

Development of a real-time PCR
for *in vivo* diagnosis of feline
Borna disease

(Utveckling av en realtids-PCR för *in vivo*-
diagnostik vid vingelsjuka hos katt)

by

Jonas Johansson

Uppsala 2004

Development of a real-time PCR for *in vivo* diagnosis of feline Borna disease

**(Utveckling av en realtids-PCR för *in vivo*-
diagnostik vid vingelsjuka hos katt)**

Jonas Johansson

Supervisor: Mikael Berg, Ph.D., Associate Professor
Section of Immunology and Virology, Department of Molecular Biosciences

Assistant supervisor: Karin Hultin-Jäderlund, DVM, Dipl. ECVN
Department of Small Animal Clinical Sciences

Master's thesis 2004:8
Veterinary program
Faculty of Veterinary Medicine

SLU
ISSN 1650-7045
Uppsala 2004

Abstract

Borna disease virus (BDV) is the causative agent of a neurological disorder in mammals and ostriches called Borna disease. BDV is also of interest as infective agent in humans with psychiatric diseases, such as mood disorders and schizophrenia, as well as in experimental models of these diseases. In Sweden, the progressive and mostly fatal neurological disorder among domestic cats, known as staggering disease or feline Borna disease, is caused by BDV. Except for cats, natural BDV infection has also been described in horses and a free-ranging lynx in Sweden. Major clinical signs of staggering disease are staggering movement, alteration in behaviour, lumbosacral pain and inability to retract the claws. Today staggering disease is clinically suspected by excluding other reasons for clinical signs and the diagnosis is further established by examining the central nervous system histopathologically at autopsy. The diagnosis is finally confirmed by immunohistochemistry. In order to get a better clinical diagnostic tool, a duplex real-time polymerase chain reaction (PCR) assay was developed. This assay has been proven positive with different strains of BDV in infected cell lines and brain tissue samples of experimentally infected cats. Further evaluation of the new assay has to be done, such as testing the sensitivity and finding the best type of sample from the living patient. When the diagnostic method is finally evaluated it will also be essential in the evaluation of new therapies, as well as in epidemiological studies.

Sammanfattning

Bornavirus (Borna disease virus, BDV) orsakar en neurologisk sjukdom, som kallas bornasjuka, hos flera däggdjur och struts. BDV har också visat sig vara intressant vid vissa psykiatriska sjukdomar hos människa, såsom depression och schizofreni, eftersom virus och antikroppar har påvisats hos patienter med sådana sjukdomar. Dessutom är experimentellt infekterade försöksdjur intressanta som modeller för psykiatrisk sjukdom. I Sverige förekommer en progressiv och ofta fatal neurologisk sjukdom hos katt, som kallas för vingelsjuka. Denna orsakas av BDV. Förutom hos katter har naturlig bornavirusinfektion i Sverige även beskrivits hos hästar och ett lodjur med neurologiska symtom. Framstående symtom på vingelsjuka är vinglig gång (bakbensataxi), beteendeförändringar, smärta från länd- och korsrygg samt oförmåga att dra in klorna, framför allt på bakbenen. Idag misstänks den kliniska diagnosen genom att utesluta övriga orsaker till symtom och diagnosen kan inte styrkas förrän vid en histopatologisk undersökning av hjärna, hjärnhinnor och ryggmärg efter obduktion. Diagnos kan slutligen fastställas vid immunhistokemi. För att få en möjlighet att ställa diagnos hos den levande katten har en realtids-PCR utvecklats. Denna har visat sig vara positiv vid ett test där olika virusstammar användes, både från infekterade celler och hjärnvävnad från experimentellt infekterade katter. Fortfarande behöver flera studier göras innan den kan användas kliniskt. Känsligheten ska testas och även vilket typ av provmaterial från den levande patienten som innehåller mycket virus. När diagnostiken är utprovad kommer den även vara ett användbart verktyg vid utveckling av nya behandlingar och vidare studier av sjukdomens epidemiologi.

Contents

Introduction	9
<i>Borna disease – historical background</i>	10
<i>Borna disease virus – virus biology and genetic properties</i>	10
<i>Borna disease in Sweden – not only staggering disease in cats</i>	11
<i>Epidemiology – search for a possible natural reservoir</i>	12
<i>Diagnostic methods – advantages and disadvantages</i>	12
Materials and methods	14
<i>Cells</i>	14
<i>Animals</i>	14
<i>RNA-preparation</i>	14
<i>Nested RT-PCR assay</i>	15
<i>Real-time PCR assay</i>	15
<i>Optimisation of real-time PCR assays</i>	16
Results	16
<i>Nested RT-PCR assay</i>	16
<i>Optimisation of real-time PCR assays</i>	17
<i>Specificity test</i>	20
Discussion	21
Acknowledgments	24
References	25

Development of a real-time PCR for *in vivo* diagnosis of feline Borna disease

Introduction

Feline Borna disease (BD), or “staggering disease” (SD), is a neurological disorder in cats, mainly characterised by a staggering movement (hind-leg ataxia), behavioural changes, lumbosacral pain and an inability to retract the claws (Lundgren 1992). The disease is clinically suspected by excluding other reasons for clinical signs. Histopathological examination of the brain, meninges and spinal cord will further strengthen the diagnosis, and finally it is confirmed by immunohistochemistry (figure 1). Serological tests have been used, but not all BDV-infected cats are seropositive (Nakamura *et al.* 1996, Johansson, Berg & Berg 2002). Furthermore, antibodies have also been found in healthy cats (Nishino *et al.* 1999).

The lack of a good diagnostic tool for the living cat is a problem for the clinician and the owner but also for the authorities, since the clinical pattern is similar to feline spongiform encephalopathy (FSE), a disease regulated by the law (SFS 1999:657, SJVFS 2002:97). The possibility to make an *in vivo* diagnosis of BD will also be a breakthrough for the evaluation of therapies, as well as epidemiological studies.

The aim of this study is to develop a duplex real-time polymerase chain reaction (PCR) assay for the diagnosis of feline BD *in vivo*.



Figure 1. Perivascular, mononuclear cell infiltrate in the mesencephalon of a cat with staggering disease. Scattered mononuclear cells and neurons contain BDV p40 protein (brown staining). Polyclonal anti-p40 rabbit serum, diluted 1:10000, ABC detection system. X200.

Borna disease – historical background

The neurological disorder, known as Borna disease, has been described in German horses for several hundred years. In the late 19th century a major epidemic outbreak among military horses occurred in the city of Borna, nearby Leipzig in southeastern Germany, thereby the name of the disease. In the 1920s the viral etiology was shown and a vaccination program started in the endemic parts of Germany, although with no or little success (reviewed in Dürrwald & Ludwig 1997, Ludwig & Bode 2000).

For a long time Borna disease was thought to be endemic in horses and sheep only in certain parts of central Europe, but virus and BDV-specific antibodies have now been found all over the world. The host range has also been widened and now natural BDV infection has been found in horses, sheep, rabbits, cattle, cats, dogs, ostriches, lynx and foxes, as well as humans (reviewed in Ludwig & Bode 2000, Dauphin *et al.* 2002). In humans, BDV antibodies and antigen have been found in patients with psychiatric diseases, such as mood disorders and schizophrenia, although the significance of these data is under debate (reviewed in Carbone 2001).

