Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
Molecular and Cellular Probes 21 (2007) 47–55
Molecular and Cellular Probes
www.elsevier.com/locate/ymcpr

Reverse transcription real-time PCR assays for detection and quantification of Borna disease virus in diseased hosts

A.R. Schindler[a,1], A. Vögtlin[b], M. Hilbe[a], M. Puorger[a], K. Zlinszky[a], M. Ackermann[b,*], F. Ehrensperger[a]

[a]*Institute of Veterinary Pathology, University of Zurich, Winterthurerstrasse 266a, CH-8057 Zurich, Switzerland*
[b]*Institute of Virology, University of Zurich, Winterthurerstrasse 266a, CH-8057 Zurich, Switzerland*

Received 10 June 2006; accepted 3 August 2006
Available online 30 August 2006

Abstract

Borna disease is a severe, immunopathological disorder of the central nervous system caused by the infection with the Borna disease virus (BDV). The detection of BDV in diseased animals, mainly sheep and horses, is achieved by histological, immunohistochemical and serological approaches and/or PCR-based technologies. In the present study, reverse transcription, real-time PCR assays were established for the detection of BDV in the brain tissue from sheep and horses, using loci for the p40 (nucleoprotein) and the p24 (phosphoprotein) genes. The PCRs were equally specific and sensitive, detecting 10 target molecules per reaction and one BDV-infected cell among 10^6 non-infected cells. In tissues from BDV-diseased sheep and horses, the p24 target was detected at higher abundance than for p40. Therefore, the p24 test is suggested to be of higher value in the diagnostic laboratory. However, both assays should be useful for addressing questions in pathogenesis and for detecting BDV reservoirs in endemic areas.

Keywords: Borna disease virus; Reverse transcription; Real-time PCR

1. Introduction

Borna disease virus (BDV) is a neurotropic virus infecting a variety of mammalian hosts [1–5], potentially even humans [6–17]. The virus establishes a non-cytolytic, persistent infection, which frequently results in a severe immunopathological disorder of the central nervous system (CNS). Although sheep and horses appear to be the main hosts, many fundamental questions regarding the epidemiology of BDV remain unanswered, for example, the modes of transmission and the involvement of vectors.

BDV is the only member of the family *Bornaviridae* within the order *Mononegavirales*, comprising enveloped, non-segmented, single- and negative-stranded RNA viruses and includes the *Filoviridae*, *Paramyxoviridae*, and *Rhabdoviridae* [18]. Replication and transcription of the BDV genome occur in the cell nucleus. The genome size is 8.9 kb, which makes it the smallest of all known negative-stranded RNA viruses [19,20]. The organization of the six open reading frames (ORFs) is similar to that of other Mononegaviruses [20]. Based on their positions in the viral genome (3′-N–X–P–M–G–L-5′) and the features of the corresponding amino acid sequences, these polypeptides are counterparts of the nucleoprotein (N), phosphoprotein (P), matrix protein (M), surface glycoprotein (G) and polymerase (L), respectively, which are also found in other negative-stranded RNA viruses [21]. Negative-stranded RNA viruses initiate infection by introducing their genetic material in the form of ribonucleoprotein complexes into host cells. These complexes, which consist of the single-stranded RNA genome associated with the RNA-binding protein N, the polymerase cofactor P and the RNA-dependent RNA polymerase L, are transcriptionally active

*Corresponding author. Tel.: +41 44 635 87 01; fax: +41 44 635 89 11.
E-mail address: email@vetvir.unizh.ch (M. Ackermann).
[1]Present address: The University of Melbourne, Veterinary Science, 250 Princes Highway, Werribee 3030, Victoria, Australia.

0890-8508/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2006.08.001

and direct the synthesis of viral mRNAs [22]. Both the composition and stoichiometry of the nucleocapsid N and P are involved in the regulation of the BDV multiplication cycle [23–25].

Intra vitam diagnosis of Borna disease (BD) is difficult. Sheep and horses with BD exhibit a range of signs [26]. For instance, increased protein content and mononuclear pleocytosis may be seen in cerebrospinal fluid (CSF) due to meningoencephalitis. However, these findings are not specific for BD, rather represent non-specific indicators for viral meningoencephalitis [26]. BDV-specific antibodies in serum and or CSF are considered useful indicators, but post-mortem confirmation by histological and immunohistochemical analysis of brain tissues is required. Histologically, variable degrees of encephalitis are observed with BD [27–29], characterized by disseminated mononuclear polioencephalomyelitis, with subsequent neuronal degeneration and meningitis. Usually, areas of the limbic system, particularly the hippocampal formation, are involved, whereas the brain stem and cerebellum remain relatively unaffected [28]. Immunohistochemical analyses of paraffin-embedded brain sections using monoclonal antibodies to the major BDV antigens P38/40 (N) and P24 (P) have shown consistently that virus-infected cells are not uniformly distributed in brains from diseased animals and that antigen-positive neurons are detected most frequently in the hippocampus [3,27,28].

Recently, there has been progress on developing improved complementary tools for the specific diagnosis of BDV infection. Serological assays, such as the enzyme-linked immunosorbent assay (ELISA) [30], and molecular methods based on nucleic acid hybridization [31] or on the PCR have been evaluated for the detection of viral transcripts [2,26,30–33]. For example, highly sensitive conventional PCR assays have been developed for the detection of BDV in unfixed or formalin-fixed brain tissue [2,32,33]. While presently these PCRs are useful, they have the disadvantage that it is a 'nested' assay [33] and, for routine application, is thus prone to cross-contamination problems during the conduct of the procedure. Therefore, laboratory diagnosis, relying exclusively on RT-nested PCR results should be evaluated and appraised with caution [5]. To overcome this limitation, the present study established a rapid and effective reverse-transcription, real-time PCR for the detection and quantification of the nucleoprotein (P38/40) and the phosphoprotein (P24) transcripts of BDV in brain tissue samples from sheep and horses with BD. Furthermore, highly sensitive and specific BDV-assays are important for the detection of inapparently infected animals and epidemiological studies regarding possible vectors.

