BDV p40-specific RNA was demonstrated in PBMC obtained from 5 out of 19 horses under investigation (Table 1). All five animals hospitalized due to clinical signs of BD had developed BDV-specific antibody titers ranging from 10 to 160. One animal was positive for viral RNA in the blood. In 8 out of the 14 clinically healthy horses, neither BDV-specific antibodies, nor BDV-specific RNA were detected (three had

Table 1
Detection of BDV-specific RNA and antibodies in blood of 19 horses

Status	*n*	BDV antibodies[a]	BDV p40 RNA
Animals with BD	1	+	+
	4	+	−
Healthy animals	8	−	−
	2	−	+
	2	+	−
	2	+	+

[a] Antibody titers ranged between 10 and 160.

been in contact with horses with BD). In two animals viral RNA was detected in cells of the peripheral blood in the absence of virus-specific antibodies (one had been in contact with diseased horses). Two animals with virus-specific antibodies were negative for BDV-specific RNA in the blood. In two of the clinically healthy horses the presence of both, virus-specific antibodies as well as viral RNA in cells of the peripheral blood was demonstrated.

None of the 25 sheep showed clinical signs of BD. The animals within the herd were analyzed upon two subsequent bleedings. After the first bleeding, BDV-specific antibodies were demonstrated in two of the animals. All PBMC samples were negative for BDV p40-specific RNA. In the second bleeding, 2 months later, two additional animals had developed BDV-specific antibodies. One animal which showed a slight increase in antibody titers (160 versus 40) now was found positive for BDV p40-specific RNA in the blood (Table 2).

3.4. *Detection of BDV p40-specific RNA in human PBMC*

BDV-specific RNA was detected in 5 out of 12 patients with schizophrenia, in 5 out of 15 patients with mood or other psychiatric disorders, and in 2 out of 13 healthy volunteers (Table 3, Fig. 2B). Samples of these healthy individuals were also tested positive for BDV RNA in an earlier examination by other investigators.

Table 2
Detection of BDV-specific RNA and antibodies in blood of 25 clinically healthy sheep

	n	BDV antibodies[a]	BDV p40 RNA
First bleeding	23	−	−
	2	+	−
Second bleeding	21	−	−
	1	+	+
	3	+	−

[a] Antibody titers ranged between 20 and 160.

Table 3
Detection of BDV-specific RNA and antibodies in human PBMC

Status	$\sum n$	n	BDV antibodies[a]	BDV p40 RNA
Schizophrenic patients	12	2	+	+
		1	+	−
		3	−	+
		2	−	−
		4	n.d.	−
Mood and other psychiatric disorders	15	1	+	−
		5	−	+
		9	−	−
Healthy individuals	13	2	−	+
		7	−	−
		4	n.d.	−

[a] Antibody titers ranged between 10 and 40.

BDV-specific antibodies could be demonstrated with titers from 10 to 40, which is generally lower as compared to the investigated experimentally and naturally infected animals. Among the schizophrenic patients, two showed BDV-specific antibodies as well as viral RNA in PBMC, one patient only had BDV-specific antibodies, three showed viral RNA in the absence of virus-specific antibodies, and in two patients neither BDV-specific antibodies, nor BDV-specific RNA was detected. Among the 15 patients with mood and other psychiatric disorders, one had developed BDV-specific antibodies. This patient was negative with regard to viral RNA. The presence of the viral genome in PBMC was demonstrated in five of these patients. Nine individuals were negative for both, viral RNA and virus-specific antibodies. Among the healthy blood volunteers, none had BDV-specific antibodies, but two individuals were positive for viral RNA in the blood.

3.5. Sequence analysis

To determine the specificity of the PCR products obtained, the nucleotide sequences of the amplified fragment derived from experimentally (rat) and naturally (sheep, horse) infected animals were determined and compared with the sequence of the BDV reference strains V and He-80. Sequence divergencies to the reference strains ranged between 0.7 and 5%. The sequence obtained from the He-80 infected rat showed no nucleotide changes when compared with strain He-80; when compared with strain V, however, eight nucleotide changes were observed, which resulted in a sequence divergency of 3.2%. The two sequences derived from the horses showed sequence divergency of 0.7 and 1.4% when compared with strain V, and 3.6 and 4.3% when compared with strain He-80, respectively. The sequence derived from the sheep had a divergence of 2.1 and 5.0% when compared with the reference strains. The sequence alignment including the positions of the nucleotide changes is given in Fig. 3. None of the nucleotide changes resulted in a change of the deduced amino acid sequence.