Borna disease virus – virus biology and genetic properties

Borna disease virus (BDV) is an enveloped, negative, non-segmented, single-stranded RNA virus (Briese *et al.* 1992). It belongs to the order of *Mononegavirales* and due to its unique properties it is the sole member of the *Bornaviridae* family. The members of the order of *Mononegavirales* typically have 5-10 genes encoding for the nucleoprotein (N), phosphoprotein (P), matrix protein (M), attachment or surface glycoprotein (G) and the polymerase protein (L). The transcription is initiated by a viral RNA-dependent RNA polymerase (Pringle 1997).

The genome of BDV is about 8900 nucleotides and consists of six major open-reading frames (ORFs). These encode for six polypeptides called p40, p24, p10, p16, p56 and p180, due to their molecular weight in kilodalton (kDa). The polypeptide p40 corresponds to the viral nucleoprotein (N), p24 to the phosphoprotein (P) and p16 to the matrix protein (M), whereas p56 is thought to correspond to the surface glycoprotein (G) and p180 to the L polymerase protein. The function of the p10 polypeptide is unknown and is sometimes referred to as the X protein (Tomonaga, Kobayashi & Ikuta 2002).

BDV seems to be a conserved virus, since the different strains are genetically very similar. The two classical laboratory strains are strain V and He/80, both derived from horses. Some of the human strains, as well as other animal strains, show identical or almost identical genome sequences. Therefore, several scientists argue that the prevalence of BDV throughout the world is due to the limitation of the methods or contamination. A new variant of BDV was found in Austria (No/98) that showed more than 15% genetic variation from other strains (Nowotny *et al.* 2000).

BDV has a strict neurotropism, at least in immunocompetent animals, and the replication takes place in the nucleus of infected cells (Morales *et al.* 1988, Briese

et al. 1992). A possible entry of infection is by the olfactory receptor cells in the olfactory epithelium and then an axonal transport of virus to the olfactory bulb takes place. From the neurons of the olfactory bulb, virus infects other parts of the brain via synapses and dendrites (Morales *et al.* 1988). In persistently infected animals, virus is found in almost all organs and infectious virus is also found in the urine (Morales *et al.* 1988, Sauder & Staeheli 2003). This is probably due to an axonal transport of virus to the urogenital tract (Morales *et al.* 1988).

Borna disease in Sweden – not only staggering disease in cats

The progressive, mostly fatal, neurological disorder, known as staggering disease (SD), was described in Sweden for the first time in 1974 (Kronevi *et al.* 1974). Histopathologically, SD is characterised by a non-suppurative inflammation in the brain, meninges and spinal cord, thereby indicating a viral disease. It was not until the 1990s Borna disease virus (BDV) could be determined as the etiological agent. This was shown in several studies, both by serological and molecular biological methods, as well as in experimental infections with BDV strain V and a feline isolate from a naturally infected cat (Lundgren & Ludwig 1993, Lundgren *et al.* 1995a, Lundgren *et al.* 1995b, Lundgren *et al.* 1997).

In Sweden, feline Borna disease seems to be endemic in areas around the cities of Stockholm and Uppsala (Mälardalen and Uppland) (Lundgren 1992). Feline Borna disease has also been reported in Austria, Switzerland, United Kingdom, Japan and Germany, although BDV-specific antibodies have been found even in other countries (Nowotny & Weissenböck 1995, Bornand *et al.* 1998, Reeves *et al.* 1998, Nakamura *et al.* 1999, Huebner *et al.* 2001, Helps *et al.* 2001, Horii *et al.* 2001). More recently, Borna disease has also been described in horses and a free-ranging lynx in Sweden (Berg, Skidell & Berg 1998, Berg, Dörries & Berg 1999, Degiorgis *et al.* 2000).

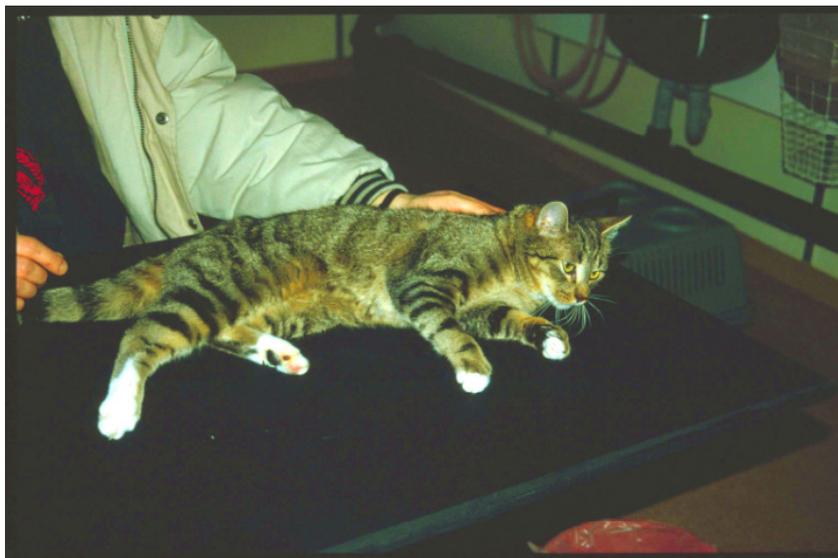


Figure 2. Cat with staggering disease, paralysed in its hind-legs.

Epidemiology – search for a possible natural reservoir

Most of the cats with feline BD start to show clinical signs (mainly staggering movement (hind-leg ataxia), behavioural changes, lumbosacral pain and an inability to retract the claws, see figure 2) from December to July (Lundgren 1992, Hultin-Jäderlund 2003). Similar seasonal patterns have been seen in naturally infected horses and sheep (Dürwald & Ludwig 1997, Vahlenkamp *et al.* 2002). This, together with the geographical localizations, indicates some kind of natural reservoir. Furthermore, feline BD is mostly seen in cats with access to rural and/or woodland environment, as well as in cats that are hunting mice (Berg *et al.* 1998).

Several possible reservoirs have been suggested, among them rodents, birds and ticks. However, serological surveys of wild rodents in Asia have not shown any signs of BDV infection (Tsujiura *et al.* 1999, Hagiwara *et al.* 2001). A Swedish BDV infected lynx was infested with ticks, but none of the investigated ticks were BDV-positive (Degiorgis *et al.* 2000). The only suggested reservoir shown to be positive for BDV has been wild birds. In 2001, faecal samples from two mallards and one jackdaw in Uppsala were reported to contain BDV-specific RNA (Berg *et al.* 2001). However, these findings do not exclude other natural reservoirs.

The interesting finding that experimentally infected neonatal rats excrete infectious BDV in their urine has raised a hypothesis of the transmission of BDV among horses and sheep (Morales *et al.* 1988). Wild rodents persistently infected with BDV may possibly contaminate hay or other feed. When horses and sheep consume it, virus can be up-taken by the neurons in the nasal or pharyngeal mucosa (Staheli *et al.* 2000). This hypothesis was tested experimentally by introducing naive rats in cages with persistently infected rats. The results show that fresh urine was probably the way of transmission between the two groups of rats. BDV-infected cells were mainly found in the olfactory pathways, indicating uptake of virus in the olfactory receptor cells (Sauder & Staheli 2003). It has also been shown that experimental vertical transmission exists in pregnant mice (Okamoto *et al.* 2003). Therefore, the natural reservoir could be a persistently BDV-infected rodent population.