2. Material and methods

2.1. Tissue samples

Ovine and equine brain samples were collected during autopsies at the Institute of Veterinary Pathology, University of Zurich. Specifically, samples from the cortex, hippocampus and cerebellum (or lobus piriformis, if no cerebellum was available) were collected from six previously BDV-diagnosed animals (three sheep and three horses) from the years 1996 to 2003. As negative controls, brain samples from two healthy, assumingly uninfected animals (one sheep and one horse) were also collected. From one-half of the brain, tissue samples (1 cm^3) were collected using a scalpel, stored in Petri dishes at −20 °C or −70 °C. The other half of the brain was fixed by immersion in 4% buffered formaldehyde for at least 48 h, sliced, trimmed and stained using haematoxilin–eosin (HE) for microscopic examination.

2.2. Immunohistochemical examination of tissue samples

Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated in xylene and alcohol. Sections were then counter-stained with haemalaun for 2 min and washed in H_2O for 3–5 min. The following incubations were at ambient temperature and, after each step, slides were thoroughly washed with phosphate-buffered saline, pH 8.0 (PBS). After digestion with 0.05% Pronase (DAKO Cytomation, Copenhagen, Denmark) for 10 min, endogenous peroxidase was blocked with H_2O_2 (3% in H_2O and supplemented with 0.2% NaN_3) for 10 min. Separate sections were incubated for 12 h in a 1:1000 dilution of monoclonal mouse-anti-BDV antibody which recognizes the proteins P38/40 (L. Stitz, Tuebingen Germany) or a 1:500 dilution of a monoclonal antibody against protein P24 (L. Stitz). Subsequently, sections were washed and incubated with a secondary anti-mouse/anti-rabbit-biotinylated antibody and then with streptavidin-peroxidase (both from the ChemMateTM-KIT, DAKO Cytomation) for 15 min each. Finally, the reaction was developed using AEC substrate (AEC-substrate chromogen kit, Zymed, San Francisco, CA, USA, 00-2007).

2.3. Cells and plasmids

Uninfected and BDV-infected MDCK cells (ATCC No. CCL-34) were grown at 37 °C using Iscove's Modified Dulbecco's Medium (IMDM), which consisted of 500 ml IMDM, of 50 ml 10% fetal bovine serum, 12.5 ml of 1 M HEPES buffer, 5 ml of 200 mM L-glutamine, and 5 ml penicillin–streptomycin solution (all from Sigma, St. Louis, USA). An aliquot of the cell suspension was stained with trypan blue and counted in a Neubauer chamber in order to normalize cell numbers for RNA isolation. BDV-infected MDCK cells (BDV isolate of the horse H1766) were a gift from Dr. S. Herzog, Institute of Virology, University of Giessen, Germany. Two plasmids containing the *p40* ORF (pI_pPG401:p40-dHLX-ABP-6xHis and pI_pCRII-ORFI) as well as a plasmid containing the *p24* ORF (pI_pATH-ORFII:BDV ORFII) were gifts from Dr. Philip Grob, Institute of Virology, University of Zurich.

2.4. RNA extraction and reverse transcription

Total RNA was extracted from either Porcine epidemic diarrhea virus (PEDV, coronavirus)-infected Vero cells [34], BDV-infected MDCK cells or vaccine suspensions containing either a mixture of canine distemper virus (CDV, strain Onderstepoort), canine adenovirus type 2 (CAV-2, strain Manhattan), canine parvovirus type 2 (CPV-2, strain 154), and canine parainfluenzavirus type 2 (PIV-2, strain Cornell) (Vetamun Live Standard, Intervet International B.V., Boxmeer, The Netherlands) or rabies virus (Rabdomun, ESSEX, Tierarznei, Germany) using the RNeasy Mini Kit (QIAGEN AG Basel, Switzerland). Brain tissue samples (30 mg per 40 µl) were disrupted and homogenized with QIAshredder, and total RNA was subsequently isolated using the RNeasy Mini Kit. Lysates were digested with 1 µl RNase-free DNase (Promega, Madison, USA) for 1 h at 37 °C, followed by a deactivation step at 95 °C for 5 min. Isolated and purified RNA was reverse transcribed into cDNA using the random primer Reverse Transcriptase System (Promega, Madison, USA). Briefly, total RNA was incubated with random primer and 30 units of reverse transcriptase for 1 h at 42 °C. The enzyme was inactivated for 5 min at 95 °C. The cDNA was stored at −20 °C.

2.5. Primers and probes

Quantification of the *p40* and *p24* transcripts of BDV and the corresponding genomic RNA, which was also subject to reverse transcription, was carried out with specific double-fluorescently labeled probes in a ABI Prism 7700 sequence detector (Applied Biosystems, Rotkreuz, Switzerland). TaqMan® probes were labeled with FAM (5-carboxyfluorescein) as the 5′ fluorescent reporter and TAMRA (6-carboxytetramethylrhodiamine) as the 3′ quencher. For the specific amplification of BDV-specific cDNA, primers and an internal oligonucleotide as a probe were designed based on the ORFs of *p40* and *p24* from the four known BDV virus strains Borna V, Borna HE/80, Borna No/98 and Borna H1766.

