Staggering disease in cats in Sweden: Development of molecular methods for detection and genetic characterization of Borna disease virus strains

Jessica Schenck

Main supervisor: Jonas J. Wensman PhD student
Assistant supervisor: Anne-Lie Blomström PhD student

Master thesis, 30 HEC
Department of Biomedical Sciences and Veterinary Public Health
Division of Parasitology and Virology
SLU, Uppsala, 2008

Abstract

The aim of this project was to get clearer knowledge about the spread of Borna disease virus (BDV) by comparing sequences from BDV infected cats from different regions in Sweden. BDV is associated with staggering disease (SD), a feline neurological disorder that causes non-suppurative meningoencephalomyelitis. However, cats showing signs for SD are not always confirmed to be BDV infected. In order to get a broader picture of the possible etiological cause/s of SD, molecular screening of viruses using random amplification and sequencing was performed. This study includes eight cats, whereas four were previously positive for BDV in nested RT-PCR and real-time RT-PCR analysis and four cats, only histopathology examined, where three showed signs for SD. Molecular epidemiology approach with RT-PCR and nested RT-PCR for previously positive cats gave PCR products at incorrect as well as correct sizes. However, sequencing showed only feline origin. Since real-time RT-PCR also was negative, one hypothesis is that the RNA had degraded or disappeared through the RNA extraction process. Nested PCR was also performed for the four new cats. Also here the sequencing showed only presence of feline origin. However, for these cats, real-time RT-PCR was positive for the pooled PCR product. Molecular screening of viruses for these four cats showed as before only sequences of feline origin. This project has identified some of the problems of studying RNA viruses present in small amounts in brain tissue. There is always a risk that the RNA is lost during the RNA extraction process. Therefore, further studies are needed to provide more information about the BDV molecular epidemiology in Sweden.

Sammanfattning

Syftet med projektet var att få mer kunskap om spridning av bornavirus (BDV) genom att jämföra sekvenser från BDV-infekterade katter från olika regioner i Sverige. BDV är associerat med vingelsjuka hos katt, en neurologisk sjukdom som karakteriseras av en non-purulent meningoencefalomyelit. Dock kan vingelsjuka inte alltid kopplas till BDV-infektion. För att få en bredare bild av tänkbara etiologiska orsaker till vingelsjuka utfördes även molekylär screening av virus med slumpmässig amplifiering och sekvensering. Här studeras åtta kattprover, varav fyra tidigare positiva för BDV vid PCR och realtids-PCR analyser. De resterande fyra katterna hade enbart undersökts histopatologiskt där tre visade tecken på vingelsjuka. PCR-analysen för tidigare positiva katter gav band vid fel eller korrekta storlekar. Sekvensering visade dock enbart PCR-produkt från katt. Eftersom även resultatet från realtids-PCR var negativt finns det risk för att RNA har degraderat eller försvunnit under extraheringen. PCR-analys utfördes för de fyra nya katterna som enbart studerats histopatologiskt. Även här visade sekvensering enbart RNA från katt. För dessa katter gav emellertid realtids-PCR ett positivt resultat för poolad PCR-produkt. Molekylär screening av virus för dessa fyra katter visade bara på värdprodukt för både DNA och RNA. Detta projekt visar problematiken med RNA-virus, som finns i små halter i hjärnvävnad. Det finns även en risk att RNA försvinner vid extrahering av RNA. Därför krävs ytterligare studier för att ge mer information om molekylär epidemiologi av BDV i Sverige.

Content

Introduction

Viruses affect a wide variety of organisms including animals, plants, bacteria and humans. There are more than 4000 different viruses and 30 000 strains that could affect a living organism. Since viral infections in humans and animals often results in an immune response and disease there is a great deal of research going on within this area (Murphy *et al.,* 1999).

One viral infection that causes a neurological disease is Borna disease virus (BDV). It affects mainly horses and sheep in central Europe and occurs in endemic waves. Signs include behavioural changes and difficulty moving extremities leading to ataxia. Studies based on thousands of clinically diagnosed horses in Germany indicated that additional signs such as depression, excitation and sensory disturbances in motility could also be observed. The infection lasts between one to three weeks and when signs develop, the outcome is generally fatal. However, it has been found that animals could carry BDV without developing observable signs (Ludwig & Bode, 2000).

BDV is associated with staggering disease (SD) also named feline Borna disease (BD), a feline neurological disorder showing histopathological characteristics of a viral infection in the central nervous system (CNS) (Lundgren, 1992; Lundgren *et al.,* 1997). In Sweden almost 100 diagnosed cases of SD have been reported in the last ten years. Most cases are in the middle of Sweden but sporadic findings of the southern and northern parts have also been observed (J. Wensman, unpublished data).

Since BDV infected cats probably occur in endemic areas, a deeper knowledge of molecular epidemiology of BDV is needed and therefore phylogenetic studies of BDV could be one approach. However, cats showing signs for SD are not always associated and confirmed to be BDV affected (Staeheli *et al.,* 2000). Therefore another approach could be to detect unknown viruses and/or BDV using a broader molecular screening of viruses. However, this approach has some technical drawbacks and there are some problems with host ribosomal RNA when searching for RNA viruses. Furthermore, there are some bioinformatics problem, such as how to handle unknown sequences and incorrect annotations in databases (Allander *et al*., 2005).

Borna disease virus (BDV)

History

Clinical signs of Borna disease (BD) was probably first described in 1660, where horses with pain were described as dumb and dull. The name Borna has its origin from the city of Borna in south-eastern Germany where a severe outbreak of the disease occured around 1900. Since the disease had an economic impact it neccessitated extensive veterinary investigations, showing that the diseased horses had inflammatory reactions in the midbrain. Intranuclear inclusion bodies, a characteristic sign of certain viral infections were later found in the brain of horses and these were named Joest-Degen after the authors of this discovery. In the 1930s, a German veterinarian named Zwick, succeded to isolate the virus by adapting it into rabbits (Ludwig & Bode, 2000). However, it was not until early 1994 that the full-length genome of BDV was sequenced (Cubitt *et al.,* 1994).

Genome

BD is caused by a RNA virus. Related families are *Paramyxoviridae*, *Rhabdoviridae* and *Filoviridae*, therefore BDV was classified as the family *Bornaviridae* in the order of *Mononegavirales* (Murphy *et al.,* 1999). The virus genome is around 8.9 kilo bases and single-stranded of negative-sense and non-segmented (Cubitt & de la Torre, 1994).

The spherical virions of BDV are around 90 nm in diameter and the core is around 50-60 nm in diameter. BDV is transcribed in the nucleus of the host cells and there the mRNA, that code for more than one protein (polycistronic) is produced in high levels (Murphy *et al.,* 1999). Within animals, BDV is the only known negative-strand RNA virus that has this characteristic property (de la Torre, 2006). BDV contains six open reading frames (ORFs) that encode the following proteins: nucleoprotein, phosphoprotein transcriptional activator, matrix protein, surface glycoprotein, RNA-dependent RNA polymerase, polypeptides and the p10, see Figure 1 (de la Torre, 2006; Kolodziejek *et al.,* 2005).

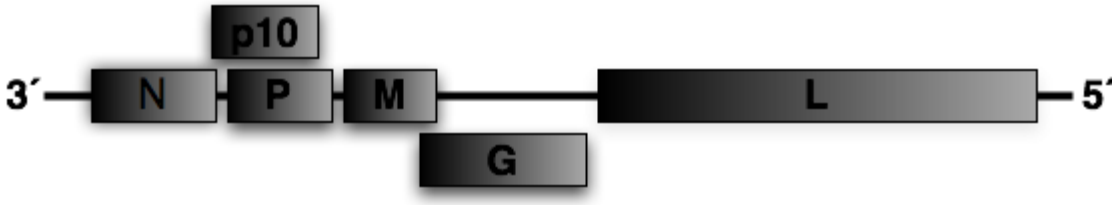

Figure 1. The organization of the BDV genome into six ORFs that encodes for proteins ordered in following direction: nucleoprotein, phosphoprotein transcriptional activator (P), matrix protein (M), surface glycoprotein (G), RNA-dependent RNA polymerase (L) and the p10 ORF, also named X that encodes a polypeptide of 10 kDa (de la Torre, 2006; Kolodziejek *et al.,* 2005).

Epidemiology

BDV infection in mammals and birds

BDV has a broad host range including predominantly horses and sheep, but it has also been shown to infect cats, dogs and cattle. In horses, BDV is endemic in primarily eastern Germany. However, affected horses have also been found in other countries in central Europe including Switzerland and regions in Austria (Ludwig & Bode 2000). Antibodies to BDV have also been reported in sporadic findings in healthy horses in the United States of America, Iran and Japan (Kao *et al.,* 1993; Bahmani *et al.,* 1996; Hagiwara *et al.,* 2002).