In cats, infected rodents and/or birds could be a possible way of infection by prey or by contaminating the surroundings. Ticks are still interesting as a reservoir, since the areas where feline BD are found in Sweden, also are known for ticks residing.

Diagnostic methods – advantages and disadvantages

As discussed in the general introduction, today the only way to make a confident diagnosis is to perform an autopsy and a histopathological examination including immunohistochemistry. Serology is not a good method, because of the high amount of false positive and false negative animals (Nakamura *et al.* 1996, Nishino *et al.* 1999, Johansson, Berg & Berg 2002).

Conventional nested reverse transcriptase polymerase chain reaction (nested RT-PCR) is a very sensitive method, but it is also very sensitive to contamination. This is because of the handling of the samples after one round of amplification, when PCR products are easily transferred to other (negative) samples. The way of

detecting PCR products by gel electrophoresis is also time-consuming and only semi-quantitative. The further developed method of real-time PCR do not have any post-PCR handling and due to that the risk of contamination is lowered, as well as the time consumed. The number of templates initially present in the sample is also possible to quantify.

Simplified, one can say that the real-time PCR machine consists of one rapid thermal cycler unit and one laser spectrophotometer unit. The fluorescence intensity increases for every cycle of amplification, as the number of templates increases. There are three different ways of getting the fluorescence: a) SYBR-green, b) molecular beacons and c) Taqman probes. All of them use different kind of fluorescent molecules binding to DNA.

SYBR-green binds into the minor grooves of double-stranded DNA, thereby increasing the intensity of fluorescence. After every cycle of amplification the template amplified will increase and so will the fluorescent emission. The SYBR-green will bind to any double-stranded DNA, and in that sense it is unspecific (Applied Biosystems 2003).

Molecular beacons are hair-pinned oligonucleotides with a fluorophore and a quencher close together. When the oligonucleotide and its specific target DNA have hybridised, the fluorophore and quencher separate from each other. The fluorophore then starts to fluoresce (Vet, van der Rijt & Blom 2002).

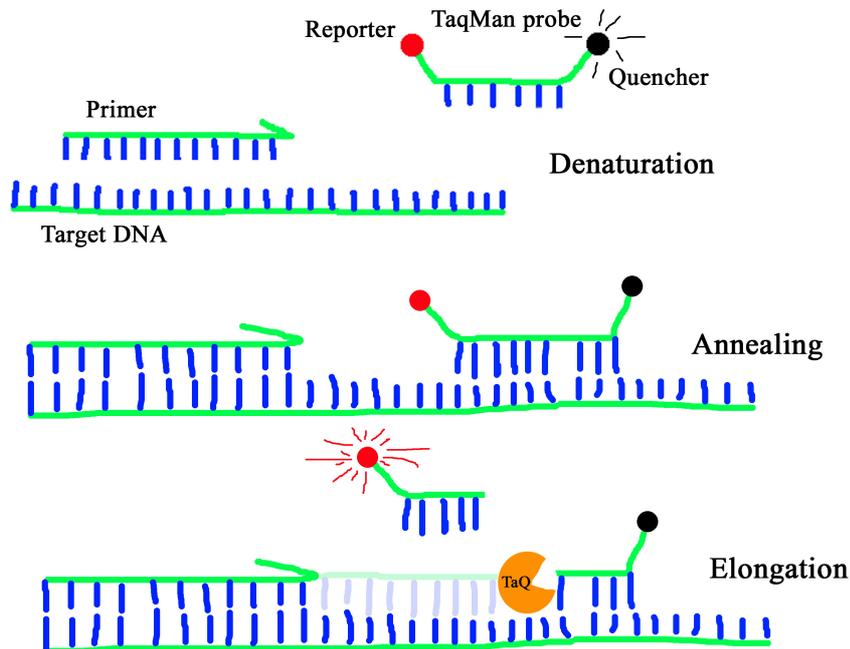


Figure 3. The principle of Taqman. During the elongation step the *Taq* polymerase cleaves the probe, and the reporter and quencher dyes separate. Now the reporter can emit its fluorescence.

Taqman probes are oligonucleotides with a high-energetic reporter dye at its 5' end and a low-energetic quencher dye at its 3' end (figure 3). As long as the probe is intact, the quencher suppresses the reporter. During the elongation step, the *Taq* (*Thermus aquaticus*) polymerase removes nucleotides in front of it by its 5'–3' endonuclease activity. When the probe is cleaved, the distance between the reporter and the quencher increases. The quencher is then no longer able to suppress the reporter and a fluorescent emission appears (Mackay, Arden & Nitsche 2002, Applied Biosystems 2003).

In this study, Taqman probes with two different kinds of reporter dyes at different wavelengths (FAM and ROX) and a so-called black hole quencher, which does not give any fluorescence background, were used.

Materials and methods

Cells

During the optimisation, with respect to primer, manganese acetate and probe concentration, of the real-time PCR assays, C6 (rat glioma) cells infected with BDV He/80 (Carbone *et al.* 1993) were used. The cells were diluted in a tenfold dilution series. In the specificity test, Vero monkey cells infected with BDV No/98 (Nowotny *et al.* 2000) also were used.

Animals

In the specificity test, brain tissue samples from cats experimentally infected with BDV strain V and a feline isolate (Lundgren *et al.* 1997), as well as from some naturally infected cats (cats with staggering disease) were used. RNA from five horses with neurological disorder (Berg, Dörries & Berg 1999) and brain tissue from a BDV-infected free-ranging lynx (Degiorgis *et al.* 2000) were also used during this test.

RNA-preparation

Total RNA from cultured cells and brain tissue samples was extracted with Trizol and Trizol LS reagents (Invitrogen Life Technologies). The manufacturer's protocol was slightly modified. Briefly, RNA preparation was done by adding 1 ml of Trizol or 0.75 ml of Trizol LS per 50-100 mg of tissue or 0.25 ml of cultured cells. The tissue was homogenised manually with a single use mortar. After an incubation of five minutes at room temperature, 0.2 ml of chloroform was added per 1 ml Trizol/ 0.75 ml of Trizol LS initially used. The samples were then incubated another 2-3 minutes at room temperature before they were centrifuged at <12000g at 2-8°C. The aqueous phase was transferred to a fresh tube and the RNA was precipitated with 0.5 ml isopropyl alcohol per 1 ml Trizol/ 0.75 ml of Trizol LS initially used. After an incubation of 10 minutes at room temperature the samples were centrifuged at <12000g at 2-8°C. The RNA was then washed once with 1 ml of 75% ethanol and then redissolved in RNase-free water. The concentration and the purity were then estimated by spectrophotometry.

