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Detection of Borna disease virus RNA in naturally infected animals by a nested polymerase chain reaction

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Summary

Borna disease virus in naturally infected horses, a donkey and sheep was detected for the first time by amplification of viral RNA using PCR. In contrast to a control group of healthy horses, brain tissue was positive by this assay in all animals with neurological symptoms. The use of a second round of PCR with nested primers following Southern hybridization confirmed the specificity and increased the sensitivity of the test. Comparison with conventional methods recommends this technique for monitoring of BDV infections at a molecular level.

Borna disease virus (BDV); Reverse transcriptase-PCR; RNA extraction

Introduction

Borna disease (BD) in naturally infected animals (horses and sheep) is characterized by a clinically defined neurological syndrome, starting with behavioural alterations, loss of coordination, followed by a severe general disease and death (Zwick, 1939). Sporadic occurrence in endemic areas of Central Europe is still observed. This persistent virus infection of the central nervous system (Ludwig et al., 1988) has been studied in many experimental models (Ludwig et al., 1885). The diagnosis of natural and experimental infection is mainly based on histopathology with perivascular infiltrates in the limbic system (Zwick, 1939), intranuclear Joest-Degen inclusions (Joest and

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Degen, 1909; Gosztonyi et al., 1993), specific antigen in the brain (Sprockhoff, 1956; Gosztonyi and Ludwig, 1984; Ludwig et al., 1985; Carbone et al., 1987; Ludwig et al., 1988) and viral antibodies (Ludwig and Thein, 1977; Ludwig et al., 1993).

The isolation and preliminary characterization of cDNA clones inferred to encode the 24 kD and 40 kD protein, respectively, enabled further characterization of the virus (Lipkin et al., 1990; VandeWoude et al., 1990). Infectious viral particles contain a negative polarity 8.5 kb RNA which is transcribed in the nucleus of the cells (Briese et al., 1992).

Using the sequence information of the cDNA clone encoding the 40 kD protein (Lipkin et al., 1990, McClure et al., 1993), we adapted the polymerase chain reaction (PCR) to search for BDV RNA in naturally infected animals. The isolation of RNA from tissue samples obtained under field conditions with sufficient quality for PCR and the use of a second nested primer pair opens new possibilities for a rapid and sensitive detection of BDV in animals.

Materials add Methods

Animals and tissue samples

Horses, sheep and a donkey with classical clinical signs of Borna disease (Ludwig et al., 1985) originating from endemic areas in Saxony, Thuringia and Saxony-Anhalt, Germany, were used for this study. The animals were autopsied under field conditions, tissue samples were collected from different brain regions and stored at $-20°C$, $-70°C$ and in 4 M guanidinium thiocyanate at $-70°$, respectively (see Table 1).

Tissue culture and virus assay

Young rabbit brain (YRB) cells were grown in EDM (Flow Laboratories) supplemented with 5% fetal calf serum, and kept at 5% CO_2. For infectivity studies the second passage of the cells was seeded in 24-well tissue culture plates (Nunc). Infection was done with a 10% (w/v) suspension of the tissue samples in EDM, which had been sonicated with a Branson sonifier (20 cycles/min, 40 mA) for 2 min (Gosztonyi and Ludwig, 1984; Ludwig et al., 1993). In order to estimate the sensitivity of the RT-PCR assay YRB cells cultivated in 100 mm culture plates (Nunc) were infected with 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} focus forming units (ffu) of BDV, respectively. The cells were harvested 48 hours later and used for RNA extraction.

Indirect Immunofluorescent Test (IFT)

YRB cells infected after one passage with 5×10^{-3} ffu of BDV per cell were seeded on coverslips. The cells were fixed 5 days later with cold acetone and

stored at −20°C before use. The IFT was carried out as described earlier (Bode et al., 1992; Ludwig et al., 1993). In brief, the cells were incubated simultaneously with equal volumes of horse serum and the BDV-specific monoclonal antibody KFu2 diluted 1:100 in PBS with 1% FCS. After washing, the cells were incubated with FITC labelled goat anti-horse IgG (Dianova) and TRITC labelled F(ab')2 rat anti-mouse IgG (Dianova). The fluorescent staining of the cells was examined with a Zeiss ICM 405 microscope.