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BDV strain V   TTACGGGGAA AAGACGACGC AGCGTGATCT CACCGAGCTG GAGATCTCCT CTATCTTCAG CCATTGTTGC TCATTACTAA   80

BDV He-80      .......... .....A..A. .......... .......... .......... ....A..... .......... ..........   80
pR-1           .......... .....A..A. .......... .......... .......... ....A..... .......... ..........   80
pR-2           .......... .....A..A. .......... .......... .......... ....A..... .......... ..........   80
pH(A)-1        .......... .......... .A........ .......... ........T. .......... .......... ..........   80
pH(A)-2        .......... .......... .A........ .......... ........T. .......... .......... ..........   80
pH(B)-1        .......... .......... .A........ .......... ........T. .......... .......... ..........   80
pH(B)-2        .......... .......... .A........ .......... ........T. .......... .......... ..........   80
pS-1           .......... .......... .A........ .......... ........T. .......... .......... ........G.   80
pS-2           .......... .......... .A........ .......... ........T. .......... .......... ........G.   80


BDV strain V   TAGGGGTTGT GATAGGATCG TCGTCTAAGA TCAAAGCAGG AGCCGAGCAG ATCAAGAAAA GGTTTAAAAC TATGATGGCA  160

BDV He-80      .T........ .......... ..A....... .T........ .......... .......... .......... ..........  160
pR-1           .T........ .......... ..A....... .T........ .......... .......... .......... ..........  160
pR-2           .T........ .......... ..A....... .T........ .......... .......... .......... ..........  160
pH(A)-1        .......... .......... .......... .......... .......... .......... .......... ..........  160
pH(A)-2        .......... .......... .......... .......... .......... .......... .......... ..........  160
pH(B)-1        .......A.. .......... .....C.... .......... .......... .......... .......... ..........  160
pH(B)-2        .......A.. .......... .....C.... .......... .......... .......... .......... ..........  160
pS-1           .......... .......... .......... .......... .......... .......... .......... C.........  160
pS-2           .......... .......... .......... .......... .......... .......... .......... C.........  160


BDV strain V   GCCTTAAACC GGCCATCCCA TGGTGAGACT GCTACACTAC TCCAGATGTT TAATCCACAT GAGGCTATAG ATTGGATTAA  210

BDV He-80      .......... .......... .......... .......... .T........ .......... .......... ..........  210
pR-1           .......... .......... .......... .......... .T........ .......... .......... ..........  210
pR-2           .......... .......... .......... .......... .T........ .......... .......... ..........  210
pH(A)-1        .......... .......... .......... .......... .......... .......... .......... ..........  210
pH(A)-2        .......... .......... .......... .......... .......... .......... .......... ..........  210
pH(B)-1        .......... .......... .......... .......... .......... .......... .......... ..........  210
pH(B)-2        .......... .......... .......... .......... .......... .......... .......... ..........  210
pS-1           .......... .......... .......... ......T... .......... .......... .....C.... ..........  210
pS-2           .......... .......... .......... ......T... .......... .......... .....C.... ..........  210
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Fig. 3. Nucleotide sequence alignment of BDV p40 among BDV strain V, BDV He-80, and cDNA clones derived from BDV infected rat (pR), horse (pH), and sheep (pS). Partial p40 sequences correspond to nucleotides 407–687 of the BDV genome (antigenomic polarity). Dots indicate nucleotide identity with respect to BDV strain V.

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BDV strain V     CGGCCAACCC TGGGTAGGCT CCTTTGTGTT GTCTCTACTA A   281

BDV He-80        ......G... .......... .......... .......... .   281
pR-1             ......G... .......... .......... .......... .   281
pR-2             ......G... .......... .......... .......... .   281
pH(A)-1          .......... .......... .......... .......... .   281
pH(A)-2          .......... .......... .......... .......... .   281
pH(B)-1          .......... .......... .......... .......... .   281
pH(B)-2          .......... .......... .......... .......... .   281
pS-1             .......... .......... .......... .......... .   281
pS-2             .......... .......... .......... .......... .   281
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Fig. 3. (*Continued*).