Nested RT-PCR assay

The RT reaction was performed in a final volume of 40 µl containing 2.5 µg of total RNA. The RNA was mixed with 1.06 µg of cDNA primer and 1 mM of each dNTP and incubated at 65°C for 5 minutes. Then 1x first strand buffer, 10 mM DTT and 80 U of RNaseOUT (Invitrogen Life Technologies) were added and incubated at 42°C for 2 minutes. After adding 400 U of the reverse transcriptase, Superscript II (Invitrogen Life Technologies), the RT reaction was performed at 42°C for 50 minutes. To inactivate the enzyme the samples were incubated at 70°C for 15 minutes.

First PCR amplification was performed with 1 µl of cDNA from the RT reaction in a final volume of 25 µl. The reaction mix consisted of 1x PCR buffer with ammonium sulphate, 1x magnesium chloride, 10 pmol of each primer and 2.5 U of *Taq* polymerase (Invitrogen Life Technologies). The samples were processed through 40 cycles of 30 seconds at 95°C, 1 minute at 55°C and 1.5 minute at 72°C, with a final extension of 5 minutes at 72°C.

Nested PCR amplification was performed at the same conditions as first PCR, although the samples were processed through 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 1.5 minute at 72°C. For the nested PCR amplification 1 µl of first PCR product was used in a final volume of 25 µl.

Gel electrophoresis was performed with a 1.5% agarose gel with TAE-buffer by transferring 10 µl of each nested PCR product with 2 µl of loading dye. The gel was stained with ethidium bromide after a run of about 60-90 minutes at 150 V.

Real-time PCR assay

The probes used were designed by aligning (Multalin version 5.4.1) the sequences of the P and L genes of the strains He/80 (GenBank accession number AJ311522) and No/98 (GenBank accession number AJ311524) and by using the program Beacon Designer 2.1. Both of the probes had no mismatches for these strains. The P gene probe (5'-FAM-CAGCGAACCGGAAGGGAGCAGCTATC-BHQ-3') was 26 nucleotides in length and the L gene probe (5'-ROX-AGGGCAAGTGGGCATGATATTATGGAGCAG-BHQ-3') was 30 nucleotides in length.

The primers were designed in the same way as the probes. The P gene sense primer (5'-GAACCCCTCCATGATCTCAGAY-3') had one mismatch and the anti-sense primer (5'-CTCYGTCAGCTTCTTGGATRAG-3') had two. The L gene primers were designed as one pair of non-degenerated primers, identical to He/80, and one pair of degenerated primers, as a consensus of He/80 and No/98. The L gene sense non-degenerated primer had following sequence, 5'-GGATTCTCATTGGCAAGTCATTCT-3', whereas the anti-sense non-degenerated primer had 5'-CCTCTCCAAGTTAGCCCATGT-3'. The L gene sense degenerated primer (5'-GRATTCTYATTGGCAAGTCRTTCT-3') had three mismatches and the anti-sense degenerated primer (5'-CCKCTCMAYGTTWGCCCAYGT-3') had five.

Primers and probes were ordered from Biosearch Technologies Inc., CA, USA. The length of the PCR products in these two systems is 88 and 89 base pairs respectively.

The real-time PCR assay was prepared with special precaution for contaminations, as previously described (Belák & Thorén 2001). This was done by preparing the reaction master mix in a separate laboratory from the adding of RNA. Furthermore, when RNA was added, tube holders and openers were used to avoid cross contamination.

The chemicals used in the master mix, except for primers and probe, was *rTth* (*Thermus thermophilus*) DNA polymerase (2.5 U/ μ l), 5X EZ buffer (250 mM bicine, 575 mM potassium acetate, 40% (w/v) glycerol, pH 8.2) and 25 mM manganese acetate (all three, Applied Biosystems), 10 mM of each dNTP, 1 mg/ml of bovine serum albumin (BSA) and DMPC-treated water to a total volume of 25 μ l.

The *rTth* DNA polymerase has the ability to act as both reverse transcriptase and DNA polymerase under the right conditions, thereby allowing the use of the same enzyme in the reverse transcriptase (RT) reaction as in the PCR reaction, without changing reaction conditions. The RT reaction was performed with an initial incubation at 42°C for 5 minutes, followed by another incubation at 60°C for 30 minutes. After a third incubation at 95°C for 3 minutes the samples were processed through 40 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 45 seconds at 72°C. The data were then analysed and presented with Sequence Detection Systems 1.9 (Applied Biosystems). The real-time PCR machine in use was the ABI PRISM 7700 Sequence Detector.

Optimisation of real-time PCR assays

The real-time PCR assays were optimised with respect to primer, manganese acetate and probe concentration according to standard procedures at the Department of Virology, National Veterinary Institute, Uppsala, Sweden. To evaluate which set of primer concentrations that were best, nine different concentrations of forward/reverse primers were used in duplicates (50/50, 50/300, 50/900, 300/50, 300/300, 300/900, 900/50, 900/300 and 900/900, all concentrations in nM). Seven different concentrations of manganese acetate were used in the optimisation, ranging from 1.5 to 4.5 mM. All concentrations were run in duplicates. For the probe titration, four different concentrations (100, 200, 300 and 400 nM) were used in duplicates. The optimisation was performed for each primer pair (P gene, L gene non-degenerated and degenerated).

Results

Nested RT-PCR assay

In order to test the RNA quality after preparation, some of the samples were tested in nested RT-PCR assay with β -actin primers (figure 4). This gene is expressed in all cells and if the RNA is of bad quality, no or weak PCR product is seen. At the same time, the samples were tested if they were BDV positive using BDV-p23/P

and BDV-p40/N primers. The samples were then used in the real-time PCR assay. Samples that were BDV positive in the nested RT-PCR assay were also positive in the real-time PCR assay. However, the RNA from natural infected horses and cats, that earlier had been positive with conventional RT-PCR (Berg, Dörries & Berg 1999, M Berg unpublished results), showed to be negative with real-time PCR.

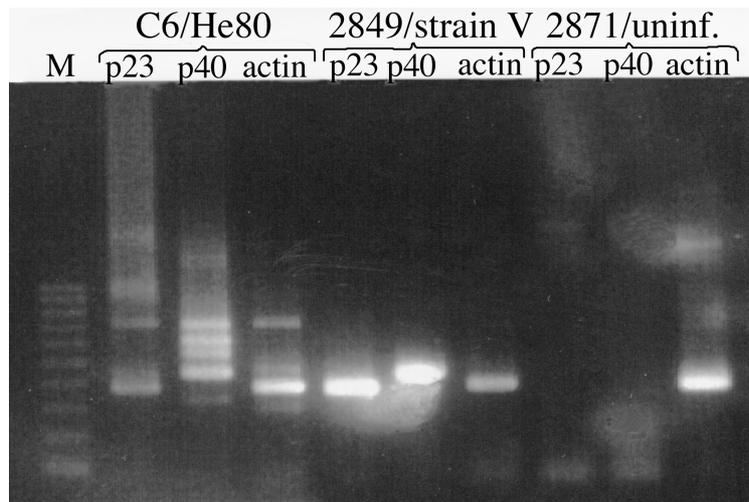


Figure 4. Gel electrophoresis of nested RT-PCR showing C6 (rat glioma) cells infected with BDV He/80, brain tissue from a cat experimentally infected with strain V and brain tissue from an uninfected cat. p23 = BDV-p23/P, p40 = BDV-p40/N, actin = β -actin. M = molecular weight marker.