The following primers and probes were used:

p40-forward primer	5′-GGTTTAAAACTATGATGGCA GCCTTA-3′
p40-reverse primer	5′-GTGGATTAAACATCTGGAGTA GTGTAGC-3′
p40-probe	5′-FAM-ACCGGCCATCCCATGGT GAGAC-TAMRA-3′
p24-forward primer	5′-TCCCTGGAGGACGAAGAAGA T-3′
p24-reverse primer	5′-CTTCCGTGGTCTTGGTGACC-3′
p24-probe	5′-FAM-CCAGACACTACGACGG GAACGA-TAMRA-3′

Amplification with the p40 and p24 primers yielded products of 78 and 69 bp, respectively. The expected PCR product was used as a query sequence in a BLAST search at GenBank (www.ncbi.nlm.nih.gov). This search revealed high specificity for BDV. As an endogenous control and calibrator, eukaryotic 18S rRNA (Applied Biosystems, Foster City, CA, USA) was amplified. The probe was labeled with VIC™ dye-MGB.

2.6. Real-time PCR and data analysis

PCR amplification was carried out in 25 µl volumes per well containing 12.5 µl of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, foster City, CA, USA), 600 nM of each primer, 80 nM (p40-probe) and 160 nM (p24-probe) of the probes, and 3.5 µl of cDNA. The 18S rRNA was amplified as an internal control to monitor the quality of the extracted RNA. The same 18S rRNA primers/probe were used for the amplification of cDNA from MDCK cells and tissues. The PCR conditions were: 2 min at 50 °C to activate the Uracil-N-Glycosilase (UNG), which detects and degrades the former PCR products, and then 10 min at 95 °C to inactivate the Uracil-N-Glycosilase and activate the polymerase, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 1 min. All experiments were conducted at least three times and the same results were obtained by independent technicians. Furthermore, known negative samples and also water was always carried through the experiments as controls. The data were analyzed using sequence detector software V1.7 (PE Systems). Absorbance was measured in a microplate reader at a wavelength of 492 nm. Signals were regarded as positive if the fluorescence intensity (increase of fluorescence of the reporter dye represented by ΔRn) exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle, CT). CT values of 40 were regarded as negative. The CT value is the cycle at which a statistically significant increase in ΔRn is first detected. For the generation of relative standard curves, the CT values of MDCK cells persistently infected with BDV were determined for p40, p24 and the 18S rRNA, using 10-fold dilution series of the cDNA template.

In order to compare (semi-quantitatively) samples of different origins, relative standard curves were established, as described previously [35]. MDCK cells persistently infected with BDV were used for the generation of relative standard curves for p40, p24, and 18S rRNA as an endogenous control and calibrator. Normalized infected MDCK cells were serially diluted in 10-fold steps. From these cells, RNA was extracted and reverse transcribed. The TaqMan results from these serial dilutions were then used to generate relative standard curves ($y = ax + b$) for the virus RNA using p40 and p24 primers and probe, and for the host cell RNA (i.e. the total RNA used for the reverse transcription reaction) using 18S rRNA primers and probe. These virus-specific standard curves were used as basis to normalize the signals (CT values) from p40 and p24 primer pairs from all experimental data and account

for differences in the kinetics of the PCR reactions between the two primers (represented by difference in the slope a). A standard curve representing the signal from 18S rRNA primers in a dilution series of uninfected MDCK cells was then used as an endogenous control to calibrate the system (hence termed the calibrator) against differences in the efficiency of the RNA extraction or cDNA synthesis of single experiments.

For all experimental samples (sheep and horse brain samples), the target quantity (p40, p24 values) was determined from the corresponding standard curve divided by the target quantity of the calibrator (18S rRNA). Therefore, the calibrator became the $1\times$ sample, and all other quantities were expressed as an *n*-fold difference relative to the calibrator.

3. Results

3.1. Specificity and sensitivity of the PCR

To determine the specificity of the two primer pairs and the corresponding probes targeting either *p40* or *p24* RNA of BDV, RNA from BDV and from more or less distantly related viruses was reverse transcribed and used as a template in the reverse transcription, real-time PCR. While preparations from BDV-infected cell cultures as well as extracts from brain samples of a sheep with previously diagnosed Borna disease yielded positive results, RNA from negative controls yielded negative results (data not shown). Negative controls included other members of the order Mononegavirales, including lyssa virus (Rhabdoviridae), PIV-2 and CDV (both Paramyxoviridae), as well as a representative of the positive polar RNA viruses, i.e. PEDV (Coronaviridae). Furthermore, samples from two DNA viruses, CAV-2 (Adenoviridae) and CPV-2 (Parvoviridae), were also tested with negative results. Thus, the two sets of primers and probes designed for detection of BDV were shown to be specific.

In order to establish the number of DNA molecules representing reverse transcribed template detectable by the PCR, 10-fold dilution series (covering the range between 10^{8} and 10^{-2} plasmid molecules per reaction) of plasmids containing either the *p40* ORF or the *p24* ORF, were tested. In six series of independent experiments, involving independent laboratory personnel, a minimum of 10 molecules per reaction could be detected with both systems. The efficiency of each PCR ranged between 99% and 100% with slopes of the standard curves of between −3.1775 and −3.471. Finally, the standard curves remained linear over a range of 6 orders of magnitude.