RNA extraction

RNA extraction was carried out according to Chomczynski and Sacchi (1987) with some modifications. Briefly, immediately after thawing the tissue was minced on ice and homogenized with the denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Sequentially, 2 M sodium acetate, pH 4, phenol and chloroform-isoamyl alcohol were added to the homogenate. Samples were centrifugated at $15\,000 \times g$ for 20 min, and the aqueous phase was used for precipitation of the RNA by isopropanol and ethanol. After determination of the concentration of the RNA, the nucleic acid was stored in 70% ethanol at −70°C. The quality of the RNA was determined by electrophoresis. RNA preparations (2 μg) were electrophoresed in 1% agarose gels containing formaldehyde and stained with ethidium bromide (Sambrook et al., 1989).

Selection of primers, probe and reagents

The oligonucleotides were designed according to published sequences from the cDNA clone pAB5 encoding the 40 kD protein (Lipkin et al., 1990; McClure et al., 1993) using the computer program OLIGO, version 4.0, and were synthesized in a Gene Assembler Plus (Pharmacia LKB). The following nucleotide sequences were chosen:

External primers:
antisense 5'-CTT CTT ACT CCA GTA AAA CGC-3'
sense 5'-GTC ACG GCG CGA TAT GTT TC-3'

These oligonucleotides were also used to prime selectively either the positive-strand viral mRNA or the negative-strand viral genomic RNA in the reverse transcriptase (RT) reaction.

Internal (nested) primers:
antisense 5'-ATT CTT TAC CTG GGG ACT CA-3'
sense 5'-GCC TTG TGT TTC TAT CTT TC-3'
β-actin primers:
antisense 5'-GTG TGG TGC CAA ATC TTC TCC-3'
sense 5'-GCG CTC GTC GTC GAC AAC GG-3'

For Southern hybridization a 800-bp *Eco*RV insert of the plasmid pAB5 was used as the probe.

The M-MLV reverse transcriptase and the corresponding buffer system were obtained from Life Technologies. All PCR reagents including nucleotides, 10 x reaction buffer and Taq polymerase (Amplitaq) were obtained from Perkin-Elmer Cetus.

RT-PCR ASSAY

Prior to cDNA synthesis the RNA (2 μg) was incubated at 75°C for 4 minutes, spun briefly and placed on ice. The reverse transcriptase reaction was performed in a final volume of 20 μl containing 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.5 mM each dNTP, 100 pmol of the appropriate external primer, 1 unit RNasin, and 10 units M-MLV reverse transcriptase at 37°C for 90 minutes. After the reverse transcriptase reaction the enzyme was inactivated by heating at 90°C for 10 min.

Non-nested PCR

PCR amplifications were carried out in 50 μl volumes containing 2–10 μl of the RT reaction. For determination of the sensitivity of the PCR serial dilutions of the plasmid pAB5 were subjected to PCR. Reaction conditions consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM of each primer, and 0.5 unit of Taq polymerase. The samples were processed through 35 cycles of 2 min at 94°C, 2 min at 54°C, and 2.5 min at 72°C, with a final extension of 10 min at 72°C.

Nested PCR

Reaction mixtures were made as above, although the samples were processed through 22 cycles only for the first PCR. Two microliters of the first reaction were used for a second round of PCR with the nested primer pair for an additional 22 cycles.

Visualisation of the PCR products

One fifth (10 μl) of each reaction volume was analysed in a 1% agarose gel in the presence of 2 μg/μl ethidium bromide. Gels were visualized and photographed under illumination using a wavelength of 302 nm (Sambrook et al., 1989).

Nucleic acid hybridization

A further identification of the PCR products was made by nucleic acid hybridization. The insert of the plasmid pAB5 was labelled with α-^{32}P dCTP

(spec. Activity 3000 Ci/mMol, Amersham) using a random labeling primers kit (Life Technologies). Southern blotting and hybridization were carried out according to standard conditions as described elsewere (Sambrook et al., 1989).

Results

Sensitivity of the assay

Dilutions of plasmid pAB5 encoding for the 40 kD protein of BDV were amplified to assess the sensitivity of PCR amplification. PCR amplification with the external primer pair for 35 cycles of as little as 60 molecules (0.5fg) of the pAB5 DNA yielded a visible 750-bp band (Fig. 1A, lane 9). The use of a second round of PCR and Southern hybridization increased the sensitivity up to a detection limit of 10 molecules (Fig. 1C, lane 11).