Optimisation of real-time PCR assays

The optimisation resulted in a reaction protocol for each system (Table 1a and 1b). The L gene non-degenerated and degenerated primer systems resulted in the same protocol. Finally, reaction efficiency were calculated for each system by running a dilution series from 1:1 to 10^{-6} and then making a standard curve. The reaction efficiency, y-intercept and correlation co-efficiency are shown in table 2, where reaction efficiency is the number of copies amplified each cycle, y-intercept corresponds to the number of cycles required to amplify one unit of template to reach the fluorescence threshold and correlation co-efficiency is a measure of how well the data correlates to the given concentrations of the standard.

Table 1a. BDV p23/P-gene real-time PCR protocol.

Reagents per reaction	1X (μ l)	Final conc.
DMPC-H ₂ O	6	-
5X EZ buffer	5	1X
Mn(OAc) ₂ (25 mM)	2.5	2.5 mM
4XdNTPs (10 mM)	1.25	0.5 mM
BSA (1 mg/ml)	2	0.08 mg/ml
p23/P-probe (10 μ M)	0.75	0.3 μ M
<i>rTth</i> polymerase (2.5 U/ μ l)	1	0.1 U/ μ l
F-primer p23/P (10 μ M)	2.25	0.9 μ M
R-primer p23/P (10 μ M)	2.25	0.9 μ M
<u>RNA: 2 μl</u>		
Total:	25 μ l	

Table 1b. BDV L-gene (non-degenerated and degenerated primers) real-time PCR protocol.

Reagents per reaction	1X (μ l)	Final conc.
DMPC-H ₂ O	7.25	-
5X EZ buffer	5	1X
Mn(OAc) ₂ (25 mM)	2.5	2.5 mM
4XdNTPs (10 mM)	1.25	0.5 mM
BSA (1 mg/ml)	2	0.08 mg/ml
L-gene probe (10 μ M)	1	0.4 μ M
<i>RTth</i> -polymerase (2.5 U/ μ l)	1	0.1 U/ μ l
F-primer L-gene (10 μ M)	0.75	0.3 μ M
R-primer L-gene (10 μ M)	2.25	0.9 μ M
<u>RNA: 2 μl</u>		
Total:	25 μ l	

Table 1c. BDV duplex real-time PCR protocol.

Reagents per reaction	1X (μ l)	Final conc.
DMPC-H ₂ O	2	-
5X EZ buffer	5	1X
Mn(OAc) ₂ (25 mM)	2.5	2.5 mM
4XdNTPs (10 mM)	1.25	0.5 mM
BSA (1 mg/ml)	2	0.08 mg/ml
p23/P-probe (10 μ M)	0.75	0.3 μ M
L-gene probe (10 μ M)	1	0.4 μ M
<i>RTth</i> -polymerase (2.5 U/ μ l)	1	0.1 U/ μ l
F-primer p23/P (10 μ M)	2.25	0.9 μ M
R-primer p23/P (10 μ M)	2.25	0.9 μ M
F-primer L-gene (10 μ M)	0.75	0.3 μ M
R-primer L-gene (10 μ M)	2.25	0.9 μ M
<u>RNA: 2 μl</u>		
Total:	25 μ l	

Table 2. Reaction efficiency (E) in percent, y-coefficient (Y) and correlation co-efficiency (C) are shown for each real-time PCR assay.

<u>Assay</u>	<u>E</u>	<u>Y</u>	<u>C</u>
p23/P gene	60	33.696	0.964
L-gene non-degenerated	82	35.034	0.963
L-gene degenerated	57	35.662	0.958

The first run with duplex real-time PCR, that is both the P and L gene systems in the same tube, was run with the individual system protocols put together. Only slight differences could be seen between the duplex and simplex systems and the duplex system seemed to be better than the simplex (Figure 5 a & b). Therefore, the duplex real-time PCR reaction protocol resulted in adding the two simplex protocols together (Table 1c).

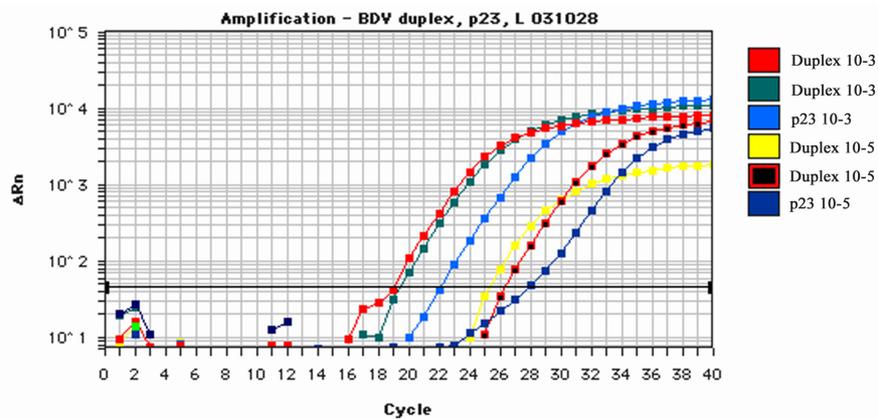


Figure 5 a). Comparison between duplex and simplex real-time PCR assay (p23/P-gene).

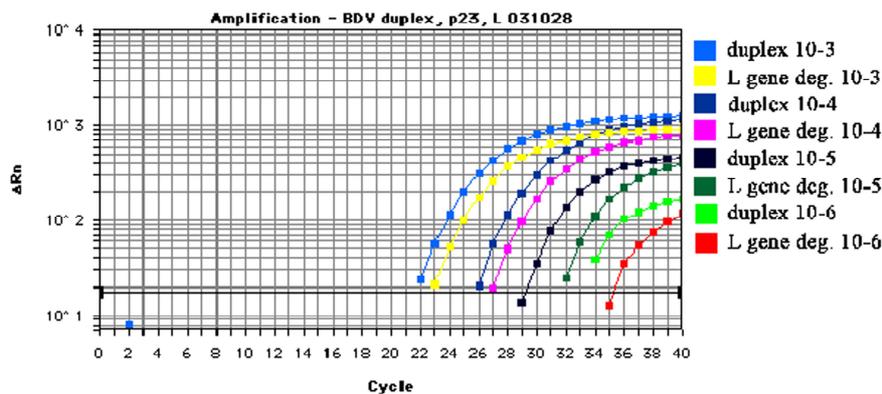


Figure 5 b). Comparison between duplex and simplex real-time PCR assay (L-gene degenerated primers).

Specificity test

To see the specificity of the real-time PCR assay, some different strains were used in a specificity test. The system was designed for He/80, but also for No/98, a strain that differs quite a lot genetically, as discussed in the introduction. The strains used for the specificity test were He/80, No/98, strain V and a feline isolate from a naturally infected cat, as well as samples from some naturally infected horses, cats and a lynx. Those positive were He/80, No/98, strain V and in some extent perhaps also the feline isolate, as shown in figure 6 a-c, but further experiments have to be performed to confirm the last observation.

