Development of a real-time RT-PCR assay for improved detection of Borna disease virus

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Abstract

Borna disease virus (BDV) is a non-segmented, negative-stranded RNA virus, which infects cells of the central nervous system (CNS) in many different species. BDV is the causative agent of the neurological disorders in horses and sheep termed classical Borna disease (BD), as well as staggering disease in cats. At present, the diagnosis staggering disease or feline BD is made by histopathology or immunohistochemistry of the CNS. In order to obtain a better clinical diagnostic tool, a duplex real-time RT-PCR assay (rRT-PCR) was developed. TaqMan® probes and primers specific for the BDV P and BDV L genes were designed by aligning the sequences of known BDV strains. After optimisation, the sensitivity and specificity of the rRT-PCR were established. The detection limit was set to 10–100 viral genomic copies per reaction and the assay detects the BDV strains V and He/80, as well as the most divergent BDV strain known so far, No/98. Furthermore, the system detected feline BDV variants in five naturally infected cats and a feline isolate used in experimental infection of cats. This rRT-PCR assay will be a powerful tool in further studies of BDV, including epidemiological screening and diagnosis.

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1. Introduction

Borna disease virus (BDV) is a neurotropic, enveloped, non-segmented, negative-stranded RNA virus that belongs to the family \textit{Bornaviridae}, genus \textit{Bornavirus} within the order \textit{Mononegavirales} (Briese et al., 1994; Cubitt et al., 1994). Borna disease, which was first described in horses in Germany in 1767, was considered as an endemic among horses and sheep in certain parts of central Europe (Ludwig and Bode, 2000). Today, however, natural infection has been confirmed in a broad range of hosts worldwide, among them cats. A fatal neurological disorder in cats, known as staggering disease, has been described in Sweden since the early 1970s (Kronevi et al., 1974). This disorder has been characterised as a non-suppurative meningoencephalomyelitis, thereby indicating a viral disease (Lundgren, 1992). However, it was not until the 1990s that the disease was linked to BDV infection (Lundgren et al., 1995, 1997).

The size of the BDV genome is approximately 8900 nucleotides and it consists of six major open reading frames (Briese et al., 1994; Cubitt et al., 1994). These encode for six polypeptides named after their molecular weight in kilodaltons (kDa). The polypeptide p40 corresponds to the viral nucleoprotein (N), p24 to the phosphoprotein (P), p16/gp18 to the matrix protein (M) and p56/gp94 to the surface glycoprotein (G), whereas p190 is thought to correspond to a RNA-dependent RNA polymerase (L). The function of the p10 polypeptide, sometimes referred to as the X protein, is largely unknown, though it has been reported to be involved in the regulation of the viral polymerase as a co-factor to the P protein (Poenisch et al., 2004).
The molecular epidemiology of BDV implicates that the viral genome is well conserved. Most BDV strains are genetically similar, and in a study where 40 field viruses, vaccine and laboratory strains were analysed, the different isolates showed a nucleotide sequence identity of 97–99% in the p24 region (Kolodziejek et al., 2005). So far, only one published BDV strain is divergent compared to the others, around 16% genetic divergence. This strain was recovered from an Austrian horse in 1998, and is called No/98 (Nowotny et al., 2000; Pleschka et al., 2001).

At present, there are no consensuses on the diagnosis of BDV. Several different serological methods, as well as molecular biological methods have been used (reviewed in Ludwig and Bode, 2000). Previously, conventional nested RT-PCR assays have been used for the detection of BDV RNA (for example, Sauer and de la Torre, 1998; Zimmermann et al., 1994). Conventional PCR, however, is difficult to use for quantitation, and it is also laborious and time-consuming. Most recently, a real-time RT-PCR (rRT-PCR) assay for the detection of BDV in horse and sheep brain has been described (Schindler et al., 2007). However, it is not established whether this assay can detect more divergent strains, like No/98.