4. Discussion

BDV is known as a neurotropic virus infecting a wide range of animal species. In vivo diagnosis of BDV infection is usually performed by the demonstration of specific antibodies. Several investigators reported the detection of BDV-specific RNA in the blood of naturally infected horses, sheep, cattle, and cats (Bahmani et al., 1996; Nakamura et al., 1996; Hagiwara et al., 1996, 1997; Reeves et al., 1998). Positive RT-PCR results were reported from cells of the peripheral blood isolated from humans with psychiatric disorders, but also from healthy individuals (Bode et al., 1995; Kishi et al., 1995a; Sauder et al., 1996; Takahashi et al., 1997; Iwata et al., 1998). Because these observations could not be confirmed by others (Kubo et al., 1997; Lieb et al., 1997; Richt et al., 1997) they were a matter of debate. Therefore, the establishment of a sensitive, reliable and reproductive assay to confidently assess the prevalence of BDV in blood cells of infected individuals is necessary. We investigated cells of the peripheral blood from experimentally and naturally BDV-infected animals and man for the presence of viral RNA. The assay used differs from published protocols by the RNA isolation method, by the selected primer sequences, and by the use of a One Tube RT-PCR system. So far, target cells of BDV in the peripheral blood are not sufficiently characterized. Recently, the granulocyte fraction of psychiatric patients was reported to harbor infectious virus (Planz et al., 1999). After collection of blood using EDTA, we preferred the isolation of RNA from whole blood using a silica based purification system and thereby excluded substances (e.g. heparin, phenol/chloroform, Fe^{2+}) known to inhibit enzyme activities (Weitjens et al., 1996).

Consistent with the replication strategy of negative stranded RNA viruses, there is evidence that BDV p40 mRNA is among the most abundant viral transcripts present in infected cells and tissues (Banerjee et al., 1991; Conzelmann, 1998). Therefore, we focused in subsequent investigations on the p40 coding region. Random hexanucleotides have been shown to reduce the sensitivity of the assay (Sauder and de la Torre, 1998). To detect also virus which is not transcriptionally active, we used specific primers annealing to both, genomic RNA and p40 mRNA in the RT-reaction. For diagnostic purposes, generation of PCR products of considerably more than 300 bp, as used in other investigations (Zimmermann et al., 1994; Richt et al., 1997), is not recommended (Dieffenbach et al., 1995). Even the 449 bp fragment generated by primers p40 s and p40 as is rather long, but it had been chosen to enable the construction of the BDV p40-comp

RNA standard. The application of a One Tube RT-PCR system allows the amplification of the synthesized cDNA without any loss of templates. Experiments using standardized reaction conditions showed that the efficiency of amplification also depends on the type of the cycler used (unpublished observations).

Using the BDV p40-wt RNA standard as well as the BDV p40-comp RNA standard, we were able to show that the assay is at least as sensitive as other published BDV-specific RT-PCR assays (Iwata et al., 1998; Sauder and de la Torre, 1998), since 10 RNA molecules, synthesized in different laboratories, were detected reproducibly. Minor differences observed in the detection of the RNA standards may be considered within the margin of experimental variation.

The nested RT-PCR was performed under strict conditions, with local separation of RNA isolation, preparation of the reaction buffers, amplification and product analysis. Each RT-PCR was screened for contaminations routinely using negative reagent controls and RT-dependent amplification controls. All of these controls were negative. In addition, the risk of contaminations was significantly reduced by using BDV p40-comp RNA instead of wild type RNA as the performance control, and by the application of the One Tube RT-PCR system.

Neurons are the primary target cells of BDV within the CNS. The distribution of BDV outside of the CNS has not been intensively studied. Obviously, the immune system has a considerable influence on the viral dissemination. During infection, BDV disseminates intra-axonally in peripheral nerves (Carbone et al., 1987; Morales et al., 1988). In experimentally infected adult rats, BDV was only detected in peripheral nerve endings, but not in parenchymal cells of visceral organs, with the exception of medullar cells of the adrenal gland (Narayan et al., 1983; Gosztonyi and Ludwig, 1984; Carbone et al., 1987). In perinatally infected rats, however, BDV spread more readily and was demonstrated in parenchymal cells of different organs and exocrine glands. In these animals BDV was excreted in urine (Herzog et al., 1984; Morales et al., 1988).