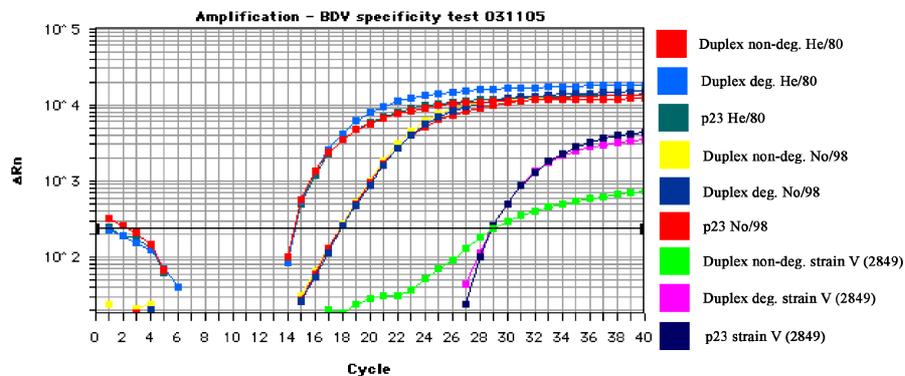


Figure 6 a). BDV specificity test, duplex and simplex real-time PCR assay (p23/P). He/80 = C6 BDV He/80 infected cells, strain V = experimentally infected cat (brain tissue), No/98 = Vero monkey cells infected with BDV No/98.

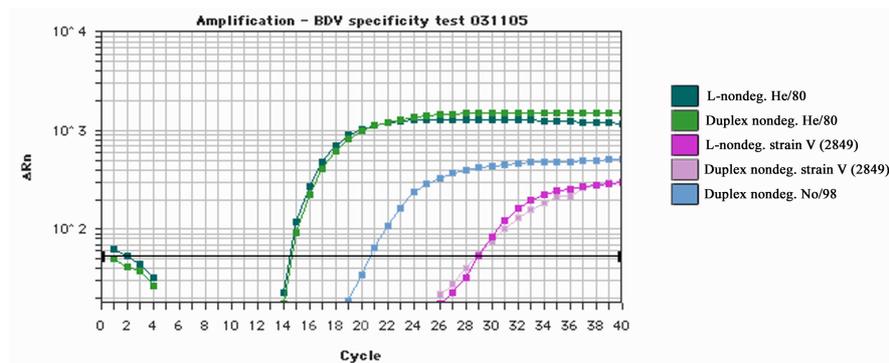


Figure 6 b). BDV specificity test, duplex and simplex real-time PCR assay (L-gene non-degenerated primers). See figure 6 a) for explanations.

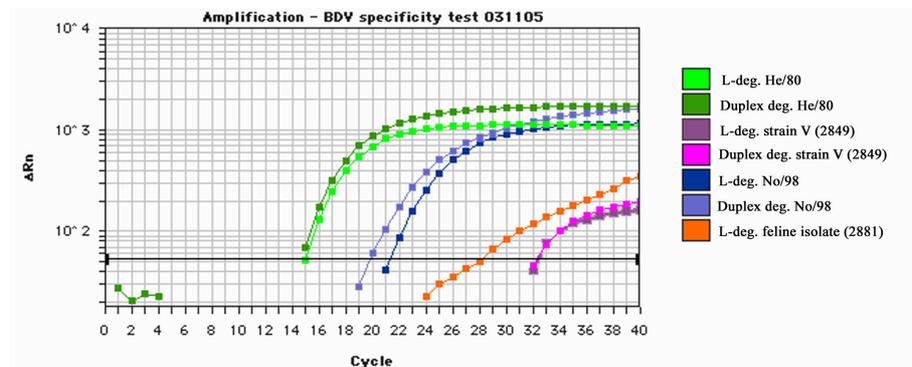


Figure 6 c). BDV specificity test, duplex and simplex real-time PCR assay (L-gene degenerated primers). Feline isolate = brain tissue from cat experimentally infected with a feline isolate from cat with staggering disease. See also figure 6 a) for explanations.

Discussion

Today there is no way to make a confident diagnosis of feline Borna disease/staggering disease in the living cat. This is of course a problem for owners and practitioners, but also for the evaluation of relevant therapies. Results of a clinical and neurological examination are not specific, and could mimic the findings in other central nervous system diseases, such as feline spongiform encephalopathy (FSE). Serology is not reliable, as discussed earlier, and neither haematology nor serum chemistry is diagnostic either. Recently, a study showed that an enzyme immune assay directed towards BDV-specific circulating immune complexes (antibodies bound to antigen) was a better method than other serological assays. The authors suggest that three different immune assays towards circulating immune complexes, plasma antigen and antibodies would be a powerful diagnostic tool. However, these have only been used for humans and horses, but could in principle be adapted for cats (Bode *et al.* 2001). The presence of antibodies in cerebrospinal fluid is probably more adequate than in blood, but such a sample is relatively difficult and risky to obtain and the cat has to be anaesthetised. Therefore, a simply taken sample, like blood or conjunctival fluid, would be convenient for subsequent search for viral RNA. A modern variant of RNA detection is the real-time PCR assay. It is a sensitive method and not as time-consuming as nested RT-PCR, where the detection of PCR products is done by gel electrophoresis. Real-time PCR also has the advantage of not including any post-PCR handling, and therefore the risk of cross contamination is decreased.

In order to develop such a diagnostic method, certain criteria have to be fulfilled. It has to be specific and sensitive, which means that it has to detect different strains of the virus, but no other virus, and also detect the virus of interest in low concentration.

Firstly, probes and primers were designed by aligning two different BDV strains, He/80 and No/98, to find the most homologous regions. Most strains are genetically very similar to the standard laboratory strains He/80 and strain V, both derived from horses. However, No/98 is the strain with most variations genetically, compared to other strains. Because of the difficulty to find homologous regions in the L gene that were sufficiently long for a probe and two primers to fit, two pairs of primers were designed. One pair was non-degenerated and homologous to He/80. The other pair was degenerated and homologous to both He/80 and No/98. Two different gene systems (L and p23/P genes) were used to decrease the risk of contamination. The probability for contaminating the same sample with both genes or PCR products of those genes should be quite low, especially since only the p23/P and p40/N genes have been used in conventional RT-PCR at this laboratory.

The two systems were then individually optimised with respect to primer, manganese acetate and probe concentrations, according to standard protocols at the Department of Virology, Swedish National Veterinary Institute, Uppsala. These two systems were put together in a duplex assay, by just adding the two individual protocols. The duplex and simplex assays seemed to work equally well when compared.

In order to test the specificity of this new real-time PCR assay, some different strains were used. The strain used in the optimisation was He/80, and apart from that strain, No/98, strain V and a feline isolate from a naturally infected cat were used, as well as material from some naturally infected horses, other cats and a lynx. The He/80 and strain V strains were positive in all systems used. The No/98 were positive in all systems except for the non-degenerated L gene system. Probably this is due to the number of mismatches in the non-degenerated primers. The feline isolate seemed to be positive in the degenerated L gene system only. This has to be repeated in order to know whether or not it is a true result. The naturally infected horses, cats and lynx were all negative in this test, although they had been positive with conventional nested RT-PCR earlier. This was possibly because of degradation of RNA during storage, PCR inhibitors or low sensitivity of the real-time PCR assay.