Staggering disease or feline Borna disease is a fatal neurological disorder in cats characterised by a staggering movement (hind-leg ataxia), behavioural changes, lumbosacral pain and an inability to retract the claws (Lundgren, 1992). The disease is suspected clinically by excluding other reasons for clinical signs. At present, the diagnosis is often made by histopathology or immunohistochemistry of the central nervous system (CNS). However, serological methods have been developed and used both for epidemiological screening and to diagnose cats with neurological symptoms (reviewed in Kamhieh and Flower, 2006). The seroprevalence of BDV-antibodies in cats showing symptoms of unspecified neurological disease is ranging from 0 to 66.6%, depending on method used and number of animals studied (Helps et al., 2001; Horii et al., 2001; Ouchi et al., 2001; Reeves et al., 1998). The seroprevalence in the normal cat population has not yet been fully characterised, but studies performed indicates highly variable results (2–41.6%) (Helps et al., 2001; Horii et al., 2001; Nakamura et al., 1996; Nishino et al., 1999; Ouchi et al., 2001; Reeves et al., 1998). In staggering disease, however, the humoral response seem to be weak or not present in naturally infected cats (Johansson et al., 2002; Lundgren and Ludwig, 1993). Experimentally infected cats on the other hand show high titres (Johansson et al., 2002).

The variation of seroprevalence in different studies suggest the presence of false-positive results, as a study using three different serological methods including specificity tests showed a significantly lower seroprevalence of BDV in humans compared to other studies (Fukuda et al., 2001). In the same study, no correlation between immunological and BDV RNA findings using nested RT-PCR of PBMCs were seen.

The lack of a good diagnostic tool for the living animal is a problem for the clinician and the owner. Apart from the clinical situation, the study of the epidemiology of BDV, as well as therapy evaluation is difficult without a clinical diagnostic tool. The current study presents a new, rapid, sensitive and specific duplex, one-step rRT-PCR for the simultaneous detection and quantification of the p24 and the L polymerase genes of BDV. This novel tool could be useful for future diagnostic methods and for epidemiological screening of different populations.

2. Materials and methods

2.1. Cells

C6 (rat glioma) cells infected with BDV He/80 (Cubitt et al., 1994) were used during the optimisation of the rRT-PCR assays. In the specificity test, Vero monkey cells infected with BDV No/98 (Nowotny et al., 2000) were also used.

2.2. Animals

In the specificity test, brain tissue samples from cats experimentally infected with BDV strain V and a feline isolate (Lundgren et al., 1997) were used. Brain tissue samples of five naturally infected cats, showing symptoms of staggering disease, as well as asymptomatic cats were also investigated. These cats have been autopsied and sampled at the Department of Pathology, Swedish University of Agricultural Sciences (SLU) from 1993 to 2005 (Table 1).

<table>
<thead>
<tr>
<th>Cat no. (year of sampling)</th>
<th>Breed/sex/age (years)</th>
<th>Duration of signs</th>
<th>Signs</th>
<th>Serology</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (1993)</td>
<td>DSH/NM/9</td>
<td>3 weeks</td>
<td>Depression, hindleg incoordination, fever</td>
<td>FIV, FeLV, Borrelia, Ehrlichia (all negative)</td>
<td>Non-suppurative meningoencephalomyelitis</td>
</tr>
<tr>
<td>B (1994)</td>
<td>DSH/F/3</td>
<td>1.5 years</td>
<td>Incoordination, loss of appetite</td>
<td>Not done</td>
<td>Non-suppurative encephalitis</td>
</tr>
<tr>
<td>C (1996)</td>
<td>DSH/NF/2</td>
<td>6 weeks</td>
<td>Incoordination, vocalisation, inability to retract the claws, staring gaze</td>
<td>No data</td>
<td>Non-suppurative meningoencephalomyelitis</td>
</tr>
<tr>
<td>D (1996)</td>
<td>DSH/NM/3</td>
<td>4 weeks</td>
<td>Incoordination</td>
<td>No data</td>
<td>Non-suppurative meningoencephalomyelitis</td>
</tr>
<tr>
<td>E (2005)</td>
<td>DSH/NF/8</td>
<td>6 months</td>
<td>Incoordination, loss of weight, increased vocalisation</td>
<td>No data</td>
<td>Non-suppurative meningoencephalomyelitis</td>
</tr>
</tbody>
</table>