In sheep, BDV is endemic in primarily Germany, and in Switzerland there have been finding of BDV in sheep and goat. Furthermore, BDV antibodies have also been found in Japan, where Hagiwara *et al*. (1997) found presence of antibodies in healthy sheep. Ludwig & Bode, (2000) have also reported prevalence of BDV antibodies in two herds of healthy sheep in Germany. The signs of BDV infection in sheep are the same as in horses, i.e., disturbances in the CNS. However, sheep show less significant signs as compared to horses.

Few cases of BD in cattle have been reported in Germany and Switzerland (Bode *et al.,* 1994). The cattle showed neurological signs as progressive paralysis and the clinical picture resembles bovine spongiform encephalopathy (BSE) (Ludwig & Bode, 2000). Antibodies to BDV have also been found in healthy dairy cattle in Japan (Hagiwara *et al.* 1996). Hagiwara

et al., (2008) also reported BDV in wild Japanese macaques that were captured due to agricultural damage.

Feline BDV infection has not only been reported in Sweden, there have also been cases in Austria, Germany, Japan and Australia (Nowotny & Weissenbock, 1995; Huebner *et al.,* 2001; Nakamura *et al.,* 1999; Kamhieh *et al.,* 2006). Reports of BDV-antibodies in Japan by serology studies in house cats, with or without neurological signs was obtained by Nakamura *et al.,* (1999).

BDV infection has also been reported in humans with psychiatric disorders where antibodies, antigen and viral RNA were detected (for example Bode *et al.,* 2001 & Nakamura *et al.,* 2000) However, these results have later been disputed (Dürrwald *et al.,* 2007).

Findings of BDV in different species and regions of the world indicate that BDV may be more widespread in a sub-clinical form, with probably long incubation periods. Furthermore, BDV may not necessarily be restricted to historically endemic areas as in eastern Germany (Ludwig & Bode, 2000).

Genetic variation

Several BDV strains have been sequenced from different animals as for example horses and sheep. Comparison of different BDV sequences revealed that most of the strains are closely related and that the nucleotide sequences do not differ more than 5 % (Kolodziejek *et al.,* 2005). European BDV sequences have non-random distribution of base exchange, indicating that the strains probably originate from a stable progenitor. However, some of the strains from horses, sheep and cat in Japan, Iran and United States are almost identical to the laboratory strain, V. Therefore there have been discussions of accidental contamination (Staeheli *et al.,* 2000).

BDV infection in Sweden

In Sweden, reports of BDV infection in horses, lynx and cats have been reported (Berg *et al.,* 1999; Degiorgis *et al.,* 2000; Lundgren *et al.,* 1997). A study of clinically healthy racing

horses and racing horses with diffuse neurological signs, showed higher BDV seroprevalence in the group with neurological signs and BDV RNA in blood samples was also found. These results indicate an association with BDV infection and atypical disease pattern as diffuse neurological signs in horses. This study included only 23 horses showing neurological signs, therefore further studies are needed to get a clearer knowledge about the epidemiology of BDV in horses in Sweden (Berg *et al.,* 1999).

In 1999, a free-ranging lynx was shot outside Gävle because of abnormal behaviour. Histopathological examination showed that the lynx suffered from non-suppurative meningoencephalitis, noticeable similar to SD, associated with BDV infection in cats (Degiorgis *et al.,* 2000). The presence of BDV infection in the brain was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) analysis, immunohistochemistry and *in situ* hybridization. This is the only known case of BDV in lynx.

The presence of BDV in Swedish cats has been confirmed by Lundgren *et al.* (1995), Wensman *et al.* (2007) and others. Most feline BD cases are reported around "Mälardalsregionen", in the middle of Sweden. However, there have also been sporadic findings of BDV outside this region. Since the disease probably is wide-spread a deeper knowledge of molecular epidemiology of BDV is needed.

Reservoirs unknown

Based on the geographical localisation, seasonal pattern and animal species infected there is a hypothesis of a natural reservoir of BDV (Dürrwald *et al.,* 2006). Furthermore, wild birds are known as a natural reservoir of viruses and therefore Berg *et al.* (2001) performed studies on faecal samples from wild birds in Sweden. The study showed that jackdaws and mallards could be infected carriers of BDV and thereby a reservoir of BDV. Recently, viral RNA was found in a bicoloured white-toothed shrew in endemic BD area in Switzerland (Hilbe *et al.,* 2006) and BDV-specific antibodies were found in voles in Finland (Kinnunen *et al.,* 2007).

Staggering disease

History

In cat, non-suppurative meningoencephalomyelitis of unknown etiology was first reported by Kronevi *et al.* (1974) where thirty cats with neurological disorders were examined. Changed behaviour as anxiety, more affection to the owner and muscular weakness in the hind-legs causing ataxia was observed. Within one to four weeks most of the cats died due to severe neurological signs. Lymphocytes and histiocytes, involved in the immune system were found in the spinal cord, meninges and brain. It has been confirmed that SD is not caused by other virus infections such as feline infectious peritonitis virus, feline leukaemia virus or feline immunodeficiency virus (Lundgren, 1992).

Since the disease still had an unknown etiology the research continued and Lundgren & Ludwig (1993) established that the cause probably was BDV. It was confirmed by antibodies in Swedish cats with SD. The results showed an antibody prevalence of 44 % in affected cats. However, further studies as for example virus isolation was needed to confirm those results since BDV at that time only had been verified in sheep and horses. Lundgren *et al*. (1995) described presence of viral antigen and RNA using RT-PCR, immunohistochemistry and antigen-ELISA. The results showed BDV-specific antigen and RNA in feline CNS samples. Furthermore, a feline variant of BDV was isolated. However, further studies have to confirm the presence of BDV since the amount of BDV antigen and RNA in horses was higher compared to cats.

An experimental infection in 1997, Lundgren *et al.* (1997) provided strong evidence that feline SD is associated with BDV. This was obtained by inoculating cats intracerebrally with feline BDV or BDV laboratory reference strain V from rabbit. The results showed that three out of eight cats developed neurological signs similar to SD as well as non-suppurative meningoencephalomyelitis. This is a strong indication that BDV is the etiological agent of SD.

Clinical and histopathological signs

SD has several clinical signs including changed behaviour like anxiety, depressive symptoms, increased mewing and more affection to the owner. Muscular weakness in the hind-legs causing ataxia, lumbosacral pain, inability to withdraw claws and a staring gaze is also observed (Kronevi *et al.,* 1974; Lundgren 1992). Within one to four weeks the disease progress from sub-acute to chronic stage where most of the cats die due to severe signs described above. Since clinical signs alone could not be used for diagnosis of the disease further analysis is needed (Kamhieh & Flower, 2006).

Histopathological findings in cats infected with BDV shows a non-purulent meningoencephalomyelit, mainly in the grey matter of the brain (mesencephalon), basal ganglia and hippocampus. Furthermore, BDV could also be found in cortex, see Figure 2 (Lundgren 1992; Lundgren *et al.,* 1995).

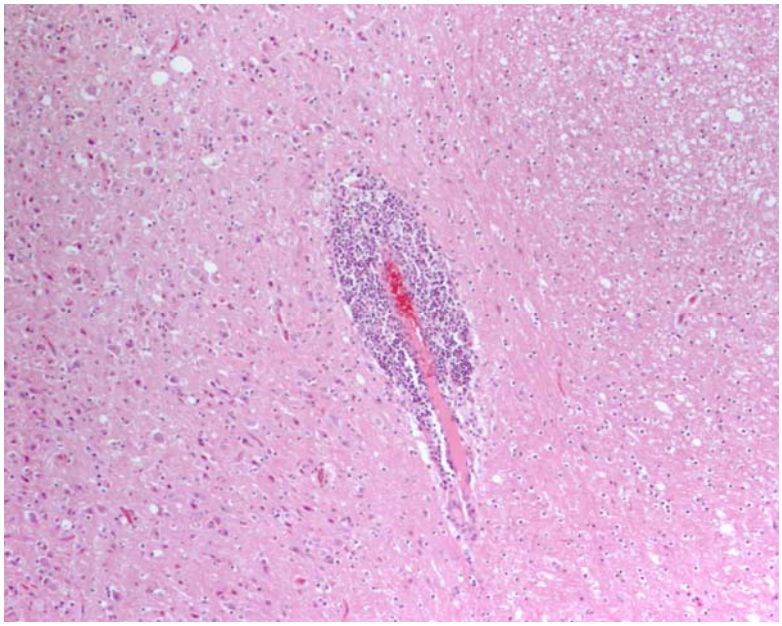

Figure 2. Histopathology of brain tissue from a cat with SD (O.55/08). A characteristic perivascular mononuclear cuff can be seen as well as diffuse inflammation in the parenchyma. Photo: Gete Hestvik, SLU

Aim

The aim of this project was to get clearer knowledge about the spread of BDV and genetic diversity by comparing sequences from BDV affected cats and horses from different areas in Sweden. The approach was first to test different primer combinations to amplify overlapping parts of the BDV genome using classical PCR. The same approach was used for samples from cats with SD that previously have been positive for BDV. In order to get a broader picture of the possible etiological cause/s of SD, molecular screening of viruses using random amplification and sequencing were also performed (Allander *et al.*, 2005).