In order to determine the sensitivity of the RT-PCR assay, total RNA was isolated from YRB cells infected with different multiplicities and serial dilutions of the RNA (1:10, 1:100, 1:1000) were subjected to RT-PCR. RNA from cells infected with 0.001 ffu/cell was successfully amplified with the PCR (Fig. 1A, lane 4). The use of a second round of PCR with the nested primer pair following Southern hybridization with pAB5 increased the sensitivity up to 100-fold (0.0001 ffu/cell, RNA diluted 1:10, Fig. 1C, lane 6,).

Detection of BDV RNA in diseased animals

Tissue samples from 36 diseased animals (32 horses, 1 donkey and 3 sheep)

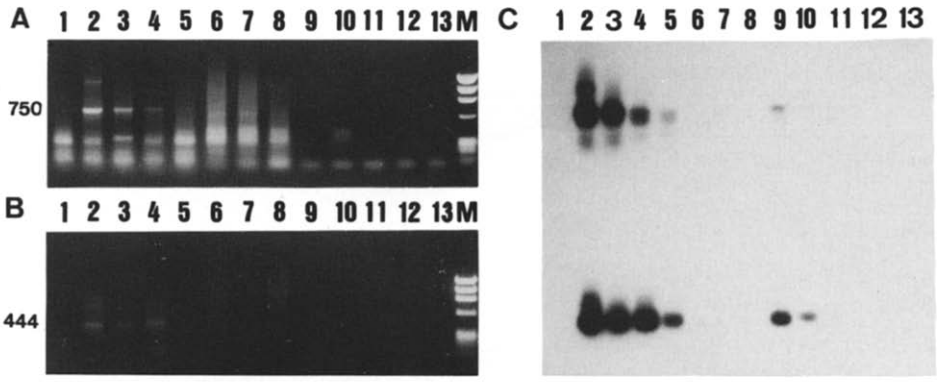

Fig. 1. PCR analysis of serial dilutions of pAB5 DNA and RNA extracted from YRB cells infected with BDV. (A) Non-nested PCR using the external primer pair, (B) nested PCR and (C) Southern hybridization of A and B. 1 uninfected YRB cells; 2 YRB cells infected with 10^{-1} ffu/cell; 3 10^{-2} ffu/cell; 4 10^{-3} ffu/cell; 5 10^{-4} ffu/cell; 6 RNA from 5 diluted 1:10; 7 RNA from 5 diluted 1:100; 8 RNA from 5 diluted 1:1000; 9 60 molecules of pAB5; 10 30 molecules of pAB5; 11 10 molecules of pAB5; 12 5 molecules of pAB5; 13 2 molecules of pAB5; M DNA marker $\times$-174-RF/*Hae*III.

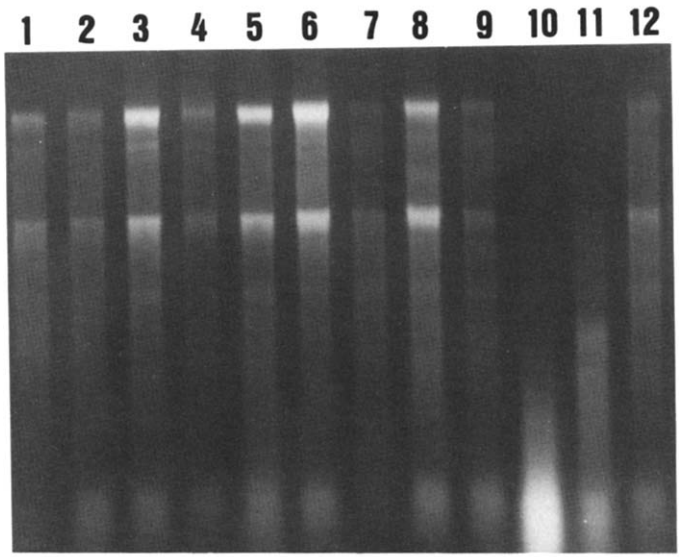

Fig. 2. Electrophoresis of RNA extracted from tissue samples stored under different conditions. If not specified, the tissue samples were cortex tissue. Samples were stored at −20°C (1–4), in 4 M guanidinium thiocyanate at −70°C (5–8), and at −70°C (9–12). 1 horse 34 med/91; 2 horse 95med/91; 3 sheep 111med/91; 4 horse 149med/91; 5 horse 06/91; 6 horse Jena 1; 7 sheep Jena 2; 8 horse B1; 9 donkey 176med/92; 10 donkey 176med/92, retina; 11 horse S1062/92, nasal mucosa; 12 horse 51062/92.

collected between February 1991 and June 1992 in areas endemic for BD were included in this study. Samples from 3 healthy horses and 4 horses with an anamnestic report of lameness from the same area served as negative controls.