DSH: domestic shorthair; NM: neutered male; NF: neutered female; F: female; FIV: feline immunodeficiency virus; FeLV: feline leukemia virus.
2.3. RNA extraction

Total RNA was extracted from cultured cells and brain tissue samples with Trizol LS and Trizol reagents (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. The tissue was homogenised manually with single use pellet pestles (Kontes Glass Company, Vineland, NJ, USA). The concentration and the purity were estimated by spectrophotometry or measured by NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

2.4. Primers and TaqMan® probes

The primers and TaqMan® probes were designed by aligning (Multalin version 5.4.1, France) the sequences of the P and L genes of the BDV strains V (GenBank accession no. U04608), He/80 (GenBank accession no. AJ311522), H1766 (GenBank accession no. AJ311523), CRNP5 (GenBank accession no. AJ311524) and No/98 (GenBank accession no. AJ311524). The most conserved regions were chosen for primer and probe design using the Beacon Designer 2.1 software (Biosearch Technologies, USA). The primers were designed in order to have minimal mismatches for the strains He/80 and No/98. For sequences of the primers and probes, as well as corresponding mismatches compared to different BDV strains, see Tables 2 and 3. The PCR product size of P gene is 88 bp and covers the nucleotides 1428–1515 of He/80. The amplicon of L gene is 89 bp and covers the nucleotides 7481–7569 of He/80. The P gene and L gene probes were labelled at the 5′-ends with 6-carboxyfluorescein (FAM) and ROX, respectively. Both of the probes utilised the non-fluorescent Black Hole Quencher Dye at the 3′-ends (Biosearch Technologies Inc., CA, USA).

2.5. Optimisation of the one-step rRT-PCR assays

The optimisation of the rRT-PCR assays was done separately for each primer pair. Titration series of primer, manganese acetate and probe concentration were performed. Ten-fold dilutions of template (RNA extracted from C6 cells (rat glioma), persistently infected with BDV He/80) were used for optimisation. Additionally, a field strain obtained from brain tissue of a cat with staggering disease was used for primer titration. All titration series were done in duplicates using MicroAmp® optical tubes (Applied Biosystems, Foster City, CA, USA) or 0.1 mL PCR tubes (Corbett Research, Australia).

2.6. BDV rRT-PCR assay

The rRT-PCR assay was prepared with special precaution for contaminations, as previously described (Belák and Thorén, 2001). Each reaction was carried out in a total volume of 25 μl, containing 2 μl of extracted RNA eluted in dimethyl pyrocarbonate (DMPC) water, 0.7 μM of each BDV P primer, 0.4 μM of each BDV L primer, 0.3 μM of the BDV P probe, 0.4 μM of the BDV L probe, 0.5 mM of dNTPs (GE Healthcare, Uppsala, Sweden), 2.5 mM of Mn(OAc)₂ (Applied Biosystems), 1× EZ buffer (Applied Biosystems), 0.08 mg/ml of bovine serum albu-
Table 3

Sequences of primers and probe of BDV L compared to different BDV strains submitted to GenBank

<table>
<thead>
<tr>
<th>Genebank accession</th>
<th>Forward (BDV 1–21 bp)</th>
<th>Reverse (BDV 1–21 bp)</th>
<th>Probe (BDV 30 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ152152 (He/80)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
</tr>
<tr>
<td>AJ152153 (VR37)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
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<tr>
<td>AJ152155 (VR37)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
</tr>
<tr>
<td>AJ152156 (CRA95)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
</tr>
<tr>
<td>AJ152157 (CRA95)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
</tr>
<tr>
<td>AJ152158 (CRA95)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
</tr>
<tr>
<td>AJ152159 (He/80)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
</tr>
<tr>
<td>AJ152160 (He/80)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
</tr>
<tr>
<td>AJ152161 (He/80)</td>
<td>GACAAGCTTCTATTTGCTT</td>
<td>CTTTTGCCTCTGACTTCT</td>
<td>AGGAGGACAGAGCAG</td>
</tr>
</tbody>
</table>