Materials and methods

Infected cells

RNA from BDV infected C6 cells (C6BDV from strain He/80) were used for the optimisation of the different primers.

Animals and tissue

In total, seven of eight Swedish cats, clinically diagnosed with SD and non-suppurative meningoencephalomyelitis, were studied; O.69/93, O.423/92, O.388/96, O.65/07, O.55/08 and O.65/08. The sample ID translates: identification number/year collected. Furthermore one cat (O.67/07) that showed no signs of infection in the CNS was also included since it had the same owner as O.65/07.

Four of the cats; O.69/93, O.423/92, O.407/94 and O.388/96 had previously been positive at least once in nested PCR and real-time RT-PCR assays. Four cats; O.65/07, O.67/07, O.55/08 and O.65/08 had only been histopathology examined. For detailed information of the cats, see Table 1. All samples were stored in -70°C freezer (Johansson *et al.,* 2002; Wensman *et al.,* 2007; M Berg & J Wensman, unpublished data).

Table 1. Clinically diagnosed SD cats from Sweden and earlier results from ELISA, real-time RT-PCR for BDVp23 and nested PCR for BDVp23 and BDVp40 (Johansson *et al.,* 2002; Wensman *et al.,* 2007; M Berg & J Wensman, unpublished data).

Cat ID: identification number/year collected:part of brain.	**Year collected**	**Histopathology**	**Serology**	**Real- time RT-PCR BDVp23**	**Nested PCR BDVp23**	**Nested PCR BDVp40**
Molecular detection and epidemiology						
O.69/93: cortex	1993	Non-supp meningoencephalomyelitis	FIV, Borrelia and Ehrlichia – all neg	Positive	Positive	Positive
O.423/92: cerebellum	1992	Non-supp meningoencephalomyelitis	Borrelia and Ehrlichia – neg	Negative	Positive	Positive
O.407/94: cortex	1994	Non-supp encephalitis	Not tested	Positive	Negative	Positive
O.388/96: mesencephalon	1996	Non-supp meningoencephalitis	BDV – pos by IFA, neg by ELISA	Positive	Positive	Positive
Detection of unknown DNA or RNA viruses and/or BDV						
O.65/07: cortex	2007	Non-supp meningoencephalitis	Not tested	Not tested	Not tested	Not tested
O.67/07: brain stem	2007	Negative section, no signs of infl in CNS	Not tested	Not tested	Not tested	Not tested
O.55/08: mesencephalon	2008	Non-supp meningoencephalomyelitis	Not tested	Not tested	Not tested	Not tested
O.65/08: mesencephalon	2008	Non-supp meningoencephalomyelitis	Not tested	Not tested	Not tested	Not tested

Molecular methods for detection and genetic characterization of BDV

RNA extraction

Total RNA was extracted from brain tissue samples; O.69/93, O.423/92, O.407/94 and O.388/96 according to RNeasy Lipid Tissue Mini Kit manual (Qiagen).

Brain tissue samples of sizes about 0.5 cm^3 were cut out and homogenized in 250 µl Qiazol (Qiagen) and thereafter further 500 µl Qiazol was added. The samples were incubated at room temperature for 5 minutes and then 150 µl of chloroform was added. The samples were shaken vigorously for 15 seconds and incubated for 2-3 minutes in room temperature and

centrifuged at 12 000 g for 15 min at 4°C to get three different phases in the following order, from upper phase to lower phase: colourless aqueous phase containing RNA; a white interphase containing DNA; red phase containing organic material. The aqueous phase volumes were estimated and then the same volume of 70% ethanol was added and the samples were mixed by vortexing. The samples were collected in an RNeasy Mini Spin Column (Qiagen) and centrifuged at ≥8000 g for 15 seconds at 25°C. To wash the columns, 700 µl buffer RW1 was added to the columns respectively, and centrifuged for 15 seconds at ≥8000 g. The column were washed with additional 500 µl buffer RPE and centrifuged for 15 s at ≥8000 g. Further 500 µl buffer RPE was added to each of the columns and the samples were centrifuged for 2 minutes at ≥8000 g to dry the RNeasy silica-gel membrane. The RNA was eluted by adding 30 µl RNase-free water and centrifuge for 1 minute at ≥8000 g. The elution procedure was carried out twice to get the total of 60 µl of RNA sample.

The RNA concentration and purity were measured by NanoDrop ND-1000 spectrophotometer at 260/280 ratio. The ratios were over 1.8-1.9 suggesting that the samples were purified and clean. Therefore the procedure continued with cDNA synthesis.

cDNA synthesis

The RNA was converted to full-length cDNA using reverse transcriptase (RT) from SuperScript™ III cDNA Synthesis System (Invitrogen). The mixture consisted of a total reaction of 40 µl where mixture I contained 2.5 mM random primer oliogonucleotides (pdN6), 0.5 mM dNTP mix (Fermentas) and 500 ng of total RNA. Mixture II contained 1× First Strand buffer (Invitrogen), 5 mM DTT (Invitrogen), 0.25 U ribonuclease (RNase) Out (Invitrogen) and 500 U of SuperScript III (Invitrogen). The reactions were incubated at 65°C for 5 minutes followed by 4°C for 5 minutes and during this time the samples were on ice for 1 minute and thereafter mixture II was added. The reaction continued at 25°C for 5 minutes, 50°C for 30 minutes, 55°C for 30 minutes and 70°C for 15 minutes. The cDNA synthesis was performed on a thermal cycler, Minicycler (MJ Research).

Primer design

Different primer pairs were selected according to Kolodziejek *et al.* (2004). The primers were BDV1f, BDV218r, BDV587f, BDV652r, BDV778f, BDV796f, BDV1161r, BDV1327f, BDV1518r, BDV1695f, BDV1837r, and BDV2138r where the number indicate the nucleotide position in the BDV genome (strain V, accession number U04608). Furthermore, using primer design tools from Cybergene (Cybergene´s homepage, 2008) one more primer, BDV2383r was designed to overlap the wanted region. Other primer sequence pairs BDVp23A and BDVp23B, BDVp23C and BDVp23D, BDVp40A and BDVp40B, BDVp40C and BDVp40D, BDV gp18f and BDV gp18r have been used previously (Berg *et al.,* 2001 and M Berg, unpublished data). See Appendix 1, Table A and Table B for primer sequences and primer pairs. All primers were ordered from DNA technology A/S.

All primers were tested in C6BDV and thereafter used for classical PCR by using Phusion™ DNA polymerase (Finnzymes). Primer sequence pairs used for the first run in the nested PCR were BDVp23AB, BDVp40AB with BDVp23CD and BDVp40CD as inner pair for the second PCR, respectively.

PCR analyses

PCR amplification of cDNA was performed by Phusion™ DNA polymerase (Finnzymes). A master mix was prepared for a final 25 µl PCR reaction with the following components: 1× Phusion High-Fidelity (HF) Buffer, 0.2 mM dNTP mix (Fermentas), 4 U Phusion™ DNA polymerase (Finnzymes), 0.4 µM primers and 1 µl of cDNA or DEPC-H_2O as negative control. Second nested PCR was performed with the same protocol with the exception of template that was 1 µl of PCR product from first nested PCR. Cycling conditions for first round and nested PCR was 98°C for 3 minutes, 35 cycles of amplification (30 seconds at 98°C, 30 seconds at 60°C and 45 seconds at 72°C) followed by 72 °C for 4 minutes.

BDV primer pairs that were used for O.388/96 and O.423/92 were following: BDV1f and BDV652r, BDV778f and BDVp23B, BDV1327f and BDVp23B, BDVp23C and BDV2383r. Nested PCR was performed for O.69/93, O.423/92, O.407/94 and O.388/96 by following primer pairs: BDVp40AB with nested primers BDVp40CD and BDVp23AB with nested primers BDVp23CD.

Comparison of the two enzymes, AmpliTaq® (Applied Biosystems) and Phusion™ (Finnzymes) was performed by using cDNA from O.388:96 and O.407:94. PCR amplification of cDNA was performed by Phusion™ DNA polymerase (Finnzymes) as described previously and PCR amplification by AmpliTaq® DNA polymerase (Applied Biosystems) was performed as following; a master mix was prepared for a final 25 µl PCR reaction with the following components: 1× GeneAmp PCR Buffer II (Applied Biosystems), 0.2 mM dNTP mixture (Fermentas), 2.5 mM $MgCl_2$, 0.05 U/µl AmpliTaq® DNA polymerase (Applied Biosystems), 0.4 µM primers and 1 µl of cDNA sample or DEPC-H_2O as negative control. Primer pairs that were used were: BDVp40AB with nested primers BDVp40CD and BDVp23AB with nested primers BDVp23CD.