To compare the new methods to an established gold standard, extracts from BDV-infected cell cultures were serially diluted (4-fold dilutions, starting from 400 focus forming units, FFU) and separate aliquots of each dilution were subjected to virus isolation and to either one of the two novel RT-PCR assays. In two independent series of experiments, BDV was detected by the novel RT-PCR methods down to the dilution of 1:256–1:1024, which corresponded to 1.5–0.4 FFU. Notably, dilutions that were negative by RT-PCR were also negative by virus isolation and extracts from non-infected cell cultures yielded always negative results with BDV primers and probe. Interestingly, throughout these experiments, the p40 template gave always lower CT values than the p24 template, although the detection limit was at the same dilution. These observations suggested that the p40 template was more abundant in virus-producing cells than the p24 template.

To make the novel assays comparable among each other, despite of slight differences in their slopes and efficiencies, relative standard curves were generated for p40, p24, and 18S rRNA. As described in materials and methods, the standard curve for 18S rRNA was established using total RNA extracted from pre-determined numbers of infected or uninfected cells. For each sample, the result of 18S rRNA amplification was used as a calibrator for standardized amounts of cellular template in the reaction. All other quantities were then expressed in arbitrary units as an *n*-fold difference relative to the calibrator.

Since natural samples may contain very few BDV-infected cells, embedded into a vast majority of non-infected cells, the performance of the novel tests under such circumstances was evaluated. To mimic this situation in a controlled fashion, BDV-infected MDCK cells were first diluted in 10-fold dilution steps and then complemented with non-infected MDCK cells to obtain a uniform number of 10^{6} cells before RNA extraction. As a negative control and to define the threshold for detection, RNA from non-infected MDCK cells was used as a template. As shown in Fig. 1, one single infected cell in 1 million non-infected cells was consistently detected using the PCR.

3.2. Detection of BDV from sheep and horse brain by RT real-time PCR and immunohistochemical examination

The novel methods were used to conduct a comparative analysis of virus loads in brain tissues from three sheep and three horses with previously diagnosed BDV. Frozen brain samples were processed and subjected to PCR analysis. The negative threshold was calculated from brain tissue of a sheep and a horse with no history of BD. To avoid operator bias, the samples were coded before total RNA was extracted three times on different days, and the corresponding cDNA samples were analyzed at least two times and, additionally, by independent investigators. The CT values were calculated using the established relative standard curves for *p40*, *p24* and 18S transcripts.

In accordance with previous histological and immunohistochemical evidence, viral RNA was detected in all three independent brain sections from each of the three sheep. The viral load in both cortex and hippocampus was shown to be higher than in the cerebellum (Fig. 2). The *p24* transcript levels were consistently greater than those for the *p40*. Interestingly, a substantially smaller BDV load was measured for one sheep (S02-1736) than for the other two.

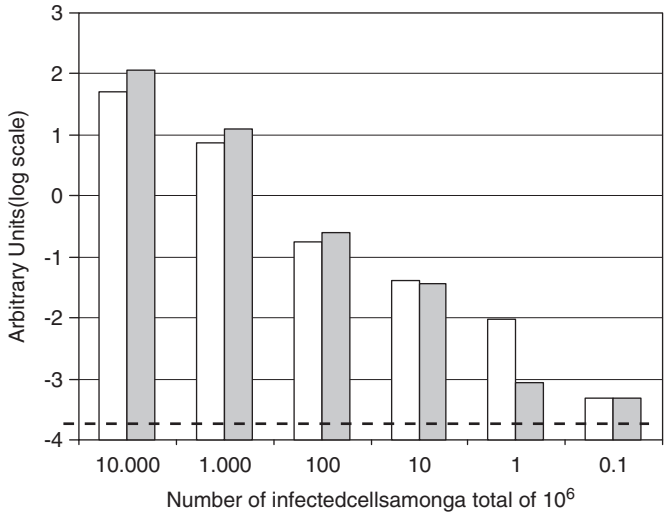

Fig. 1. Sensitivity of detection of BDV-infected MDCK cells. BDV-infected MDCK cells were diluted in 10-fold dilution steps and complemented with non-infected cells to obtain a uniform number of 10^6 cells before RNA extraction for reverse transcription, real-time PCR. The threshold for both primers was 0.00004 (dashed line), representing the normalized and calibrated value from non-infected MDCK cells (see materials and methods). White bars: amplification with p40 primers; gray bars: with p24 primers; *x*-axis: number of infected MDCK cells; *y*-axis: relative BDV copy number.

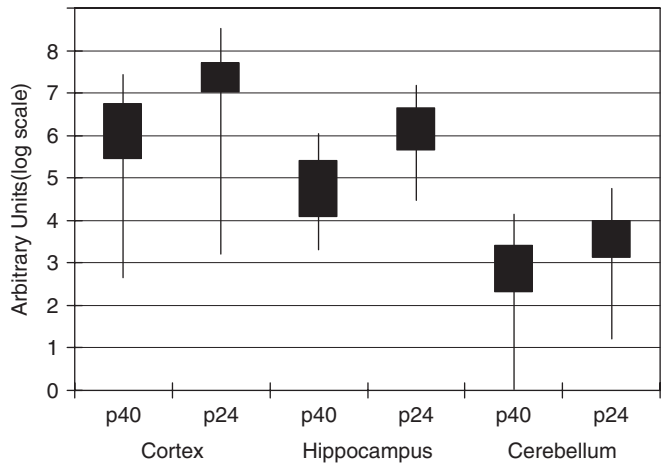

Fig. 2. Relative amounts of BDV templates in various brain regions of BDV-diseased sheep. Extracts from triplicate samples of different brain sites of three different BDV-diseased sheep (S03-0057, S02-1736 and S99-1356) were subjected to reverse transcription and real-time PCR. Box plots are shown, representing maximal, average, median, and minimal numbers of BDV-specific template detected. The signals of the BDV-specific amplifications are expressed as arbitrary units (*y*-axis) relative to the 18S rRNA signal, which normalizes the total number of cells in the assay. Amplifications for p40 and p24 were done simultaneously, using the same volume of the same template. The threshold for both primers was 0.005. *x*-axis: brain sites and PCR system; *y*-axis: relative BDV copy number.