2.7. Sensitivity tests

The sensitivity of the assays was established by using in vitro transcribed RNA of clones of PCR products as standards. For this, PCR products from C6BDV (BDV He/80) were used. The PCR products were purified by using the Wizard® PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturers’ recommendations. The purified PCR products were ligated into the pGEM®-T Easy Vector system (Promega), and transformed into competent cells (XL blue), using standard procedures. The presence of inserted PCR products was controlled by gel electrophoresis of restriction enzyme cleavage and PCR screenings. Plasmid DNA used for the in vitro transcription was purified by the Wizard® Plus Maxiprep DNA Purification System (Promega), the concentration measured by NanoDrop ND-1000 (NanoDrop Technologies), and linearised by restriction enzyme cleavage. The in vitro transcription was performed with the MEGAscript® T7/Sp6 kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocol using 1 µg of linearised plasmid DNA as template. The in vitro transcribed RNA was treated with 2 U of DNase I (Ambion) for 15 min at +37 °C. After heat-inactivation, the RNA was precipitated by adding ammonium acetate and ethanol, incubated for more than 30 min at −20 °C. The RNA pellet was dissolved in DMPC water, and precipitated once more with ammonium acetate and ethanol, as described above. The final pellet was dissolved in DMPC water and stored as aliquots at −70 °C until use. Before freezing, the RNA concentration was measured using NanoDrop ND-1000 (NanoDrop Technologies). A dilution series from 10^10 to 1 copies per 2 µl was made for each transcript, stored as aliquots at −70 °C until use in the rRT-PCR assay. As dilution buffer, DMPC water with 10 µg/ml of sheared salmon sperm DNA (Ambion) was used. Each aliquot was used only once. All of the sensitivity tests were performed using the Rotor-Gene 3000 and 0.1 mL PCR tubes (Corbett Research, Australia). At
least four replicates of each dilution were used in all the tests. The BDV P and BDV L systems were evaluated separately and in a duplex rRT-PCR assay. At least two sensitivity tests per system were performed on different occasions and the Ct-values (number of cycles for the fluorescence to reach the threshold) obtained were used for the standard curves. The threshold was set in the exponential phase of the reactions and Ct-values greater than 40 were regarded as negative.

2.8. Specificity tests

In order to test the specificity of the duplex rRT-PCR assay, a number of non-BDV viruses as well as different strains of BDV were tested. Non-BDV viruses tested were both negative-stranded (Vesicular stomatitis virus (VSV) of the New Jersey and Indiana serotypes, Porcine rubulavirus (LPMV), Canine distemper virus (CDV) and Canine parainfluenza virus (CPIV)) and positive-stranded (Feline infectious peritonitis virus (FIPV)) RNA-viruses, as well as single-stranded (Canine parovirus (CPV)) and double-stranded (Canine adenovirus (CAV)) DNA viruses. For the canineline viruses, RNA and DNA were extracted from a canine vaccine (Nobivac DHPPi vet., Intervet International B.V., Boxmeer, Netherlands) using robot extraction (MagAttract Virus Mini M48 Kit, Qiagen Inc., Valencia, CA, USA). For the BDV strains, cell lines infected with BDV He/80 and BDV No/98, as well as brain tissue from cats experimentally infected with BDV strain V and a feline isolate (Lundgren et al., 1997) were used. Brain tissue of five naturally infected cats, showing symptoms of staggering disease, was also investigated. The specificity tests were performed using ABI PRISM 7700 and Rotor-Gene 3000.