Cycling conditions for the classical PCR and the first round of nested PCR were 95°C for 2 min, 35 cycles of amplification (30 seconds at 95°C, 30 seconds at 55°C and 90 seconds at 72°C) followed by 72°C for 5 min and then 4°C. Cycling conditions for second run of the nested PCR were 95°C for 2 min, 40 cycles of amplification (30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C) followed by 72°C for 5 min.

In second nested PCR with AmpliTaq® DNA Polymerase (Applied Biosystems) the reaction was evaluated by changing the annealing temperature to following temperatures: 50.0°C, 50.8°C, 51.7°C, 52.8°C, 54.1°C, 55.2°C 55.4°C, 55.8°C 56.7°C, 57.8°C 57.9°C, 58.8°C, 59.1°C, 59.9°C, 60.4°C, 61.7°C, 62.9°C, 63.9°C and 64.9°C. The reaction was performed by using primer pairs: BDVp40CD and cDNA from O.407/94.

Nested PCR with AmpliTaq® DNA polymerase (Applied Biosystems) were performed for four cats; O65/07, O.67/07, O.55/08 and O.65/08 that only had been histopathology examined. Used primer pairs were BDVp23AB, BDVp40AB with BDVp23CD and BDVp40CD as inner pair for the second PCR, respectively.

Evaluation of cDNA synthesis

Evaluation of cDNA samples for O.69/93, O.423/92, O.407/94 and O.388/96 were performed with real-time RT-PCR by using Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), see

Appendix, Table A for sequences (Leutenegger *et al.,* 1999). A master mix was prepared for a final 25 µl reaction with the following components: 1×Buffer II (Applied Biosystems), 4 mM $MgCl_2$ (Applied Biosystems), 0.4 µM primer pairs *GAPDH* (BioSearch Technology), 0.5 µl Oligo nucleotide probe, 2.5 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems) and 1 µl of cDNA. As a positive control, cDNA from a feline mesenteric lymphnode was used and DEPC-H_2O was used as negative control. Cycling conditions for real-time RT-PCR were 95°C of 15 minutes followed by 45 cycles of amplification (95°C of 10 seconds and 60°C of 45 seconds). The evaluation of cDNA was performed on a Rotorgene 3000, Corbett Research.

Spiked RNA from C6BDV and infected cat

Synthesis of cDNA was performed according to the protocol previously described. In cDNA synthesis reaction, RNA from the cats, O.388/96 and O.407/94 were spiked with RNA from C6BDV in different concentrations where C6BDV RNA was diluted in dilution buffer containing DEPC-H_2O and 10 µg of sheared salmon sperm DNA (Ambion). A 10-fold dilution series from 1:100 to $1:10^{10}$ were made. Nested PCR with AmpliTaq® DNA polymerase (Applied Biosystems) was performed as previously described and primer sequence pairs were BDVp23AB, BDVp40AB with BDVp23D and BDVp40CD as inner pair respectively.

Analysis of classical and nested PCR products

PCR products from first round and nested PCR were analysed with gel electrophoresis. The products were purified using QIAquick PCR Purification Kit Protocol, (Qiagen) and products from gel electrophoresis were purified using QIAquick Gel Extraction Kit Protocol, (Qiagen). Thereafter 2 µl of purified PCR product was analysed on a gel to determine amount of product for cycle sequencing.

Sequence analysis

Cycle sequencing was performed by preparing a master mix for a final volume of 20 µl containing: 2 µl BigDye (Applied Biosystem), 3 µl sequencing buffer (Applied Biosystem), 2 µM primer and 3-10 ng of PCR product. Cycling conditions for cycle sequencing was 25

cycles of amplification (95°C for 15 seconds, 50°C for 10 seconds and 60°C for 4 minutes). Cycle sequencing was followed by precipitation in room temperature by adding 2 µl of 3M natrium acetate (NaAc) and 40 µl of 99 % ethanol followed by vortex. The samples were kept in room temperature for 20 minutes and centrifuged at 13 000 rpm for 20 minutes. All ethanol was removed and 250 µl of 75 % ethanol was added. The samples were centrifuged at 13 000 rpm for 20 minutes in room temperature. All ethanol was removed and the pellet was dried up for 10-15 minutes.

Sequences were analysed with BioEdit sequence aligment editor and/or SeqMan from DNA-Star Lasergene 7 (DNA-Star) and thereafter BLASTed at NCBIs homepage (www.ncbi.nlm.nih.gov/) by choosing nucleotide blast with highly similar sequences or more dissimilar sequences (2008-02-29; 2008-03-13; 2008-04-25; 2008-05-12)

Detection of unknown viruses

Molecular screening of DNA and RNA viruses using random amplification and sequencing were performed according to Allander *et al.* (2005) to detect possible unknown viruses. Four cats; O.65/07, O.67/07, O.55/08 and O.65/08, only histopathologically studied showing signs for SD, see Table 1.

DNA and RNA extraction

Brain tissue samples of sizes about 0.5 cm^3 were cut out from each of the four cats and homogenized with a single-used pestle (Kontes Glass Company) and then homogenized with 2 ml 1× DNase buffer (Roche). The homogenate was centrifuged with 4000 rpm. for 10 minutes and thereafter the liquid phase was filtrated through a 0.45 µm syringe filter (Millipore). Ten µl of DNase I (10 U/µl) (Roche) and 4 µl of RNase (1µg/µl) were mixed with around 200-250 µl of homogenate.

The DNA extraction was performed using QIAmp DNA Mini Kit according to the Blood and Body Fluid Spin Protocol (Qiagen). The RNA extraction was performed as described previously from the step where chloroform was added. The DNA and RNA concentrations and its purity were measured for each cat sample, by NanoDrop ND-1000 spectrophotometer.

DNA and RNA synthesis

The extracted DNA and RNA samples were processed separately. Labelling and 2nd strand synthesis of the DNA samples were performed by preparing a master mix with a final volume of 15 µl containing: 10 µM primer FR26RV-N (DNA Technology), Neb 2 Buffer (New England Biolabs) and 10 mM of dNTP mixture (Fermentas), see Appendix, Table A for primer sequence. Template was 10 µl of pooled DNA from the four cats, using 2.5 µl from each cat. The sample was run at 94 °C for 2 minutes and incubated on ice for 2 minutes. Thereafter 0.5 µl of 3-5 exo-Klenow fragment of DNA polymerase (New England Biolabs) was added and sample run at 37 °C for 60 minutes, 94°C for 2 minutes and incubated on ice for 2 minutes. Thereafter additional 0.5 µl of 3-5 exo-Klenow fragment of DNA polymerase (New England Biolabs) was added and sample run on 37 °C for 60 minutes and 75 °C for 10 min. Samples were stored at -20°C.

cDNA synthesis and labelling of the RNA samples was performed by preparing a master mix with a final volume of 20 µl, where mixture I contained: 10 µM primer FR26RV-N (DNA Technology) and 1 of mM dNTPs mixture (Fermentas). Mixture II contained: 4 µl 5× First Strand Buffer (Invitrogen), 1 µl DTT (0.1 M) (Invitrogen), 1 µl Rnase out inhibitor (Invitrogen) and 1 µl of SuperScript II reverse transcriptase (Invitrogen). Ten µl of RNA from the four cats, 2.5 µl from each cat was used as template. The template was mixed with mixture I and run at 65°C for 5 minutes. The sample was kept on ice and mixture II was added and run at 25°C for 5 minutes, 50°C for 60 minutes and 70°C for 15 minutes. 0.5 µl of 3-5 exo-Klenow fragment of DNA polymerase (New England Biolabs) was added and the sample was run at 37°C for 60 minutes and at 75°C for 10 min. Samples were stored at -20°C. Both syntheses of DNA and RNA samples reactions were performed on a thermal cycler, Minicycler (MJ Research).

Pooled PCR

Amplification was performed separated for DNA and RNA samples by preparing a master mix with a final volume of 50 µl PCR reaction with the following components: 1× GeneAmp PCR Buffer II (Applied Biosystems), 2.5 mM of $MgCl_2$, 0.2 mM of dNTP mixture

(Fermentas), 0.8 µM pmol primer FR20RV (DNA Technology), 0.05 U/µl AmpliTaq Gold® DNA Polymerase (Applied Biosystems) and 5 µl of template, see Appendix, Table A for primer sequence. Cycling conditions for PCR was 95°C for 11 minutes and 40 cycles of amplification (95°C for 1 minute, 65°C for 1 minute and 72°C for 2 minutes). The amplified products were run on a 1.5 % agarose gel and thereafter purified by QIAquick PCR Purification Kit Protocol (Qiagen) and run on a gel again.