Although immunohistochemistry is not considered quantitative, the results (Table 1) revealed similar differences in the staining between the different brain sites.

Similarly, BDV RNA was detected by PCR in the brains of the three BDV-positive horses. The negative threshold of 0.0004 (for both the *p40* and *p24* loci) was calculated as for the sheep, using the CT values from cortex, hippocampus and cerebellum from a horse with no history of BD (Table 2). The viral RNA loads in the different brain regions for the three horses (Fig. 3) were comparable to the loads detected in sheep. Again, a higher load was detected for p24 compared with p40. In one BDV-positive horse (S00-0913), the BDV loads in the cortex and hippocampus were greater than in the cerebellum, supporting the trend for sheep. For the other two, lobus piriformis showed similar (S96-0868) or even higher (S99-0598) BDV loads compared with cortex or hippocampus, which may be explained by the lobus piriformis (belonging to the bulbus olfactorius) proposed to being the host entry point for the virus [36,37].

4. Discussion and conclusions

In this study, two reverse transcription, real-time PCR assays were evaluated for the detection of BDV-specific nucleic acids in tissue samples from diseased animals. One assay detected and quantified the *p40* locus of the viral RNA, which represents either the gene or the transcript for the nucleoprotein N (ORF1), whereas the other assay targeted the phosphoprotein P locus (p24, ORF2). Relative quantification was achieved by using the signal from 18S rRNA as a calibrator for the amount of extracted cells in the assay. The primer and probe sequences for *p40* and *p24*, respectively, were designed based on the sequences of four BDV strains, namely Borna V, Borna HE/80, Borna H1766 and Borna No/98 [18,38–40].

In a first set of experiments, the specificity of the novel assays was established using cloned cDNA as well as RNA extracted from persistently BDV-infected cells. The selected primers specifically amplified BDV, whereas amplification of more or less distantly related viruses, including negative and positive polar RNA viruses as well as double stranded and single stranded DNA viruses, yielded negative results. Importantly, cDNA transcribed from the genomes of other members of the order mononegavirales, including rhabdovirus and parainfluenzaviruses (PIV-2 and CDV), which probably represent the closest relatives to BDV, was not amplified by the novel methods. Both rabies and parainfluenza viruses, including morbilliviruses, may affect sheep as well as horses. Depending on the prevalence of specific other neuropathogenic ovine or equine viruses in specific countries, other users of our methods may chose to test for specificity of the present system against other viruses of interest.

The sensitivity of the two novel PCRs was equal, reaching the level of approximately 10 target molecules and 0.4–1.5 FFU per assay. Interestingly, one single positive cell could be detected among 10^6 uninfected cells. This speaks for the potential of these assays for further epidemiological studies to detect possible vectors or reservoirs. For such studies it should be considered that strain No/98, which is 80% identical in sequence to the

Table 1
Comparison of BDV detection in sheep[a] by reversed transcription real-time PCR and immuohistochemical (IHC) examination

Sheep	Brain site	Reverse transcription real-time PCR[b]		IHC	IHC
		Primer p40	Primer p24	P38/40	P24
S03-0057	Cortex	1703	13 938	++[c]	++–+++[c]
	Hippocampus	65	851	+++	++
	Cerebellum	0.76	2.48	+	+
S02-1736	Cortex	0.07	0.54	+	(+)–+
	Hippocampus	0.32	5.46	+	+
	Cerebellum	0.008	0.005	–	–
S99-1356	Cortex	23	1678	+++	++
	Hippocampus	13	444	+++	+++
	Cerebellum	0.009	0.50	(+)–+	(+)–+
S02-0857	Threshold	0.005	0.005	–	–

[a]Results, normalized and calibrated, of the three BDV infected and one control sheep. Threshold calculated from the results of the negative control (sheep S02-0857). Values represent the average from three independent experiments.
[b]Relative BDV copy number per cell.
[c]Key for IHC: no antigen (–) or low (+), moderate (++) and large amount (+++) of BDV antigen detected.

Table 2
Comparison of BDV detection in horses[a] by reverse transcription real-time PCR and immuohistochemical (IHC) examination

Horse	Brain site	Reverse transcription real-time PCR[b]		IHC	IHC
		Primer p40	Primer p24	P38/40	P24
S00-913	Cortex	37	8873	+++[c]	+–++[c]
	Hippocampus	68	35 343	+++	++–+++
	Cerebellum	0.22	82	–	–
S99-0598	Cortex	0.05	0.63	–	–
	Hippocampus	10	106	++	+–++
	L. piriformis	568	10 378	Not available	Not available
S96-0868	Cortex	8.31	12	+++	+++
	Hippocampus	6.29	2008	+++	+++
	L. piriformis	57	14 248	Not available	Not available
S01-1038	Threshold	0.0003	0.0003	–	–

[a]Results, normalized and calibrated, of the three BDV infected and one control horses. Threshold calculated from the results of the negative control (horse S01-1038). Values represent the average from three independent experiments.
[b]Relative BDV copy number per cell.
[c]Key for IHC: no antigen (–) or low (+), moderate (++) and large amount (+++) of BDV antigen detected.

three other BDV strains, might be missed with the designed primer/probe combination, since No/98 differs in one nucleotide position compared with the forward primer of p40, the probe and the reverse primer of p24, respectively [38].