3. Results

3.1. Efficiency, linearity and sensitivity

First, sensitivity tests were performed in order to establish efficiency, linearity and sensitivity of the optimised rRT-PCR assays by using in vitro transcribed RNA from plasmids as standards, as described in Section 2.7. The reaction efficiency \( E \) was calculated from the standard curves established at the sensitivity tests, using the formula \( E = e^{(-\alpha)} - 1 \), where \( \alpha \) is the slope. Ct-values far away from the standard curve were not included, but at least three values were included from each dilution. The simplex BDV P and BDV L assays were linear in a range from 10 to \( 10^{10} \) copies per reaction and the reaction efficiency was 103 and 93%, respectively (Fig. 1). The BDV P assay could detect some but not all (17%) of the 1 copy per reaction dilution. Since the results were divergent and not reproducible at that dilution, the detection limit of the BDV P and BDV L simplex assays were approximately 10 copies per reaction. The duplex rRT-PCR assay had different range of linearity for the BDV P and BDV L, respectively (Fig. 2). For the BDV P in the duplex assay, the linearity ranged from 10 to \( 10^7 \) copies per reaction. As with the simplex assay, the one copy per reaction dilution was sometimes detected (two out of eight samples). For the BDV L in the duplex assay, the linearity ranged from 100 to \( 10^7 \) copies per reaction. The detection limit for BDV L in the duplex assay is therefore reduced 10-fold, compared to the simplex BDV L assay. The reaction efficiencies of the duplex assay were similar to the simplex assays (BDV P duplex 106% and BDV L duplex 84%).

3.2. Specificity tests

A specificity test using different characterised strains of BDV, including the most genetically divergent strain, No/98, as well as a number of non-BDV viruses was performed. Furthermore, brain tissue from experimentally infected cats and cats showing symptoms of staggering disease were analysed. The duplex rRT-PCR assay detected both BDV P and L of the strains He/80 and No/98 from infected cells, as well as strain V from experimentally infected cat brain (Fig. 3). Furthermore, the assay detected the P gene of five BDV variants in brain tissue of naturally infected cats and a feline BDV isolate used for experimental infection (Fig. 4), as well as BDV L in four of the naturally infected cats (cats A–D, data not shown). None of the non-BDV viruses included in the test, as well as negative controls (asymptomatic cats and uninfected cells), were detected by the assay. Similar results were obtained using both the ABI PRISM 7700 and Rotor-Gene 3000.

4. Discussion

BDV causes neurological disorders in several different species all around the world. In Sweden, BDV infection has primarily been seen in cats, where it causes the neurological disorder known as staggering disease (Lundgren et al., 1995). The diagnosis is made at autopsy by histopathology and immunohistochemistry of the CNS. Serological methods have been developed in order to diagnose staggering disease, but a study showed that naturally infected cats do not produce high titres of antibodies or no titres at all (Johansson et al., 2002). This could be due to the fact that the virus causes a persistent infection within the CNS (Ludwig et al., 1988). The duration of the BDV infection is also not known in the naturally infected cat. Therefore, it has to be determined when in the course of the disease the cats develop antibodies and when it is suitable for a serological test.

Previously, a nested RT-PCR assay to detect the p24 and p40 genes of BDV in naturally infected animals has been used in Sweden (Berg et al., 2001). This assay, however, has several drawbacks. It is a laborious method where the samples and PCR products are handled after each step, which makes the risk of cross-contamination high. The last step, gel electrophoresis, includes the use of the carcinogenic chemical, ethidium bromide, which puts the laboratory staff in hazard. For research purposes, and for diagnosis, it is also valuable to be able to quantify the number of viral genomic copies to establish the viral load. The nested RT-PCR assay is difficult to use for this application, since it lacks a good possibility for quantification. In order to overcome these drawbacks, without losing the sensitivity of the nested RT-PCR assay, an rRT-PCR assay detecting the P and L genes of BDV were developed. The rRT-PCR assay is a closed-tube, one-step reaction, where the rTth enzyme is
used both for the RT-step and the PCR without any addition or change of the reaction environment. This method is also suitable for automation, by using robots for extraction of nucleic acids, as well as preparing the reaction mix. Such handling will further decrease the risk of cross-contamination between positive and negative samples, and accelerate the obtaining of final results.