Cloning

Cloning was performed, separated for DNA and RNA samples, using pGEM®-T Easy Vector System I Kit (Promega) according to the manufacturer's instruction by preparing a master mix of a total volume with 10 µl ligation product containing following components: 1× Rapid Ligation Buffer, 50 ng pGEM®-T Easy Vector, 0.5 µl T4 DNA Ligase and 4 µl of PCR product. The mixture was incubated at room temperature for one hour and thereafter 4 µl was used for ligation and the rest was incubated at 4°C for 16 hours. Ligation products were stored at -20°C.

Four µl of ligation product was mixed carefully with 30 µl of competent cells (XL Blue), incubated on ice for 30 minutes followed by 42°C water bath for 45 seconds and one minute on ice. The cells were mixed with 0.5 ml of SOC Buffer (Invitrogen) and on shake for one hour at 37°C. Fifty µl of cells were growing on one LB/amp plate containing ampicillin (LB/amp). The rest of the ligation products were centrifuged for 3 minutes at 10.5 rpm, most of the supernatant discarded and the pellet dissolved in the remaining supernatant and put on a LB/Amp plate. The plated where placed in an incubator over night at 37°C. Single colonies on the LB/amp plates were picked and put in 2 ml of LB medium containing ampicillin and grown in a 37°C shaker for a maximum 15 hours. The samples were stored at 4°C.

PCR screening

Bacteria cells from cloning were used for PCR screening by preparing a master mix with a total volume of 25 µl containing following components: 1× GeneAmp PCR Buffer II (Applied Biosystems), 0.2 mM dNTP mixture (Fermentas), 2.5 mM MgCl2, 0.05 U/µl AmpliTaq® DNA polymerase (Applied Biosystems), 0.4 µM primer T6 and T7 (DNA

Technology) and 1 µl of grown bacteria. Cycling conditions for the PCR screening was 95°C for 12 minutes, 35 cycles of amplification (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds) followed by 72°C for 5 minutes. The PCR products were purified using Exo, SAP (Fermentas). Cycle sequencing and precipitating were performed as described previously with accept ion of a kit from Millipore. Sequences were edited in DNAStar Lasergene 7 (DNAstar) and thereafter BLASTed on NCBIs homepage (2008-04-25; 2008-05-12).

Real-time RT-PCR

Real-time RT-PCR was performed according to Wensman *et al.* (2007) (BDVp23) and Schindler *et al.*, (2007) (BDVp40) with minor modifications, by preparing a master mix for a final volume of 25 µl containing: 1× PCR Buffer (Applied Biosystems) 2.5 mM MgCl, 0.5 mM dNTPs mixture (Fermentas), 0.08 mg/ml BSA, 0.7 µM primers, 0.3 µM probe for p23 or p40 gene and 0.1 U/µl of rTh-polymerase (Applied Biosystems). One µl of the first PCR product was used as template for primer pairs BDVp23AB, BDVp40AB with cDNA from O.407/94 and O.388/96. RNA and cDNA from O.407/94 and O.388/96 was also tested in the real-time RT-PCR.

The following templates from O.65/07, O.67/07, O.55/08 and O.65/08 were also used: RNA from each of the four cats, pooled cDNA sample, pooled RT-PCR sample, purified pooled PCR sample and positive control in two different concentration of BDV infected C6 cells: C6BDV$\times 10^{-5}$ and C6BDV$\times 10^{-6}$. Cycle conditions were following: 5 minutes at 42°C and 20 minutes at 60°C and followed by 55 cycles of amplification (5 seconds at 95°C and 30 seconds at 50°C). The real-time RT-PCR of first nested PCR products, RNA and cDNA was performed on a Rotorgene 3000, Corbett Research.

Results

Molecular methods for detection and genetic characterization of BDV

Primer design

To be able to amplify larger segment suitable for sequencing, different primer pairs were selected according to Kolodziejek *et al.* (2004), Berg *et al.* (2001) and M Berg (unpublished data). All primers were tested in C6BDV cells and thereafter used for classical PCR by using Phusion DNA polymerase (Finnzymes). All primer sequences and primer pairs that worked on C6BDV by using this enzyme are described in the Appendix, Table B. However, some of the primer combinations described by Kolodziejek *et al.* (2004) did not work and those are marked in *italics* in the Appendix, Table B. PCR products were purified and thereafter prepared for sequencing. Sequences were BLASTed at NCBIs homepage (2008-02-05) and revealed BDV sequences, strain He/80.

PCR analysis

First round and nested PCR with Phusion™ DNA polymerase were performed for samples O.69/93, O.423/92, O.407/94 and O.388/96 with different primer pairs, see Appendix, Table B, marked bold. Results from PCR with Phusion™ DNA polymerase showed no bands or bands at wrong sizes for BDVp23CD (391 bases) and BDVp40CD (429 bases). Therefore, a comparison of PCR with Phusion™ DNA polymerase and AmpliTaq® DNA polymerase were performed. Results from PCR with AmpliTaq® DNA polymerase showed band at correct sizes as well as wrong sizes. Several bands were seen in some reactions, see Figure 3A and 3B for a representative example.

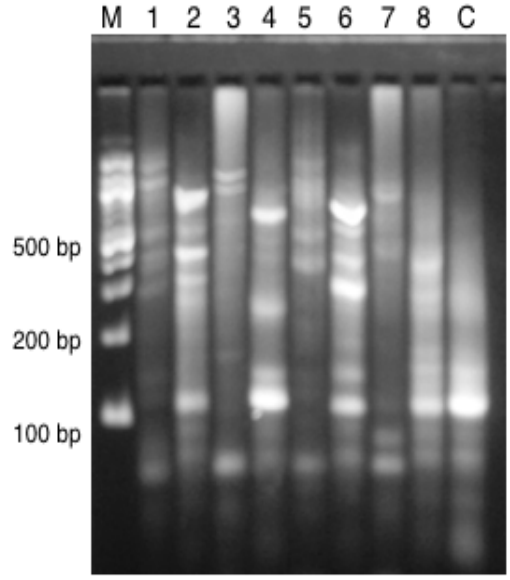

Figure 3A. PCR products with AmpliTaq® DNA polymerase were analyzed on a 1.5 % agarose gel. From left to right: M; DNA size marker (0.1 – 2 kb), 1-4: Feline sample O.407/94 with primer pair BDVp23CD for 1 and 3 and BDVp40CD for 2 and 4, 5-8: Feline sample O.388:96 with BDVp23CD for 5 and 7 and BDVp40CD for 6 and 8, C: Negative control.

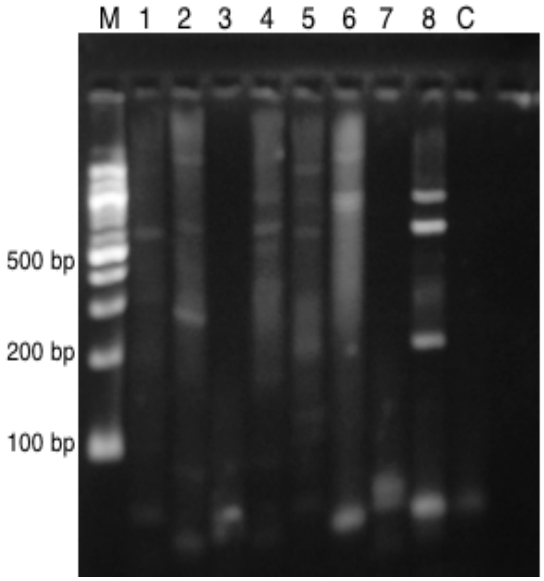

Figure 3B. PCR products with Phusion™ DNA polymerase were analyzed on a 1.5 % agarose gel: From left to right: M; DNA size marker (0.1-2 kb), Feline sample O.407/94 with primer pair BDVp23CD for 1 and 3 and BDVp40CD for 2 and 4, 5-8: Feline sample O.388:96 with BDVp23CD for 5 and 7 and BDVp40CD for 6 and 8, C: Negative control.

Sequences from all PCR products were BLASTed at NCBIs homepage (2008-02-29) and all were found to be of feline origin with no presence of BDV. Evaluation of annealing temperature in second nested PCR with AmpliTaq® DNA polymerase was performed at temperatures between 50.0°C to 64.9°C and showed no significant difference between temperatures. Therefore the procedure continued with an evaluation of cDNA samples for O.69/93, O.423/92, O.407/94 and O.388/96 with real-time RT-PCR of feline *GAPDH*. All samples showed a positive result for *GAPDH* at 82 bases, except for the negative control, indicating that the cDNA quality is acceptable.

Since the PCR analysis showed multiple unspecific bands, we wanted to evaluate the method. In order to do this, RNA from the cats, O.388/96 and O.407/94 was spiked with diluted C6BDV RNA, followed by nested PCR. Sequences from all bands were BLASTed at NCBIs

homepage (2008-04-25) and yielded BDV for cat O.407/94 spiked with C6BDV cells diluted 1:10^6 (1A and 2A) for primer pair BDVp23CD and diluted 1:10^5 for primer pair BDVp40CD. The remaining PCR products yielded feline mRNA from the host and thereby no presence of BDV, see Figure 4.