Considering the potential for cross-contamination during opening of tubes following cDNA synthesis, as required in a two-step RT-PCR assay, the use of a real-time-one-step-RNA-PCR kit was also evaluated (data not shown). Unfortunately, the sensitivity of detection was severely diminished and this approach was not pursued any further.

When viral RNA was extracted from infected cell cultures, the p40 template gave always lower CT values than the p24 template, which suggested that the p40 template was more abundant in virus-producing cells than the p24 template. As discussed below, this was not the case when tissues from Borna-diseased patients were analyzed.

BDV was readily detectable by the novel PCR tests in brain samples from animals with BD. The brain sites cortex and hippocampal formation were chosen according to the predilection sites of BDV in the brain. As expected, the relative virus loads in cortex and hippocampus were higher than in the cerebellum, which is usually also less affected by the infection [28,29,41,42]. Interestingly, the relative virus load in lobus piriformis was similar to that of the hippocampal formation. The data obtained by the PCR correlated, on a qualitative level, with the immunohistochemical findings of higher virus load in cortex and hippocampus compared to the cerebellum [28,29,41,42].

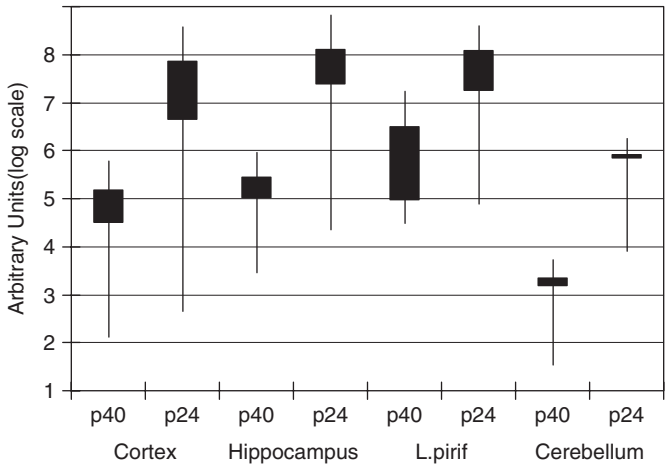

Fig. 3. Relative amounts of BDV templates in various brain regions of BDV-diseased horses. Extracts from triplicate samples of cortex and hippocampus of three different BDV-diseased horses (S00-0913, S99-0598 and S96-0868) as well as lobus piriformis from two horses and cerebellum from one horse were subjected to reverse transcription and real-time PCR. Box plots are shown, representing maximal, average, median, and minimal numbers of BDV-specific template detected. The signals of the BDV-specific amplifications are expressed as arbitrary units (*y*-axis) relative to the 18S rRNA signal, which normalizes the total number of cells in the assay. Amplifications for p40 and p24 were done simultaneously, using the same volume of the same template. The threshold for both primers was 0.0004. *x*-axis: brain sites and PCR system; *y*-axis: relative BDV copy number.

However, since different dilutions of the monoclonal antibodies had to be used for optimal immune staining, the viral protein loads (P38/40 and P24) could not be directly compared between signals arising from the anti-P24 monoclonal antibody or the anti-P38/40 monoclonal antibody, respectively. However, comparison between signals from the same tool in different locations in the brain was possible. Immunohistology gives a representation about the local distribution of infected cells in the present section, whereas our newly established methods give the average of template copies in a specific sample relative to the amount of cells used for extraction. Finally, the variation in stability of BDV antigens and RNA in formaldehyde fixed or frozen tissue has to be considered as an unreliable factor. For these reasons, consistent quantitative correlations between immunohistology and quantitative RT-PCR could not be expected.

Antibodies against P24 and P38/40 of BDV can be detected in the sera from infected animals [43]. In addition, the corresponding antigens are the main products in BDV-infected cell cultures and can be detected in brain samples from BDV-infected animals not only by immunohistochemistry, but also Western blot [44,45]. According to the literature, the ratio between P38/40 and P24 changes in the course of infection, in as much as acutely infected cells show a higher level of P38/40 antigen, whereas persistently infected cells show higher levels of P24 [23–25,30].

The present results indicate that the *p40* and the *p24* RNA targets were not present in equal numbers in brain sections from diseased animals. While varying BDV RNA loads were detected in individuals and in different brain areas, the loads associated with *p24* were consistently greater than those linked to the *p40* locus. A hallmark of the virus producing (productive) infection of cells with members of the Mononegavirales consists of the formation of a gradient of mRNAs with synthesis of the highest copy number of mRNA at the 3′-end of the negative sense genomic RNA, followed by increasingly attenuated mRNA synthesis towards the other end of the genome [46]. It had been expected that, similar to infected cell cultures, an equal or even greater number of *p40* targets would be present in productively infected cells of patient tissue. Indeed, studies have demonstrated that BDV replication is optimal when P38/40 antigen was in a 30-fold excess compared with P24 [23–25].

The present outcome with a greater number of p24 targets in brain samples, as opposed to the findings in cell cultures, was somewhat surprising. A technical explanation may be that p24 targets were reverse transcribed at greater efficiency than p40 targets, although, due to the opposing observation in cell culture extracts, it seems more plausible that more p24 than p40 targets were present in those samples. Indeed, we did not use in vitro transcribed RNA to compare the efficiency of individual cDNA synthesis in our system. However, Watanabe and others made the same observation in BDV-infected neonatal gerbils [47]. Interestingly, both the p24 mRNA and the P24 protein were more abundant than their p40 and P38/40 counterparts in diseased gerbils as opposed to healthy gerbils infected with the same dose of BDV. Together, these two independent observations suggest that the amount of p24 mRNA could be used as a marker for the progression of disease and that the gerbil model reflects the situation in the natural host. Diagnostically, these observations suggest that detection of the p24 target is the more sensitive tool for routine applications with patient materials.