TABLE 1

Detection of BDV infections in naturally diseased animals by different techniques

	Species/tissue[a]	IFT	Virus assay	PCR	Storage
34med/91	horse	–	+	+	
95med/91	horse	+	+	+	–20°C
111med/91	sheep	+	–	+	
149med/91	horse	+	–	+	
06/91	horse	–	+	+	
Jena 1	horse	+	–	+	GTC[b]
Jena 2	sheep	–	–	+	–70°C
B 1	horse	–	–	+	
176med/92	donkey	+	+	+	
176/med/92	donkey/retina	ND	–	–	–70°C
S1062/92	horse	ND	+	+	
S1062/92	horse/nasal nucosa	ND	+	+	

[a]If not specified, the tissue samples were cortex tissue. [b]Samples stored in 4 M guanidinium thiocyanate at –70°C. ND = not done.

All 36 animals with symptoms of BD were positive for viral RNA whereas in the control group no specific amplification could be detected. These results were compared to those obtained with two conventional diagnostic methods, the IFT and the virus assay. Whereas in the brain of all diseased animals BDV-specific RNA could be found, only in approximately 30% of the cases antibodies detectable by IFT or infectious virus could be demonstrated (detailed data not shown).

Clearly differences in the amount of amplified sequences could be shown in some of the tissue samples (see Fig. 3). The reason for this is not due to the varying quality of the RNA obtained from such tissues collected and stored under non-optimal conditions. A comparison of RNAs extracted from tissue samples which had been stored under different conditions is shown in Fig. 2. In 10 of 12 cases RNA with a sufficient quality (visible bands of the ribosomal RNAs) could be isolated independendly from storage conditions (see Table 1).

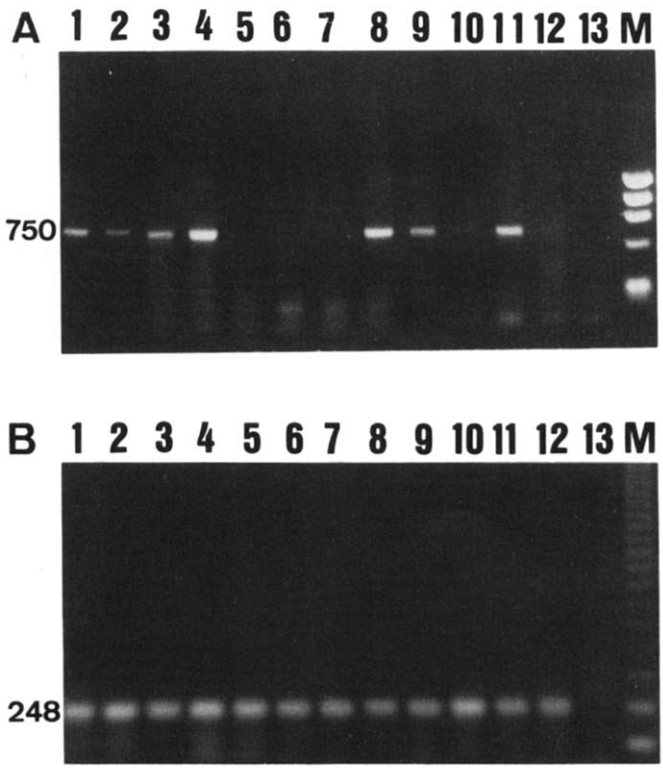

Fig. 3. (A) Detection of BDV-specific mRNA in tissue samples of different infected and control animals. If not specified the tissue samples were cortex tissue. (B) Amplification of a 248-bp (β-actin band as an internal control. 1 horse 95/92; 2 horse 34med/91; 3 horse 104/92; 4 horse B1; 5–7 healthy control horses; 8 sheep 111med/91; 9 donkey 176med/92, hippocampus, right hemisphere; 10 donkey 176med/92, hippocampus, left hemisphere; 11 donkey 176med/92, olfactory bulb, right hemisphere; 12 donkey 176med/92, olfactory bulb, left hemisphere; 13 buffer control; M DNA marker $\times$-174-RF/*Hae*III.

