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

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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.

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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
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 Born 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 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 cm³) 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 H₂O 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 H₂O₂ (3% in H₂O and supplemented with 0.2% NaN₃) 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 (pL_pPG401:p40-DHLX-ABP-6xHis and pL_pCRII-ORFI) as well as a plasmid containing the p24 ORF (pL_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 l) were disrupted and homogenized with QIAshredder, and total RNA was subsequently isolated using the RNeasy Mini Kit. Lyases were digested with 1 l 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-carboxytetramethylrhodamine) 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 cDNA, primers and an internal oligonucleotide as a probe. These virus-specific standard curves were used 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 ARn) 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 ARn 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 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 l volumes per well containing 12.5 l of 2 x 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 l 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 ARn) 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 ARn 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.

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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 (Paroviridae), 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 $10^0$ 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.
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 choose 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
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].

Table 1
Comparison of BDV detection in sheep* by reversed transcription real-time PCR and immunochemical (IHC) examination

| Sheep | Brain site | Reverse transcription real-time PCRb | IHC | IHC |
|-------|------------|-------------------------------------|-----|-----|
|       |            | Primer p40 Primer p24               | P38/40 | P24 |
| S03-0057 | Cortex | 1703 13938 | ++ ++ ++ ++ | ++ ++ ++ + |
|         | 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 | – | – |

*aResults, 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.

bRelative BDV copy number per cell.

cKey for IHC: no antigen (–) or low (+), moderate (++) and large amount (+++) of BDV antigen detected.

Table 2
Comparison of BDV detection in horses* by reverse transcription real-time PCR and immunochemical (IHC) examination

| Horse | Brain site | Reverse transcription real-time PCRb | IHC | IHC |
|-------|------------|-------------------------------------|-----|-----|
|       |            | Primer p40 Primer p24               | P38/40 | P24 |
| S00-913 | Cortex | 37 8873 | ++ ++ + | ++ ++ + |
|         | Hippocampus | 68 35343 | + + + | + + + |
|         | Cerebellum | 0.22 82 | – | – |
| S99-0598 | Cortex | 0.05 0.63 | – | – |
|         | Hippocampus | 10 106 | + | + |
|         | L. piriformis | 0.568 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 | – | – |

*aResults, 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.

bRelative BDV copy number per cell.

cKey for IHC: no antigen (–) or low (+), moderate (++) and large amount (+++) of BDV antigen detected.
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.

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