In conclusion, two reverse transcription, real-time PCR assays have been established for the detection of Borna disease virus. The primers detected BDV transcripts with high specificity and the approach also proved to be highly sensitive. In addition, the PCRs also allowed the relative quantification of virus loads from different sites in horse and sheep brain. These data were in good agreement with previous immunohistochemical studies using the same samples. The results described here and the high specificity and sensitivity of the PCRs established herein indicate that this approach will not only be a valuable diagnostic tool but may also be useful for studies of the epidemiology, ecology and pathogenesis of BDV.

Acknowledgments

The authors thank L.Stitz, Tuebingen, Germany for monoclonal antibodies against the BDV nucleoprotein and phosphoprotein, S. Herzog, Institute of Virology, University of Giessen, Germany, for the BDV-infected MDCK

cells, Ph. Grob, Institute of Virology, University of Zurich, Switzerland, for the plasmids containing the *p40* and *p24* ORFs, respectively.

References

[1] Lundgren AL, Czech G, Bode L, Ludwig H. Natural Borna disease in domestic animals others than horses and sheep. Zentralbl Veterinarmed B 1993;40:298–303.
[2] Zimmermann W, Dürrwald R, Ludwig H. Detection of Borna disease virus RNA in naturally infected animals by a nested polymerase chain reaction. J Virol Methods 1994;46:133–43.
[3] Lebelt J, Hagenau K. [Distribution of Borna disease virus in naturally infected animals with clinical disease]. Berl Munch Tierarztl Wochenschr 1996;109:178–83.
[4] Caplazi P, Melzer K, Goetzmann R, Rohner-Cotti A, Bracher V, Zlinszky K, et al. [Borna disease in Switzerland and in the principality of Liechtenstein]. Schweiz Arch Tierheilkd 1999;141:521–7.
[5] Staeheli P, Sauder C, Hausmann J, Ehrensperger F, Schwemmle M. Epidemiology of Borna disease virus. J Gen Virol 2000;8(21):23–35.
[6] Rott R, Herzog S, Fleischer B, Winokur A, Amsterdam J, Dyson W, et al. Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. Science 1985;228:755–6.
[7] Bode L, Riegel S, Ludwig H, Amsterdam JD, Lange W, Koprowski H. Borna disease virus-specific antibodies in patients with HIV infection and with mental disorders. Lancet 1988;2:689.
[8] Bechter K, Schuttler R, Herzog S. Borna disease virus: possible causal agent in psychiatric and neurological disorders in two families. Psychiatry Res 1992;42:291–4.
[9] Waltrip II RW, Buchanan RW, Summerfelt A, Breier A, Carpenter Jr WT, Bryant NL, et al. Borna disease virus and schizophrenia. Psychiatry Res 1995;56:33–44.
[10] De La Torre JC, Gonzalez-Dunia D, Cubitt B, Mallory M, Mueller-Lantzsch N, Grasser FA, et al. Detection of borna disease virus antigen and RNA in human autopsy brain samples from neuropsychiatric patients. Virology 1996;223:272–82.
[11] De la Torre JC, Bode L, Dürrwald R, Cubitt B, Ludwig H. Sequence characterization of human Borna disease virus. Virus Res 1996;44:33–44.
[12] Sauder C, Muller A, Cubitt B, Mayer J, Steinmetz J, Trabert W, et al. Detection of Borna disease virus (BDV) antibodies and BDV RNA in psychiatric patients: evidence for high sequence conservation of human blood-derived BDV RNA. J Virol 1996;70:7713–24.
[13] Bode L, Durrwald R, Rantam FA, Ferszt R, Ludwig H. First isolates of infectious human Borna disease virus from patients with mood disorders. Mol Psychiatry 1996;1:200–12.
[14] Gonzalez-Dunia D, Sauder C, De la Torre JC. Borna disease virus and the brain. Brain Res Bull 1997;44:647–64.
[15] Planz O, Rentzsch C, Batra A, Winkler T, Buttner M, Rziha HJ, et al. Pathogenesis of borna disease virus: granulocyte fractions of psychiatric patients harbor infectious virus in the absence of antiviral antibodies. J Virol 1999;73:6251–6.
[16] Schwemmle M, Jehle C, Formella S, Staeheli P. Sequence similarities between human bornavirus isolates and laboratory strains question human origin. Lancet 1999;354:1973–4.
[17] Nakamura Y, Takahashi H, Shoya Y, Nakaya T, Watanabe M, Tomonaga K, et al. Isolation of Borna disease virus from human brain tissue. J Virol 2000;74:4601–11.
[18] Briese T, Schneemann A, Lewis AJ, Park YS, Kim S, Ludwig H, et al. Genomic organization of Borna disease virus. Proc Natl Acad Sci USA 1994;91:4362–6.
[19] De la Torre JC. Molecular biology of borna disease virus: prototype of a new group of animal viruses. J Virol 1994;68:7669–75.
[20] Schneemann A, Schneider PA, Lamb RA. Lipkin WIThe remarkable coding strategy of borna disease virus: a new member of the nonsegmented negative strand RNA viruses. Virology 1995;210:1–8.