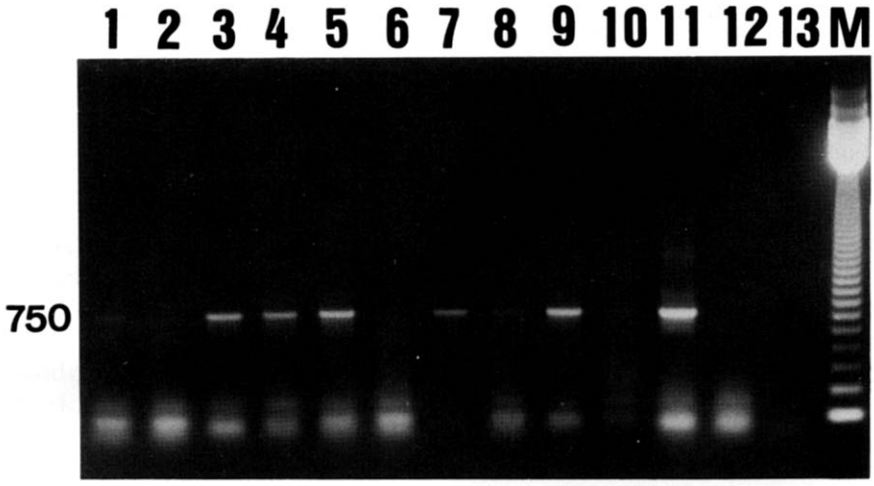

Fig. 4. Amplification of BDV-specific mRNA in different tissue samples from the right (1, 3, 5, 7, 9, 11) and the left brain hemisphere (2, 4, 6, 8, 10, 12) of the brain of donkey 176med/92. 1 and 2 cortex; 3 and 4 cerebellum; 5 and 6 hippocampus; 7 and 8 white substance, lateral ventrical; 9 and 10 nucleus caudatus; 11 and 12 olfactory bulb; 13 buffer control; M DNA marker 123-bp ladder.

The degradation of the RNA in lane 10 (retina of donkey 176 med/92) resulted in the failure of amplification of BDV RNA in this tissue sample, whereas the partial degradation of the RNA in lane 11 (nasal mucosa of horse 1062/92) had no influence on the amplification of BDV sequences (see table 1). To exclude the possible influence of degraded RNA, the amplification of a 248-bp β-actin mRNA served as an internal control.

The external antisense and sense oligonucleotides were used to prime selectively either the positive-strand viral mRNA or the negative-strand viral genomic RNA in the RT reaction. A 750-bp band is seen in all diseased animals using the antisense primer in the RT reaction for the selective amplification of BDV mRNA (Fig. 3A). The length of the PCR product correlates with the theoretical length derived from the sequence data (McClure et al., 1993). No amplification products were detected in control animals (Fig. 3A, lanes 5, 6, 7).

Differences in the level of BDV-specific amplification in samples collected from the right and left hemisphere (olfactory bulb, hippocampus) from donkey 176med/92 (Fig. 3A, lanes 9–12) with almost equal ampification of β-actin mRNA (Fig. 3B) initiated the analysis of further brain areas. It was of interest that all samples from the right hemisphere had higher amounts of mRNA then the ones from the left side (Fig. 4). The BDV mRNA expression reached its peak in samples from the limbic system (hippocampus), the nucleus caudatus, and the olfactory bulb as demonstrated in lanes 5, 9, 11.

In order to determine the amount of viral genomic RNA in the same samples the external sense primer was used to prime the RT reaction. The use of the sense primer resulted in an amplification of BDV RNA at a distinctly decreased