One way to overcome the problems with not knowing the quality and quantity of RNA in the samples, and thereby not knowing if a negative sample is truly negative, is to add another system in the assay. A possible way is to use primers and probe for a feline ribosomal RNA (rRNA). This has been done in detection of *Chlamydophila felis* and feline herpesvirus by a multiplex real-time PCR (Helps *et al.* 2003). If the sample contains low amounts of feline cells, the amount of feline rRNA is low. The reason could be an incorrect technique of taking the sample and therefore the sample should be retaken. This approach is useful in a clinical diagnostic test and the test result will be easier to interpret for the clinician.

Next step in evaluating the new assay is to perform a sensitivity test. This will be done with known concentrations of templates of the PCR products, such as *in vitro* transcribed plasmid DNA. To be a useful diagnostic tool this assay has to detect even low concentrations of viral RNA. To see if the assay only detects the virus of

interest (BDV), samples known to be positive for other viruses of the same order (*Mononegavirales*), like canine distemper virus, will be tested.

When the specificity and sensitivity of the assay are known, time has come to test the clinical samples. For this purpose, different types of material (conjunctival fluid, saliva, nasal fluid, blood, urine, faeces and cerebrospinal fluid) have been collected from cats with suspected feline BD or staggering disease at the Department of Small Animal Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala. Most of them have also been autopsied and examined histopathologically, where the diagnosis has been confirmed. At autopsy, different parts of the brain and olfactory epithelium have been collected. Earlier studies have shown that nasal and conjunctival fluids, as well as saliva in seropositive horses contain BDV-specific RNA (Richt *et al.* 1993). This was also the case in naturally infected sheep (Vahlenkamp *et al.* 2002). The entry of infection is probably by the olfactory pathway, as discussed earlier. Possibly, the replication is initiated in the olfactory epithelium before virus is transmitted to the olfactory bulb and other parts of the brain. This could be a reason for finding viral RNA in nasal fluid. As discussed in the introduction, persistently infected rats shed infectious virus in their urine, probably due to a transport of virus via peripheral nerves to the urine bladder wall. However, if this also is the case in other infected animals, as the cat, is not known.

This study shows that a duplex real-time PCR assay works with different strains of BDV, not only with infected cell lines, but also with brain tissue samples. In the only previous study where real-time PCR assay has been used for BDV, it was used to quantify the number of BDV mRNAs present in different parts of the brain (Watanabe *et al.* 2001). Thereby, this project is the first step towards a clinical diagnostic method using a real-time PCR assay, but further studies have to be done. However, it has to be investigated whether naturally infected cats shown positive with conventional nested RT-PCR also are positive with the new real-time PCR assay, as well as finding out which clinical samples contain most quantities of viral RNA and therefore would be the most suitable sample to take.

A new clinical diagnostic tool will not only be useful in diagnosing feline Borna disease, but also for epidemiological studies in search for the still unknown natural reservoir, as well as evaluating new relevant therapies. Screenings of the prevalence of BDV in different species will also be interesting. Considering the results of this study, real-time PCR assay will be a powerful tool for diagnosing Borna disease virus infections, not only in the living cat, but also in other species including man.

Acknowledgments

There are several persons I would like to thank for helping me out during this project:

- My supervisor Mikael Berg for giving me the opportunity to do this project and for valuable discussions
- My assistant supervisor Karin Hultin-Jäderlund for giving me the clinical point of view
- Mikhayil Hakhverdyan and Peter Thorén for introducing me to the real-time PCR technique, both theoretically and practically; (Peter also for designing the primers and probes)
- Anna-Lena Berg for providing me with tissue samples
- The staff at the Immunology section at the Department of Molecular Biosciences for answering all my questions & being a good company: Gunnar, Caroline, Maija-Leena, Eva, Anne, Bettan, Tanja, Dieter, Frida, Sirje, Carolina & Anders, and my fellow students Anne-Li, Karin, Lisa, Rebecka & Lina
- Sándor, Alia, Karin, Carlos & Frederik at the Virology department, Swedish National Veterinary Institute, and Sara at the Department of Ruminant Medicine
- My sister Maria for giving me new inputs
- Helena for helping me with Photoshop and Powerpoint
- Julius the cat, for waking me up in the mornings

References

- Applied Biosystems. 2003. *Real-time PCR vs traditional PCR*.
- Belák, S. & Thorén, P. 2001. *Molecular diagnosis of animal diseases: some experiences over the past decade. Expert review of molecular diagnostics 1*, 434-443.
- Berg, A.-L., Skidell, J. & Berg, M. 1998. *Bornasjuka hos häst i Sverige: första verifierade fallet. Svensk veterinärtidning 50*, 133-136.
- Berg, A.-L., Reid-Smith, R., Larsson, M. & Bonnett, B. 1998. *Case control study of feline Borna disease in Sweden. The veterinary record 142*, 715-717.
- Berg, A.-L., Dörries, R. & 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. Epidemiology and infections 127*, 173-178.
- 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*, 481-491.
- Bornand, J.V., Fatzer, R., Melzer, K., Jmaa, D.G., Caplazi, P. & Ehrensperger, F. 1998. *A case of Borna disease in cat. European journal of veterinary pathology 4*, 33-35.
- Briese, T., de la Torre, J.C., Lewis, A., Ludwig, H. & Lipkin, W.I. 1992. *Borna disease virus, a negative-strand RNA virus, transcribes in the nucleus of infected cells. Proceedings of National Academy of Sciences 89*, 11486-11489.
- Carbone, K.M., Rubin, S.A., Sierra-Honigmann, A.M. & Lederman, H.M. 1993. *Characterization of a glial cell line persistently infected with Borna disease virus (BDV): Influence of neurotrophic factors on BDV protein and RNA expression. Journal of virology 67*, 1453-1460.
- Carbone, K.M. 2001. *Borna disease virus and human disease. Clinical microbiology reviews, 14*, 513-527.
- Dauphin, G., Legay, V., Pitel, P.-H. & Zientara, S. 2002. *Borna disease: current knowledge and virus detection in France. Veterinary research 33*, 127-138.
- Degiorgis, M.-P., Berg, A.-L., Hård af Segerstad, C., Mörner, T., Johansson, M. & Berg, M. 2000. *Borna disease in a free-ranging lynx (Lynx lynx). Journal of clinical microbiology 38*, 3087-3091.
- Dürwald, R. & Ludwig, H. 1997. *Borna disease virus (BDV), a (zoonotic?) worldwide pathogen. A review of the history of the disease and the virus infection with comprehensive bibliography. Journal of veterinary medicine B 44*, 147-184.
- Hagiwara, K., Asakawa, M., Liao, L., Jiang, W., Yan, S., Jie Chai, J., Oku, Y., Ikuta, K. & Ito, M. 2001. *Seroprevalence of Borna disease virus in domestic animals in Xinjiang, China. Veterinary microbiology 80*, 383-389.
- Helps, C.R., Turan, N., Bilal, T., Harbour, D.A. & Yilmaz, H. 2001. *Detection of antibodies to Borna disease virus in Turkish cats by using recombinant p40. The veterinary record 149*, 647-650.
- Helps, C., Reeves, N., Egan, K., Howard, P. & Harbour, D. 2003. *Detection of Chlamydomphila felis and feline herpesvirus by multiplex real-time PCR analysis. Journal of clinical microbiology 41*, 2734-2736.
- Horii, Y., Garcia, J.N.P., Noviana, D., Kono, F., Sawada, T., Naraki, T. & Yamaguchi, K. 2001. *Detection of anti-Borna disease virus antibodies from cats in Asian countries, Japan, Philippines and Indonesia using electrochemiluminescence immunoassay. Journal of veterinary medical sciences 63*, 921-923.
- Huebner, J., Bode, L. & Ludwig, H. 2001. *Borna disease virus infection in FIV-positive cats in Germany. The veterinary record 149*, 152.
- Hultin-Jäderlund, K. 2003. *Feline Borna disease – a nervous system disorder in cats. Proceedings of 13th ECVIM-CA congress*, 112-114.