In this study, two different systems were used, detecting the BDV P and BDV L genes, respectively. The reason for this approach was to decrease further the risk of contamination, as well as increasing the specificity and sensitivity of the assay. It seems unlikely to be due to contamination, if a sample is positive for both the BDV P and L genes. The L polymerase gene is considered to be a conserved gene, also within the order Mononegavirales. If the unknown field strain tested would be genetically divergent, it would be likely that at least one of the two systems would detect the strain, thereby increasing the likelihood of detection.

The initial optimisation of the rRT-PCR assays was performed individually, as simplex assays, i.e., detecting one of the two genes BDV P or L. Then the two assays were put together as a duplex assay, i.e., both the BDV P and L genes were detected simultaneously. An initial test showed that there was no need for further optimisation of the duplex assay before the sensitivity test (data not shown). The sensitivity was then tested using in vitro transcribed RNA of the BDV P and L real-time PCR products (BDV He/80). In this way, the efficiency of both the RT-step and the cycling reaction was investigated. The sensitivity and efficiency of the simplex assays were compared to the duplex assay (Figs. 1 and 2). For the BDV P gene the detection limit and reaction efficiency were similar for the simplex and duplex assay (detection limit 10 copies per reaction, reaction efficiency 103 and 106%, respectively). For the BDV L gene, the sensitivity of the duplex assay was 10-fold decreased compared to the simplex assay. The reaction efficiency was however similar in the both assays (93 and 84%, respectively). The decreased sensitivity of BDV L in the duplex assay could be due to competition between the two PCR systems. This is a well-known phenomenon when using multiplex PCR, and in this assay it could be explained
by the fact that both the BDV P and L rRT-PCR uses degenerated primers (Tables 2 and 3). The BDV L assay has a forward primer containing three degenerated nucleotides, and the reverse primer contains five. This means that the forward and reverse primer of BDV L are a cocktail of several variants of the primers, thereby decreasing the concentration of each individual variant of primer. In the primer titration of the BDV L assay, the best results were given at the concentration of 0.4 \( \mu \text{M} \), compared to 0.7 \( \mu \text{M} \) for the BDV P primers. Since the tests were optimised as simplex assays, the competition between the two systems was not seen. The initial testing of the duplex assay gave similar results as the simplex assays, using the same template RNA, thereby indicating no need for further optimisation of the duplex assay (data not shown). In the sensitivity test of the duplex assay, the same number of copies per reaction for the BDV P and BDV L in vitro transcribed RNA was used. However, it is considered that the number of copies of the BDV transcripts is decreasing in a 3′–5′ manner, similar to other viruses in the order Mononegavirales (Schwemmle et al., 2005). When BDV-infected cells have been analysed, the Ct-value of BDV P is approximately two to three cycles lower than the corresponding Ct-value of BDV L using the duplex assay. However, when using the simplex assay the Ct-values differ around 1.5 cycles. Since the assay will detect both genomic BDV-RNA, as well as BDV transcripts, this would indicate that there indeed is a difference in the number of copies of the BDV transcripts, even though the exact ratio between the two genes has not been further established.

When designing the assays, the sequences of known BDV strains were aligned, including He/80 and No/98. BDV No/98 is the most divergent strain yet known compared to the others, and has an overall genetic divergence of 16% compared to BDV He/80 (Nowotny et al., 2000). The primers and probes of the BDV P and L genes designed, where chosen from conserved regions. In order to be able to detect both strains, degenerated primers where designed to minimise the number of mismatches compared to BDV He/80 and No/98. However, the BDV P probe
Fig. 3. BDV duplex rRT-PCR assay of three BDV strains. RNA from cells infected with BDV strains He/80 and No/98 were used, as well as brain tissue from a cat experimentally infected with strain V. The upper graph is showing the results of BDV P and the lower BDV L. The figure shows the results from ABI PRISM 7700 Sequence Detector. Similar results have been obtained with Rotor-Gene 3000. Negative controls (non-BDV viruses, uninfected cells and asymptomatic cats) are not shown.