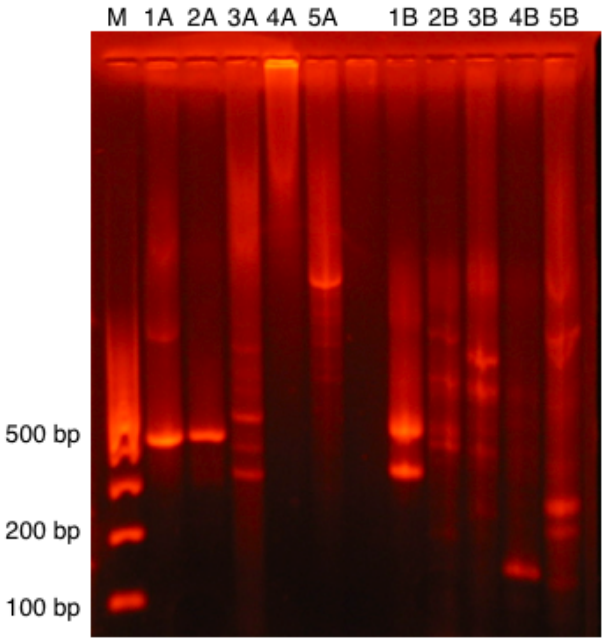

Figure 4. Cat O.407/94 spiked with C6BDV cells in different concentrations. Second nested PCR with AmpliTaq® DNA polymerase were analyzed on a 1.5 % agarose gel. From left to right: L; DNA size marker (0.1 – 2 kb), 1A-5A: Primer pair BDVp23CD with following concentration of C6BDV 1A: 1:10^5, 2A: 1:10^6, 3A: 1:10^7, 4A: 1:10^8, 5A: 1:10^9, C: negative control. 1B-5B: Primer pair BDVp40CD with following concentration of C6BDV 1B: 1:10^5, 2B: 1:10^6, 3B: 1:10^7, 4B: 1:10^8, 5B: 1:10^9.

Nested PCR for BDVp23 and BDVp40 with AmpliTaq® DNA polymerase was also performed for three cats, which had previously only been examined histopathologically; O65/07, O.55/08 and O.65/08. Furthermore, one more cat, O.67/07 that had the same owner as cat O65/07 was included. Sequences were BLASTed at NCBIs homepage (2008-04-25; 2008-05-12) and showed feline mRNA from the host and thereby no presence of BDV.

Detection of unknown viruses

Since molecular detection of BDV failed, another approach that detects unknown viruses was used on only histopathologically examined cats, showing signs for SD. Three cats; O65/07, O.55/08 and O.65/08 showing SD was chosen. Furthermore, one more cat, O.67/07 that came

from the same owner as cat O65/07 was included, see Table 1. All samples were used for RNA and DNA extraction and the cDNA for RNA viruses and DNA for DNA viruses were pooled for all four cats. The DNA and the cDNA samples were used for random PCR amplification and followed by cloning of the purified PCR products. Finally the cloned PCR products were prepared for sequencing which resulted in a total of 113 RNA sequences and 41 DNA sequences. Sequences were edited in DNAStar Lasergene 7, showing 15 contigs (74 sequences between 240-560 bases) for RNA and 4 contigs (8 sequences between 200-430 bases) for DNA. The consensus sequences from all contigs were BLASTed at NCBIs homepage (2008-04-25; 2008-05-12) by using BLASTn. All sequences were defined as host for DNA sample and host mRNA for RNA samples (for example ribosomal RNA, *Felis catus* mitochondrion).

Real-time RT-PCR

Real-time RT-PCR is considered to be a more sensitive method and therefore real-time RT-PCR for BDVp23 and BDVp40 were performed, using cDNA, first PCR product and RNA as template from O.69/93, O.423/92, O.407/94 and O.388/96. Real-time RT-PCR did not reveal the presence of BDV.

Since both nested PCR and detection of unknown viruses showed no presence BDV, real-time RT-PCR was performed to confirm that one or several of the cats had BDV infection. Real-time RT-PCR with BDVp23 was performed for the following templates from O.65/07, O.67/07, O.55/08 and O.65/08. As templates, extracted RNA from each of the four cats, pooled cDNA sample, pooled PCR sample, purified pooled PCR sample and positive control in two different concentrations of C6BDV were used. The real-time RT-PCR yielded positive results for the purified pooled PCR product in the curve between the two positive controls C6BDV $1{:}10^5$ and C6BDV $1{:}10^6$, see Figure 5.

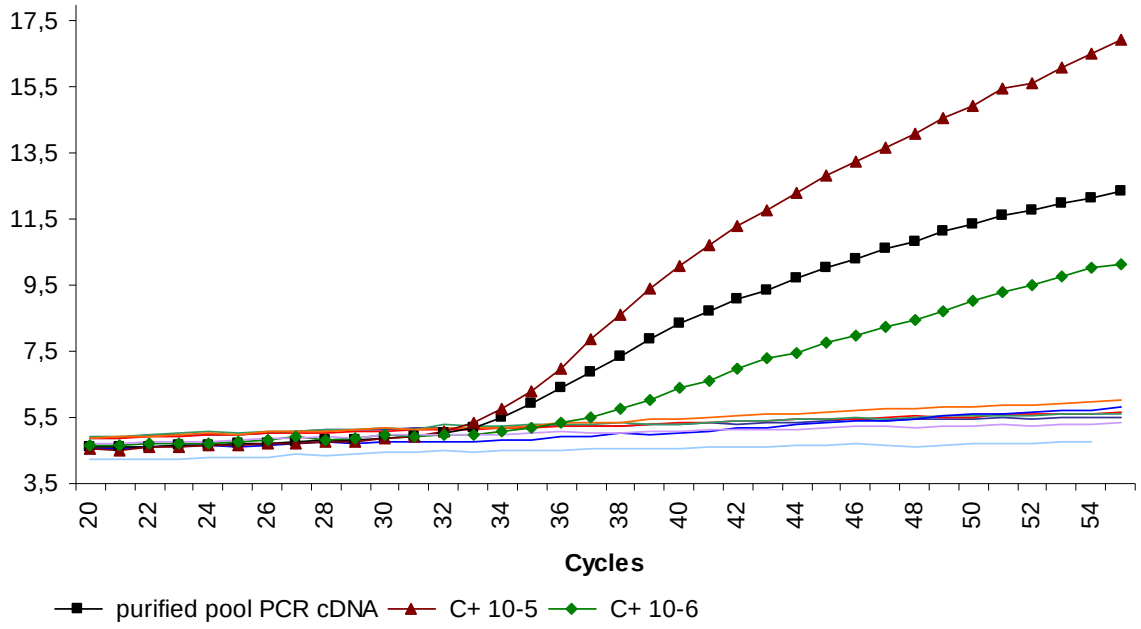

Figure 5. Real-time RT-PCR results. Starting from the uppermost curve: C6BDV 1:10^5, purified pooled PCR samples and C6BDV 1:10^6. The remaining curves correspond to extracted RNA from O.65/07, O.67/07, O.55/08 and O.65/08 and pooled RT-PCR sample.

The positive real-time PCR products were confirmed by cloning PCR products and thereafter sequencing. Sequences were BLASTed at NCBIs homepage and showed BDV for all products (2008-06-04).

Discussion

The aim of this project was to gain clearer understanding about the spread and the genetic diversity of BDV by comparing sequences from BDV infected cats and horses from different areas in Sweden. BDV is associated with SD, a feline neurological disorder. However, cats showing signs of SD are not always confirmed to have BDV infection. In general, these types of chronic infections are extremely difficult to confirm. Therefore, another approach that detects unknown viruses and/or BDV using a broader molecular screening of viruses was evaluated.

Different primer pair was tested in C6BDV using first and nested PCR with Phusion™ DNA polymerase. However, some of the primer pairs from Kolodziejek *et al.* (2004) failed (see Appendix, Table B). There is no obvious explanation why these primer combinations failed. Kolodziejek *et al.* did use another polymerase enzyme but one would not expect that to have such a dramatic affect on the success of the PCR amplification. Another explanation, though unlikely, could be that the synthesis of the primers has failed. All PCR products were sequenced and all of them showed as expected BDV, strain He/80.