In our experiments, BDV p40-RNA was detected in cells of the leukocyte-fraction of the peripheral blood in a small number of investigated adult mice as well as in immunocompetent and immunosuppressed rats. These results indicate that detection of p40 RNA in blood is not only possible in persistently infected newborn rats (Sierra-Honigmann et al., 1993) but also that the spread of BDV in blood might be an additional way of viral distribution throughout the body. In persistently infected newborn rats, the number of BDV-infected cells in the peripheral blood has been estimated to be between one cell in 10^5 or 5×10^6 cells, respectively (Rubin et al., 1995; Sauder and de la Torre, 1998). In rats infected as adults and in naturally BDV-infected animals, the number of infected cells might even be lower and therefore, a single RT-PCR (Kubo et al., 1997) might not be sensitive enough to detect such small amounts of viral RNA.

BDV-specific RNA was detected in the blood of naturally infected horses, sheep and man. The sheep were investigated by two bleedings, in spring and summer, the seasons when most BD cases are observed. The number of animals with BDV-specific antibodies increased from two to four, and one animal which showed a slight increase in antibody titers (160 versus 40) was positive for viral RNA in the peripheral blood in the second bleeding. None of the animals showed clinical signs of BD, despite the fact that these animals had been in contact with the virus within the period of observation.

Unfortunately, the peripheral blood cell RT-PCR positive animal was removed from the herd without our knowledge prior to further investigations. It would have been of great interest, whether this animal, which obviously had an active BDV replication also sheds the virus via secretions to the environment.

The highest antibody titers were found in the blood of the experimentally infected mice and rats, whereas generally low antibody titers were detected in the human blood samples. High antibody titers detected in experimentally BDV-infected mice were also reported by others (Rubin et al., 1993).

Sequence conservation between known tissue culture isolates and RT-PCR products derived from cells of the peripheral blood have been used as evidence to argue that they might represent contaminants. However, the finding of sequence conservation is consistent with previous analyses of well-characterized isolates disparate by host species and geography (Binz et al., 1994; Schneider et al., 1994; Lipkin et al., 1997). We performed sequence analysis of the obtained short nested PCR-products of 281 bp, and verified the specificity of the PCR-products. The sequence divergencies ranged from 0.7 to 5% when compared with the reference strains V and He-80. This is in accordance with the general finding of sequence divergencies among BDV field isolates of more than about 2%. In addition, we lowered the risk for contaminations by the use of a competitive RNA-template as positive control and also performed reagent and RT-dependent amplification controls, which all remained negative.

The human PBMC samples collected in different geographical regions of Germany were obtained and investigated in the framework of a double-blinded multicenter study. The detection of viral RNA in these samples was not consistent among the participating laboratories. Due to the use of different protocols for RNA isolation, reverse transcription and amplification, a number of technical details, as described above, might explain these discrepancies.

Analyzing blood samples derived from horses and men we recognized individuals which either (i) had BDV-specific antibodies in the absence of viral RNA, (ii) were positive for viral RNA in the absence of BDV-specific antibodies, or (iii) were positive for both, BDV-specific antibodies and viral RNA. At present, the reasons for these conditions, only observed under natural conditions of infection (Kishi et al., 1995b; Bahmani et al., 1996; Sauder et al., 1996; Berg et al., 1999) remain unknown. It may be speculated that they are due to (i) different routes of infection, (ii) the amount of virus at infection (iii) delayed onset of the immune response after infection, (iv) immunotolerance at infection early in life. The investigation of brain and bone marrow of healthy individuals harboring BDV-specific RNA in cells of the peripheral blood, without BDV-specific antibodies, would allow more insights in the mechanisms involved.

5. Conclusions

To confidently assess the prevalence of BDV in cells of the peripheral blood, sensitive, reliable and reproducible assays are necessary. We have detected BDV p40-specific RNA in cells of the peripheral blood in experimentally infected mice and rats, in naturally infected horses and sheep, as well as in PBMC derived from humans with various

psychiatric disorders and from healthy individuals. The selection and the numbers of animals and men under investigation does not allow to draw conclusions for the prevalence of BDV-specific RNA in blood cells of these species, but may point to an incidental but relevant role of blood for the spread of BDV in the infected organism, as well as for the transmission of the virus to other individuals.

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