has a perfect match compared to No/98, and the BDV L probe has one mismatch (also see Tables 2 and 3). The specificity test showed that both BDV P and BDV L genes of the strains He/80 and No/98, as well as another reference strain V, were detected by the rRT-PCR assay (Fig. 3). As expected, none of the non-BDV viruses were positive. Brain regions from two cats infected experimentally with a feline isolate (Lundgren et al., 1997), also was positive for BDV P, but not for BDV L. Furthermore, five brain samples of naturally infected cats were tested (Table 1), all with the typical clinical signs as well as histopathological lesions associated with staggering disease or feline Borna disease (Lundgren, 1992). All were positive for BDV P (Fig. 4) and four of them (cats A–D) were positive for BDV L (data not shown). RNA from the samples was extracted several times and they have been positive in at least more than one extract. The reason why the L polymerase gene was not always detected could be due to the low number of copies, as well as the reduced sensitivity in the duplex assay. As discussed earlier, the number of copies of the L polymerase gene is lower compared to the p24 gene. Another reason why the L polymerase gene was not always detected could be due to sequence divergence. The sequence variation of the L polymerase gene is not much studied, and so far no BDV L gene from any Swedish isolate has been sequenced.

Previous studies using rRT-PCR for the detection of BDV have been used for quantifying number of viral copies in brain tissue of experimentally infected animals (Porombka et al., 2006; Watanabe et al., 2001). However, in a recent publication a newly developed rRT-PCR has been used for the detection of BDV in naturally infected horse and sheep brains (Schindler et al., 2007). The primers and probes for the BDV p24 and p40 genes in this study are consensus sequences after aligning
Fig. 4. Naturally and experimentally infected cats analysed by the BDV duplex rRT-PCR assay. The graph shows the results of the BDV P gene. Cat 1 is experimentally infected with BDV strain V, while cats 5 and 6 are infected with a feline isolate (Lundgren et al., 1997). Cats A–E: Brain tissue from naturally infected cats collected at Dep of Pathology, SLU 1993–2005 (for clinical and pathology information, see Table 1). Cat numbers refer to Lundgren et al. (1997). The analysis was performed using the Rotor-Gene 3000. Negative controls (asymptomatic cats) are not shown.

the BDV strains V, He/80, No/98 and H1766. However, these rRT-PCR assays have never been proven to detect BDV No/98. Thereby, this study describes the first rRT-PCR assay that is able to detect the BDV strains V and He/80, as well as the most divergent BDV No/98 strain. Furthermore, the assay detects five field strains and a feline isolate used for experimental infection. The assay has a high sensitivity both for the BDV P and BDV L genes, respectively. In the duplex assay, detecting both genes simultaneously, the sensitivity of the BDV L gene is decreased 10-fold. This indicates that the assays are used preferably separately. Other assays in this laboratory have at first been designed as multiplex assays, but due to the competition between the primer systems, the sensitivity has been low for diagnostic applications. Therefore, they are instead used as separate assays run at the same time side by side. If robots are used for nucleic acid extraction and reaction mix preparation, this approach will not be much more time-consuming than a multiplex assay approach.

Next step to establish a clinical diagnostic test for BDV infection in the living cat will be investigation of different types of sample material. BDV RNA has been found in nasal and conjunctival fluids, saliva and PBMCs in horses and sheep (Berg et al., 1999; Dauphin et al., 2001; Nakamura et al., 1995; Richt et al., 1993; Vahlenkamp et al., 2002). The distribution of BDV in naturally infected cats is still unknown. However, BDV RNA has previously been found in PBMCs of cats (Nakamura et al., 1996; Nishino et al., 1999; Reeves et al., 1998).

In summary, this study presents a highly specific and sensitive rRT-PCR assay for the detection of two BDV genes, p24 and L polymerase. The assay enables a rapid detection (less than two hours using the Rotor-Gene 3000) and the ability to quantify the number of copies. This is the first published rRT-PCR assay that detects both the standard BDV strains V and He/80, as well as the most divergent BDV strain known so far, No/98. Considering the results of this study, rRT-PCR will be a powerful tool in the further studies of BDV, including epidemiological screening, diagnosis and future therapy evaluations.

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