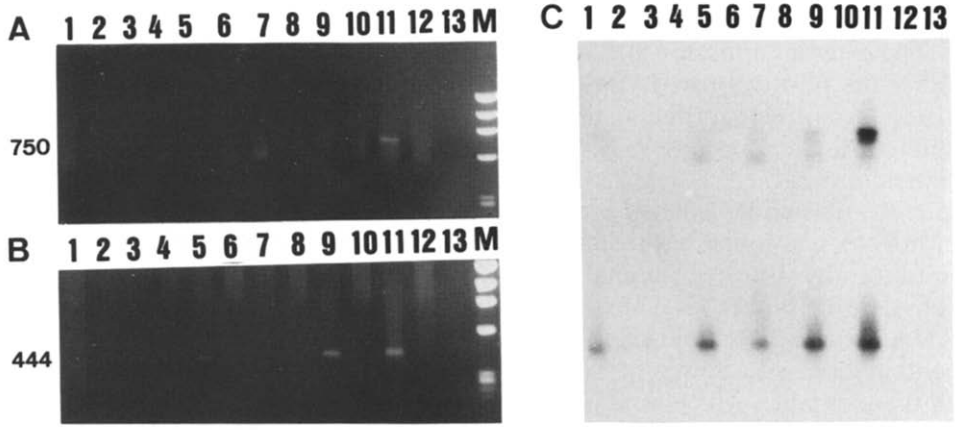

Fig. 5. Detection of viral genomic RNA in tissue samples from the right and left hemisphere of the brain of donkey 176med/92. (A) RT-PCR using the external primer pair resulting in the amplification of a 750-bp band. (B) Nested PCR amplification of a 444-bp fragment. (C) Southern hybridization of A and B. 1 and 2 cortex; 3 and 4 cerebellum; 5 and 6 hippocampus; 7 and 8 white substance, lateral ventrical; 9 and 10 nucleus caudatus; 11 and 12 olfactory bulb; 13 buffer control; M DNA marker $\times$-174-RF/*Hae*III.

level. Only in the right olfactory bulb a weak 750-bp band could seen (Fig. 5A, lane 11). Analysis of these samples in a second round of PCR using the nested primer pair revealed the amplification of the expected 444-bp BDV-specific band in almost all samples from the right hemisphere (Fig. 5B).

The specificity of the PCR was examined by Southern blotting the amplified DNA with hybridization to the ^{32}P-labelled 800-bp insert of plasmid pAB5 (McClure et al., 1993). As shown in Fig. 5C, this step increased the sensitivity of the technique enabling the detection of viral genomic RNA even in some brain samples from the left hemisphere (lanes 2, 8, 12).

Discussion

The PCR has been applied to study several animal viruses, e.g., equine herpesvirus type 1, pseudorabies virus and FMDV (Ballagi-Pordány et al., 1990; Lokensgard et al., 1991, Laor et al., 1992). Nucleotide sequence data of the cDNA clone pAB5 which encodes for the 40 kD protein of BDV (Lipkin et al., 1990; McClure et al., 1993) were the basis for the development of a highly sensitive RT-PCR assay in the Borna-system. Since the molecular information is based on a cDNA clone derived from infected rat brain of an adapted strain (Lipkin et al., 1990), we established the assay system using naturally infected animals with a defined disease. In all animals with BD-symptoms BDV-specific amplification was could be demonstrated. Failure of amplification in some of these samples (eg. retina of donkey 176med/92) is probably caused by the difficulties in obtaining RNA with sufficient quality under the conditions used

for taking and storage of the samples. Under the assay conditons used, specific amplification was not observed in any of the control horses.

The external antisense and sense primers permit a selective amplification of either the positive-strand viral mRNA or the negative-strand genomic RNA. Using the antisense primer a high amplification of BDV mRNA was obvious in almost all tissue samples. In the majority of the diseased animals mRNA expression reached its highest level in the limbic system. It is of considerable interest, that under otherwise constant conditions, an asymmetric distribution of mRNA was found in the infected donkey brain. An application of this PCR to naturally infected animals also shows, that mRNA is present at a considerable higher level than genomic RNA. The low amount of the latter RNA became detectable using a second round of PCR with nested primers. In combination with Southern hybridization using ^{32}P-labelled probes in almost all tissue samples which were positive for BDV mRNA genomic RNA was also detected. The minimum detection level of the assay in infected YRB cells is defined by a specific amplification in 200 ng RNA from cells infected with 10^{-4} ffu. For a most sensitive detection of a BDV infection, tissue samples should be taken from the limbic system and the olfactory bulb of both hemispheres of the brain. The RNA should be subjected to RT-PCR and an additional nested PCR with Southern hybridization.

In comparison with antibody detection and infectivity assay in tissue culture, which often failed the above technique was succesful in all clinically and histologically diagnosed cases, even when material was collected under suboptimal field conditions.

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