The project continued with first and nested PCR for four samples, previously positive in nested PCR and real-time RT-PCR (see Table 1). However, this study showed either none or several bands within one PCR product and/or bands at wrong sizes. Since the cats had been previously positive, a comparison of the enzymes Phusion™ DNA polymerase and AmpliTaq® DNA polymerase, with higher precision, was performed. The comparison indicated that AmpliTaq® DNA polymerase was more reliable (Figure 3A and 3B). However, sequencing of PCR products at correct sizes showed only host RNA and thereby no presence of BDV. Therefore an evaluation of annealing temperature in second nested PCR was performed. The temperature showed no significant difference in bands, indicating that the annealing temperature is correct. Consequently an evaluation of the cDNA product was performed where all products were positive, indicating that the cDNA quality is acceptable. Pooled cDNA samples used for detection of unknown viruses approach (see Table 1) were also used in nested PCR, with PCR products at correct sizes. Again, sequencing showed only feline protein.

Because BDV occurs at low levels in brain tissue, a more sensitive method like real-time RT-PCR also was performed. However, real-time RT-PCR was negative for the old samples used in molecular epidemiology (Table 1).

Since nested PCR failed, no phylogenetic analysis on BDV was able to be performed as initially planned. The lack of results from first and nested PCR as well as real-time RT-PCR could be explained as being due to BDV infected cats having a small amount of virus in brain compared to horses. However, the cats that had previously shown positive results for nested PCR (M. Berg, unpublished data) as well as real-time RT-PCR (Wensman *et al.,* 2007) should yield positive results. Therefore, another explanation could be that the RNA from the virus had degraded through thawing and freezing since the brain samples had been used several times. Another reason could be that the RNA extraction method is not optimal. There is always a risk that the RNA disappears through the extraction steps and when the sample contain small amount of RNA this step is crucial. Another reason might be that the particular piece of brain tissue chosen did not contain BDV.

Because there were no BDV sequences in the molecular epidemiology approach a broader approach was used, according to Allander *et al.* (2005), to detect unknown DNA and/or RNA viruses, and maybe BDV. The samples were pooled and contained brain tissue from four new cats only examined by histopathology, where three showed signs for SD (Table 1). The pooled samples, one for detection of RNA viruses and one for DNA viruses were used for PCR and followed by cloning. Sequencing of cloned PCR products resulted in a total of 113 RNA sequences and 41 DNA sequences with 15 and 4 contigs, respectively. However, all sequences were defined as feline host DNA for DNA samples and feline host RNA for RNA samples.

Since the approach to detect unknown DNA or/and RNA viruses involves sequencing several samples to get even just a single virus, more sequencing of clones has to be performed to confirm the presence of viruses. This requires more laboratory work, and another issue could be cost of multiple sequencing. It is also difficult to get rid of host DNA and RNA. In particular, host ribosomal mRNA is an issue for detection of RNA viruses. Therefore an optimization of this method is needed. There are also some bioinformatics problems as how to

continue with unknown sequences not found in the available databases. The databases could also contain incorrect annotations.

Real-time RT-PCR was negative for the old samples used in molecular epidemiology although positive for the pooled sample used in detection of unknown viruses (see Table 1 and Figure 5). The positive real-time RT-PCR shows that BDV is a contributory factor for SD in cats. There is probably a need of more sensitive methods to perform molecular genetics studies on BDV infected cats. One method to improve the effectiveness of PCR by BDV may be to isolate virus from cat brain in cell cultures to increase the amount of virus, thereby there yielding more material to work with.

Conclusion

This project has identified some of the problems of studying RNA viruses in small amounts in brain tissue. There is always a risk that the RNA is lost during the RNA extraction process and, when the sample contains very low RNA levels to begin with, the extraction step is particularly crucial. Furthermore, RNA can degrade when the sample is thawed and frozen several times. Another factor may be that the causative virus, due to intense immune reaction, has disappeared. Since molecular epidemiology of Borna disease in Sweden remains largely unknown, further studies are needed.

Acknowledgments

Jonas J. Wensman, SLU

Mikael Berg, SLU

Anne-Lie Blomström, SLU

Rest of virology employee at SLU & SVA

Financial support: Agria

References

ALLANDER T., TAMMI M., ERIKSSON M., BJERKNER A., TIVELJUNG-LINDELL A. AND ANDERSSON B. (2005) Cloning of a human parvovirus by molecular screening of respiratory tract samples. *PNAS* 36:12891-12896

BAHMANI M.K., NOWROUZIAN I., NAKAYA T., NAKAMURA Y., HAGIWARA K., TAKAHASHI H., RAD M.A. & IKUTA K. (1996) Varied prevalence of Borna disease virus infection in Arabic, thoroughbred and their cross-bred horses in Iran. Virus Research 45(1):1-13

BERG A.-L., DÖRRIES R. AND BERG M. (1999) Borna disease virus infection in racing horses with behavioral and movement disorders. Archives of Virology 144:547-559

BERG M., JOHANSSON M., MONTELL H. & BERG A.L. (2001) Wild birds as a possible natural reservoir of Borna disease virus. Epidemiol Infect. 127(1):173-178

BODE L., DÜRRWALD R. & LUDWIG H. (1994) Borna virus infections in cattle associated with fatal neurological disease. Vet Rec 135:283-284

BODE L., RECKWALD P., SEVERUS W.E., STOYLOFF R., FERSZT R., DIETRICH D.E. & LUDWIG H. (2001) Borna disease virus-specific circulating immune complexes, antigenemia, and free antibodies--the key marker triplet determining infection and prevailing in severe mood disorders. Molecular Psychiatry 6(4):481-491

CUBITT B. & DE LA TORRE J.C. (1994) Borna Disease Virus (BDV), a Nonsegmented RNA Virus, Replicates in the Nuclei of Infected Cells Where Infectious BDV Ribonucleoproteins Are Present. Journal of Virology 68(3):1371-1381

CUBITT B., OLDSTONE C. & DE LA TORRE J.C. (1994) Sequence and Genome Organization of Borna Disease Virus. Journal of Virology 68(3)1382-1396

DE LA TORRE J.C. (2006) Reverse-genetic approaches to the study of Borna disease virus. Nature Review - Microbiology 4:777-783

DEGIORGIS M.P., BERG A-L., HÅRD AF SEGERSTAD C., MÖRNER T., JOHANSSON M. AND BERG M. (2000) Borna Disease in a Free-Ranging Lynx (Lynx lynx) Journal of Clinical Microbiology 8:3087-3091

DÜRRWALD R., KOLODZIEJEK J, MULUNEH A, HERZOG S. & NOWOTNY N. (2006) Epidemiological pattern of classical Borna disease and regional genetic clustering of Borna disease viruses point towards the existence of to-date unknown endemic reservoir host populations. Microbes and Infection 8:917-929

HAGIWARA K., NAKAYA T., NAKAMURA Y., ASAHI S., TAKAHASHI H., ISHIHARA C., IKUTA K. (1996) Borna disease virus RNA in peripheral blood mononuclear cells obtained from healthy dairy cattle. Med Microbiol Immunol 185(3):145-51

HAGIWARA K., KAWAMOTO S., TAKAHASHI H., NAKAMURA Y., NAKAYA T., HIRAMUNE T., ISHIHARA C., IKUTA K. (1997) High prevalence of Borna disease virus infection in healthy sheep in Japan. Clinical and Diagnostic Laboratory Immunology 4(3)339-344

HAGIWARA K., OKAMOTO M., KAMITANI W., TAKAMURA S., TANIYAMA H., TSUNODA N., TANAKA H., IWAI H. & IKUTA K. (2002) Nosological study of Borna disease virus infection in race horses. Veterinary Microbiology 84:367-374

HAGIWARA K., TSUGE Y., ASAKAWA M, KABAYA H., OKAMOTO M., MIYASHO T., TANIYAMA H., ISHIHARA C., DE LA TORRE J.C. & IKUTA K. (2008) Borna disease virus RNA detected in Japanese macaques (Macaca fuscata). Primates 49(1):57-64

HILBE M., HERRSCHE R., KOLODZIEJEK J., NOWOTNY N., ZLINSZKY K. & ECHRENSPERGER F. (2006) Shrews as Reservoir Hosts of Borna Disase Virus. Emerging Infectious Diseases 12(4):675-677

HUEBNER J., BBODEL., LUDWIG H. (2001) Borna disease virus infection in FIV-positive cats in Germany. Vet Rec 149(5):152

JOHANSSON M., BERG M. AND BERG A-L. (2002) Humoral immune response against Borna disease virus (BDV) in experimentally and naturally infected cats. Veterinary Immunology and Immunophatology 90:23-33

KAMHIEH S. & FLOWER R.L.P. (2006) Borna Disease Virus (BVD) infection in cats. A concise review based on current knowledge. Veterinary Quarterly 28:65-73

KAMHIEH S., HODGSON J., BODE L., LUDWIG H., WARD C. & FLOWER R.L. (2006) No evidence of endemic Borna disease virus infection in Australian horses contrasts with endemic infection in other countries. Archives of Virology 151:709-719

KAO M., HAMIR A.N., RUPPRECHT C.E., FU Z.F., SHANKAR V., KOPROWSKI H. & DIETZSCHOLD B. (1993) Detection of antibodies against Borna disease virus in sera and cerebrospinal fluid of horses in the USA. Vet Rec. 132:241-244

KINNUNEN P.M., BILLICH C., EK-KOMMONEN C., HENTTONEN H., KALLIO E., NIEMIMAA J., PALVA A., STAEHELI P., VAHERI A. & VAPALAHTI O. (2007) Serological evidence for Borna disease virus infection in humans, wild rodents and other vertebrates in Finland. Journal of Clinical Virology 38:64-69

KOLODZIEJEK J., DÜRRWALD R., HERZOG S., EHRENSPERGER F., LUSSY H. & NOWOTNY N. (2005) Genetic clustering of Borna disease virus natural animal isolates, laboratory and vaccine strains strongly reflects their regional geographical origin. Journal of General Virology 86:385-398

KRONEVI T., NORDSTRÖM M., MORENO W. AND NILSSON P. (1974) Felina Ataxia due to Nonsuppurative Meningoencephalomyelitis of Unknown Aetiology. Nord. Vet.-Med. 26:720-725

LEUTENEGGER C.M., MISLIN C.N., SIGRIST B., EHRENGRUBER M.U., HOFMANN-LEHMANN R. & LUTZ H. (1999) Quantitative real-time RT-PCR for the measurement of feline cytokine mRNA. Veterinary Immunology and Immunopathology 71:291-305.