[21] Tordo N, de Haan P, Goldbach R, Poch O. Evolution of negative-stranded RNA genomes. Semin Virol 1992;3:341–57.
[22] Conzelmann KK. Nonsegmented negative-strand RNA viruses: genetics and manipulation of viral genomes. Annu Rev Genet 1998;32:123–62.
[23] Schneider U, Naegele M, Staeheli P, Schwemmle M. Active borna disease virus polymerase complex requires a distinct nucleoprotein-to-phosphoprotein ratio but no viral X protein. J Virol 2003;77:11781–9.
[24] Geib T, Sauder C, Venturelli S, Hassler C, Staeheli P, Schwemmle M. Selective virus resistance conferred by expression of Borna disease virus nucleocapsid components. J Virol 2003;77:4283–90.
[25] Perez M, Sanchez A, Cubitt B, Rosario D, De la Torre JC. A reverse genetics system for Borna disease virus. J Gen Virol 2003;84:3099–104.
[26] Bilzer T, Grabner A, Stitz L. [Immunopathology of Borna disease in the horse: clinical, virological and neuropathologic findings]. Tierarztl Prax 1996;24:567–76.
[27] Bilzer T, Planz O, Lipkin WI, Stitz L. Presence of CD^{4+} and CD^{8+} T cells and expression of MHC class I and MHC class II antigen in horses with Borna disease virus-induced encephalitis. Brain Pathol 1995;5:223–30.
[28] Caplazi P, Ehrensperger F. Spontaneous Borna disease in sheep and horses: immunophenotyping of inflammatory cells and detection of MHC-I and MHC-II antigen expression in Borna encephalitis lesions. Vet Immunol Immunopathol 1889;61:203–20.
[29] Gosztonyi G, Ludwig H. Borna disease of horses. An immunohistochemical and virological study of naturally infected animals. Acta Neuropathol (Berl) 1984;64:213–21.
[30] Watanabe M, Zhong Q, Kobayashi T, Kamitani W, Tomonaga K, Ikuta K. Molecular ratio between borna disease viral-p40 and -p24 proteins in infected cells determined by quantitative antigen capture ELISA. Microbiol Immunol 2000;44:765–72.
[31] Carbone KM, Moench TR, Lipkin WI. Borna disease virus replicates in astrocytes, Schwann cells and ependymal cells in persistently infected rats: location of viral genomic and messenger RNAs by in situ hybridization. J Neuropathol Exp Neurol 1991;50:205–14.
[32] Richt JA, Herzog S, Haberzettl K, Rott R. Demonstration of Borna disease virus-specific RNA in secretions of naturally infected horses by the polymerase chain reaction. Med Microbiol Immunol (Berl) 1993;182:293–304.
[33] Sorg I, Metzler A. Detection of Borna disease virus RNA in formalin-fixed, paraffin-embedded brain tissues by nested PCR. J Clin Microbiol 1995;33:821–3.
[34] Utiger A, Tobler K, Bridgen A, Ackermann M. Identification of the membrane protein of porcine epidemic diarrhea virus. Virus Genes 1995;10:137–48.
[35] Hussy D, Stauber N, Leutenegger CM, Rieder S, Ackermann M. Quantitative fluorogenic PCR assay for measuring ovine herpesvirus 2 replication in sheep. Clin Diagn Lab Immunol 2001;8:123–8.
[36] De la Torre JC. Molecular biology of Borna disease virus and persistence. Front Biosci 2002;7:d569–79.
[37] Gonzalez-Dunia D, Cubitt B, De la Torre JC. Mechanism of Borna disease virus entry into cells. J Virol 1998;72:783–8.
[38] Cubitt B, Oldstone C, De la Torre JC. Sequence and genome organization of Borna disease virus. J Virol 1994;68:1382–96.
[39] Nowotny N, Kolodziejek J, Jehle CO, Suchy A, Staeheli P, Schwemmle M. Isolation and characterization of a new subtype of Borna disease virus. J Virol 2000;74:5655–8.
[40] Pleschka S, Staeheli P, Kolodziejek J, Richt JA, Nowotny N, Schwemmle M. Conservation of coding potential and terminal sequences in four different isolates of Borna disease virus. J Gen Virol 2001;82:2681–90.
[41] Caplazi P, Waldvogel A, Stitz L, Braun U, Ehrensperger F. Borna disease in naturally infected cattle. J Comp Pathol 1994;111:65–72.

[42] Hornig M, Weissenbock H, Horscroft N, Lipkin WI. An infection-based model of neurodevelopmental damage. Proc Natl Acad Sci USA 1999;96:12102–7.
[43] Briese T, Hatalski CG, Kliche S, Park YS, Lipkin WI. Enzyme-linked immunosorbent assay for detecting antibodies to Borna disease virus-specific proteins. J Clin Microbiol 1995;33:348–51.
[44] Bause-Niedrig I, Pauli G, Ludwig H. Borna disease virus-specific antigens: two different proteins identified by monoclonal antibodies. Vet Immunol Immunopathol 1991;27:293–301.
[45] Haas B, Becht H, Rott R. Purification and properties of an intranuclear virus-specific antigen from tissue infected with Borna disease virus. J Gen Virol 1986;67(Part 2):235–41.
[46] Pringle CR. The order Mononegavirales—current status. Arch Virol 1997;142:2321–6.
[47] Watanabe M, Lee B-J, Kamitani W, Kobayashi T, Taniyama H, Tomonaga K, et al. Neurological diseases and viral dynamics in the brains of neonatally Borna disease virus-infected gerbils. Virology 2001;282:66–76.