- Johansson, M., Berg, M. & Berg, A.-L. 2002. *Humoral immune response against Borna disease virus (BDV) in experimentally and naturally infected cats. Veterinary immunology and immunopathology* 90, 23-33.
- Kronevi, T., Nordström, M., Moreno, W. & Nilsson, P.O. 1974. *Feline ataxia due to nonsuppurative meningoencephalomyelitis of unknown aetiology. Nordisk veterinärmedicin* 26, 720-725.
- Ludwig, H. & Bode, L. 2000. *Borna disease virus: new aspects on infection, disease, diagnosis and epidemiology. Review of science and technology* 19, 259-288.
- Lundgren, A.-L. 1992. *Feline non-suppurative meningoencephalomyelitis. A clinical and pathological study. Journal of comparative pathology* 107, 411-425.
- Lundgren, A.-L. & Ludwig, H. 1993. *Clinically diseased cats with non-suppurative meningoencephalomyelitis have Borna disease virus-specific antibodies. Acta veterinaria scandinavica* 34, 101-103.
- Lundgren, A.-L., Lindberg, R., Ludwig, H. & Gosztonyi, G. 1995a. *Immunoreactivity of the central nervous system in cats with a Borna disease-like meningoencephalomyelitis (staggering disease). Acta neuropathologica* 90, 184-193.
- Lundgren, A.-L., Zimmermann, W., Bode, L., Czech, G., Gosztonyi, G., Lindberg, R. & Ludwig, H. 1995b. *Staggering disease in cats: isolation and characterization of 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. & Ludwig, H. 1997. *Neurological disease and encephalitis in cats experimentally infected with Borna disease virus. Acta neuropathologica* 93, 391-401.
- Mackay, I.M., Arden, K.E. & Nitsche, A. 2002. *Real-time PCR in virology. Nucleic acids research* 30, 1292-1305.
- Morales, J.A., Herzog, S., Kompter, C., Frese, K. & Rott, R. 1988. *Axonal transport of Borna disease virus along olfactory pathways in spontaneously and experimentally infected rats. Medical microbiology and immunology* 177, 51-68.
- Nakamura, Y., Asahi, S., Nakaya, T., Bahmani, M.K., Saitoh, S., Yasui, K., Mayama, H., Hagiwara, K., Ishihara, C. & Ikuta, K. 1996. *Demonstration of Borna disease virus RNA in peripheral blood mononuclear cells derived from domestic cats in Japan. Journal of clinical microbiology* 34, 188-191.
- Nakamura, Y., Tsujimoto, H., Machida, S. & Ikuta, K. 1999. *High prevalence of Borna disease virus in domestic cats with neurological disorders in Japan. Veterinary microbiology* 70, 153-169.
- Nishino, Y., Funaba, M., Fukushima, R., Mizutani, T., Kimura, T., Iizuka, R., Hirami, H. & Hara, M. 1999. *Borna disease virus infection in domestic cats: evaluation by RNA and antibody detection. Journal of veterinary medical sciences* 61, 1167-1170.
- Nowotny, N. & Weissenböck, H. 1995. *Description of feline nonsuppurative meningoencephalomyelitis ("staggering disease") and studies of its etiology. Journal of clinical microbiology* 33, 1668-1669.
- Nowotny, N., Kolodziejek, J., Jehle, C.O., Suchy, A., Staeheli, P. & Schwemmler, M. 2000. *Isolation and characterization of a new subtype of Borna disease virus. Journal of virology* 74, 5655-5658.
- Okamoto, M., Hagiwara, K., Kamitani, W., Sako, T., Hirayama, K., Kirisawa, R., Tsuji, M., Ishihara, C., Iwai, H., Kobayashi, T., Tomonaga, K., Ikuta, K. & Taniyama, H. 2003. *Experimental vertical transmission of Borna disease virus in the mouse. Archives of virology* 148, 1557-1568.
- Pringle, C.R. 1997. *The order of Mononegavirales – current status. Archives of virology* 142, 2321-2326.
- Richt, J.A., Herzog, S., Haberzettl, K. & Rott, R. 1993. *Demonstration of Borna disease virus-specific RNA in secretions in naturally infected horses by the polymerase chain reaction. Medical microbiology and immunology* 182, 293-304.
- Reeves, N.A., Helps, C.R., Gunn-Moore, D.A., Blundell, C., Finnemore, P.L., Pearson, G.R. & Harbour, D.A. 1998. *Natural Borna disease virus infections in cats in the United Kingdom. The veterinary record* 143, 523-526.
- Sauder, C. & Staeheli, P. 2003. *Rat model of Borna disease virus transmission: Epidemiological implications. Journal of virology* 77, 12886-12890.

- SFS 1999:657. *Epizootilagen. Jordbruksdepartementet, 1999-06-10.*
- SJVFS 2002:97. *Föreskrifter om ändringar i Statens jordbruksverks föreskrifter (SJVFS 1999:102) om epizootiska sjukdomar. Statens jordbruksverk, 2002-12-27.*
- Stacheli, P., Sauder, C., Hausmann, J., Ehrensperger, F. & Schwemmler, M. 2000. *Epidemiology of Borna disease virus. Journal of general virology 81, 2123-2135.*
- Tomonaga, K., Kobayashi, T. & Ikuta, K. 2002. *Molecular and cellular biology of Borna disease virus infection. Microbes and infection 4, 491-500.*
- Tsujimura, K., Mizutani, T., Kariwa, H., Yoshimatsu, K., Ogino, M., Morii, Y., Inagaki, H., Arikawa, J. & Takashima, I. 1999. *A serosurvey of Borna disease infection in wild rats by a capture ELISA. Journal of veterinary medical sciences 61, 113-117.*
- Vahlenkamp, T.W., Konrath, A., Weber, M. & Müller, H. 2002. *Persistence of Borna disease virus in naturally infected sheep. Journal of virology 76, 9735-9743.*
- Vet, J.A.M., van der Rijt, B.J.M. & Blom, H.J. 2002. *Molecular beacons: colorful analysis of nucleic acids. Expert review of molecular diagnostics 2, 77-86.*
- Watanabe, M., Lee, B.-Y., Kamitani, W., Kobayashi, T., Taniyama, H., Tomonaga, K. & Ikuta, K. 2001. *Neurological diseases and viral dynamics in the brains of neonatally Borna disease virus-infected gerbils. Virology 282, 65-76.*