LUDWIG H, BODE, L. (2000) Borna disease virus: new aspects on infection, disease, diagnosis and epidemiology. Rev. sci. tech. Off. Int. Epiz. 19:259-288

LUNDGREN A.-L. (1992) Feline Non-suppurative Meningoencephalomyelitis. A Clinical and Pathological Study. J. Comp. Path. 107:411-425

LUNDGREN, A-L. & LUDWIG, H. (1993) Clinically diseased cats with nonsuppurative meningoencephalomyelitis have BDV-specific antibodies. Acta Veterinaria Scandinavica 34:101-103

LUNDGREN A.-L., ZIMMERMAN W., BODE L., CZECH G., LINDBERG R. AND LUDWIG H. (1995) Staggering disease in cats: isolation and characterization for the feline Borna disease virus. Journal of General Virology 76:2215-2222

LUNDGREN A.-L., JOHANNISSON A., ZIMMERMANN W., BODE L., ROZELL B., MULUNEH A., LINDBERG R. AND LUDWIG H. (1997) Neurological disease and encephalitis in cats experimentally infected with Borna disease virus. Aeta Neurophatol 93:391-401

MURPHY F., GIBBS P., HORZINEK M AND STUDDERT M. (1999) Veterinary Virology. Academic Press. United States of America.

NAKAMURA Y., WATANABE M., KAMITANI W., TANIYAMA H., NAKAYA T., NISHIMURA Y., TSUJIMOTO H., MaACHIDAS. & IKUTA K. (1999) High prevalence of Borna disease virus in domestic cats with neurological disorders in Japan. Veterinary Microbiology 3:153-169

NAKAMURA Y., TAKAHASHI H., SHOYA Y., NAKAYA T., WATANABE M., TOMONAGA K., IWAHASHI K., AMERO K., MOMIYAMA N., TANIYAMA H., SATA T., KURATA T., DE LA TORRE J.C. & IKUTA K. (2000) Isolation of Borna disease virus from human brain tissue. J Virol. 74(10):4601-4611

NOWOTNY N., WEISSENBÖCK H. (1995) Description of feline nonsuppurative meningoencephalomyelitis ("staggering disease") and studies of its etiology. Journal of Clinical Microbiology 33(6):1668-1669

STAEHELI P., SAUDER C., HAUSMANN J., EHRENSPERGER F. AND SCHWEMMLE M. (2000) Epidemiology of Borna disease virus. *Journal of General Virology* 81:2123-2135

WENSMAN J.J., THORÉN P., HAKHVERDYAN M., BELAK S. AND BERG M. (2007) Development of a real time RT-PCR assay for improved detection of Borna disease virus. *Journal of Virological Methods* 143:1-10

Appendix

Table A. Primer sequences that were used in classical and nested PCR (Kolodziejek *et al.*, 2005; J. Wensman & M. Berg, unpublished data), real-time RT-PCR for BDVp23 (Wensman *et al.*, 2007), evaluation of cDNA with real-time RT-PCR for GAPDH (Leutenegger *et al.*, 1999) and PCR screening with FR26RV-N and FR20R in detection of unknown viruses (Allander *et al.*, 2005).

Classical and nested PCR	
Forward (position)	**Bases**
BDV1f	5´-GTTGCGTTAACAACMAACCA-3´
BDV218f	5´-GAACGCAGTGGCATTGTTAG-3´
BDVp40A (278)	5´-GCCTTGTGTTTCTATGTTTGCTAATC-3´
BDV587f	5´-TGGTGAGACTGCTACACTAC-3´
BDV778f	5´-AGGAGTACCTCGCAGAATG-3´
BDV1327f	5´-AGACACTACGACGGGAACGA-3´
BDVp23A (1391)	5´-TGACCCAACCAGTAGACCCA-3´
BDV1695f	5´-GATCGCTCCATGAAGACAAT-3´
BDVgp18f (1893)	5´-GAACGCAGTGGCATTGTTAG-3´
BDVp23C (1447)	5´-TCAGACCCAGACCAGCGAA-3´
BDVp40C (452)	5´-TCTCCTCTATCTTCAGCCATTGTTGC-3´
Reverse (position)	**Bases**
BDV518r	5´-AAGTTAGTGACGGACGCTGG-3´
BDV652r	5´-ATAGATTCCATTAACGGCCA-3´
BDV796r	5´-AAGGAGTACCTCGCAGAATG-3´
BDV1161r	5´-GATAGGTGTGACTGGTCT-3´
BDVp40B (1006)	5´-GGAGTAAGAAGGAAAACCCCACAATGG-3´
BDV1518r	5´-AAGTTAGTGACGGAGCTGG-3´
BDV1837r	5´-GCATTTATCCCCAGCTCCCA-3´
BDVp23B (1869)	5´-GACAACGGATGAATGGGACA-3´
BDV2138r	5´-ACTTCCAGATTGACGACTTC-3´
BDV2383r	5´-GAGATAAGGCGACTTTGCCA-3´
BDVgp18r (2321)	5´-CGCGTCGACCTAAGGCCCTGAAGATCG3´
BDVp23D (1838)	5´-CGCGCATTTATCCCCAGCT-3´
BDVp40D (881)	5´-ATGCTGACCTGTTCCCGTTTCTGG-3´
PCR screening	
FR26RV-N	5´-GCCGGAGCTCTGCAGATATCNNNNNN-3´
FR20R	5´-GCCGGAGCTCTGCAGATATC-3´
real-time RT-PCR	
BDVp23f	5´-GAACCCCTCCATGATCTCAGAY-3´
BDVp23r	5´-CTCYGTCACTAGCTTCTTGATRAG-3´
BDVp23 probe	5´-CAGCGAACCGGAAGGGAGCAGCTATC-3´
GAPDH.57f	5´-GCCGTGGAATTTGCGT-3´
GAPDH.138r	5´-GCCATCAATGACCCCTTCAT-3´
GAPDH.77probe	5´-CTCAACTACATGGTCTACATGTTCCAGTATGATTCCA-3´

Table B. Primer sequence pairs that worked in classical and nested PCR with AmpliTaq® DNA polymerase for C6BDV, He/80. Those combinations that failed are marked as *italic* and those that the project continued with are marked as **bold** (Kolodziejek *et al.,* 2005; J. Wensman & M. Berg, unpublished data).

Forward (position)	**Reverse (position)**	**Length**
BDV1f	**BDV652r**	**652**
BDV1f	BDV1161r	1161
BDV1f	BDVp23B (1869)	1868
BDV218f	BDV796r	578
BDV218f	BDV1161r	943
BDVp40A (278)	**BDVp40B (1006)**	**728**
BDV587f	BDV1161r	574
BDV587f	*BDV1518r*	*931*
BDV587f	*BDV2138r*	*1551*
BDV778f	*BDV1161r*	*383*
BDV778f	*BDV1518r*	*740*
BDV778f	**BDVp23B (1869)**	**1091**
BDV1327f	*BDV1837r*	*510*
BDV1327f	**BDVp23B (1869)**	**542**
BDVp23A (1391)	**BDVp23B (1869)**	**478**
BDVp23A (1391)	*BDV2138r*	*747*
BDV1327f	BDV2383r	1056
BDVp23C (1447)	**BDV2383r**	**936**
BDV1695r	*BDVp23B (1869)*	*164*
BDV1695r	*BDV2138r*	*443*
BDVgp18f (1893)	BDV2383r	490
BDVgp18f (1893)	BDVgp18r (2321)	428
BDVp23C (1447)	**BDVp23D (1838)**	**391**
BDVp40C (452)	**BDVp40D (881)**	**429**